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Effect of *Morinda citrifolia* (Noni)-Enriched Diet on Hepatic Heat Shock Protein and Lipid Metabolism-Related Genes in Heat Stressed Broiler Chickens

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Effect of *Morinda citrifolia* (Noni)-Enriched Diet on Hepatic Heat Shock Protein and Lipid
Metabolism-Related Genes in Heat Stressed Broiler Chickens

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

by

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Bachelor of Science in Poultry Science, 2016

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ABSTRACT

Heat stress (HS) has been reported to alter fat deposition in broilers, however the underlying molecular mechanisms are not well-defined; therefore, the objectives of the current study were to: (1) determine the effects of acute (2 h) and chronic (3 wk) HS on the expression of key molecular signatures involved in hepatic lipogenic and lipolytic programs; and (2) assess if diet supplementation with dried Noni medicinal plant (0.2% of the diet) modulates these effects. Broilers (n=480 males, 1 d) were randomly assigned to 12 environmental chambers, subjected to two environmental conditions (HS at 35°C vs. thermoneutral condition [TN] at 24°C) and fed two diets (control vs. Noni) in a 2 × 2 factorial design. Feed intake and body weights were recorded, and blood and liver samples were collected at 2 h and 3 wk post-heat exposure. HS depressed feed intake, reduced body weight, and up-regulated the hepatic expression of heat shock protein HSP60, HSP70, HSP90, as well as key lipogenic proteins fatty acid synthase (FASN), acetyl co-A carboxylase alpha (ACC α), and ATP citrate lyase (ACLY). HS down-regulated the hepatic expression of lipoprotein lipase (LPL) and hepatic triacylglycerol lipase (LIPC), but up-regulated adipose triglyceride lipase (ATGL). Although it did not affect growth performance, Noni supplementation regulated the hepatic expression of lipogenic proteins in a time- and gene-specific manner. Prior to HS, Noni increased ACLY and FASN in acute and chronic experimental conditions, respectively. During acute HS, Noni increased ACC α , but reduced FASN and ACLY expression. Under chronic HS, Noni up-regulated ACC α and FASN but it down-regulated ACLY. In cells, HS exposure to 45°C for 2 hours down-regulated ACC α , FASN, and ACLY compared to TN exposure at 37°C. Treatment with quercetin, one bioactive ingredient in Noni, up-regulated the expression of ACC α , FASN, and ACLY under TN conditions, but it appeared to down-regulate ACC α and increase ACLY levels under HS

exposure. In conclusion, our findings indicate that HS induces hepatic lipogenesis in chickens and this effect is probably mediated via HSPs. The modulation of hepatic heat-shock protein HSP expression suggests that Noni might be involved in modulating the stress response in chicken liver.

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DEDICATION

This thesis is dedicated to all my family and close friends who supported me, and guided me through graduate school.

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CHAPTER 1. REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Since the mid-1900s, the establishment of vertical integration, and improvements in genetic selection, nutrition, and on-farm management lead to today's faster growing, higher meat-yielding broiler chicken. These highly efficient broilers meet the increasing consumer preferences and economic demands for more affordable and higher protein sources (Griffen and Goddard, 1994), which can feed the dramatically growing human population in the decades to come. Studies comparing different genetic broiler lines over time have demonstrated improvements in broiler production (e.g., growth, livability, feed conversion; Havenstein et al., 1994a, 2003a, b) and carcass characteristics (e.g., carcass weight and yield, part weight and yield, abdominal fat weight and yield; Havenstein et al., 1994b, 2003c). However, these improvements have caused several negative impacts on genetically improved broilers lines, such as depressed immune function (Cheema et al., 2003, Qureshi and Havenstein, 1994), hyperphagia (Sonoda, 1983), increased occurrence of skeletal muscle myopathies (Velleman, 2015), and a hypersensitivity to a higher environmental temperature (heat stress) due to an increased metabolic activity (Berong and Washburn, 1998; Cahaner and Leenstra, 1992; Settar et al., 1999; Washburn et al., 1980).

Hans Selye indicated that the word stress is defined as “the nonspecific response of the body to a demand,” and a stressor (high environmental temperature in this case) is defined as “an agent that produces stress” (Selye, 1976). Therefore, heat stress can be defined as the response of the animal to high environmental temperatures that disrupts the animal's normal physiological homeostasis or equilibrium. Heat stress thus creates a negative balance between the amount of

heat energy from the animal's body flowing to the surrounding environment and the amount of heat energy produced by the animal (Lara and Rostagno, 2013). The negative imbalance can be caused by various environmental factors, including sunlight, thermal radiation, air temperature, and humidity (Lara and Rostagno, 2013). Heat stress represents one of the most detrimental types of stress to the poultry industry due to its many adverse effects on production, animal well-being, and economic losses. Therefore, it is critical to understand the mechanisms of heat stress response in broilers in order to develop mechanism-based strategies to improve poultry production and sustainability.

1.2 NEGATIVE IMPACTS OF HEAT STRESS ON POULTRY BEHAVIOR, AND ENDOCRINE AND IMMUNE FUNCTION

Under high environmental temperature and humidity stress conditions, avian species will alter their behavior and physiological homeostasis to lower their body temperature when actively seeking thermoregulation (Lara and Rostagno, 2013). Mack and colleagues (2013) observed behavioral changes in birds exposed to heat stress conditions such as spending less time eating and moving, while spending more time drinking, panting, resting, and keeping their wings elevated compared to birds under normal environmental conditions. By decreasing movement and eating, less body heat can be generated because an increase in heat production has been shown to be linked with feeding level (Wiernusz and Teeter, 1993; Zhou and Yamamoto, 1997); therefore, a lower food intake would lead to less metabolic heat production. Even though panting can assist birds in evaporative heat loss, the increase in panting behavior can be detrimental to the bird because panting decreases carbon dioxide levels in blood plasma which then causes a loss in hydrogen atoms. This loss of hydrogen atoms then leads to an increase in plasma pH, known as respiratory alkalosis, and induces increased organic acid bioavailability (Mongin,

1968; Richards, 1970). These factors negatively impact blood bicarbonate availability and free calcium levels in the blood for egg shell mineralization, which is extremely important for broiler breeders and laying hens as this process impacts egg shell quality of hatching and table eggs (Marder and Arad, 1989). In order for birds to utilize evaporative cooling there needs to be an increase in respiration; however, a decrease in respiration is needed to decrease alkalosis (Etches et al., 2008). Therefore, birds will also utilize the gular flutter, which is the fluttering of the upper neck muscles (gular area) to increase the evaporative loss of heat by increasing the air circulation on surfaces of the tissue of the mouth and upper respiratory tract (Dawson et al., 1982).

The neuroendocrine system of poultry species is also impacted by high environmental conditions resulting in activation of the central stress response system, hypothalamic-pituitary-adrenal (HPA) axis, and elevated plasma corticosterone (a key stress hormone) concentrations (Nathan et al., 1976; Quinteiro-Filho et al., 2010). Studies looking at the regulation and balance between triiodothyronine (T_3) and thyroxine (T_4), key thyroid hormones that regulate body temperature and metabolic activity, in laying hens and broilers have shown a consistent decrease in T_3 under heat stress conditions (Elnagar et al., 2010; Geraert et al., 1996; Mack et al., 2013; Yahav and Hurwitz, 1996), whereas T_4 concentrations under heat stress conditions revealed inconsistent results, with some reporting increases (Cogburn et al., 1987; Elnagar et al., 2010), decreases (Bobek et al., 1980), or no change in plasma T_4 (Mack et al., 2013; Mitchell and Carlisle, 1992).

In many of the studies conducted on the immune function in poultry, heat stress has been shown to have an immunosuppressing effect on different parameters measured in laying hens and broilers (Lara and Rostagno, 2013). Bursa of fabricius, thymus, and spleen weights were all decreased in heat-stressed broilers in the study done by Quinteiro-Filho and colleagues (2010),

whereas reduced lymphoid organ weights were also reported in studies with broilers (Bartlett and Smith, 2003; Niu et al., 2009), suggesting a decrease in the function of these organs to aid in their immune response functions. Interestingly, white blood cell counts, antibody production, and lymphocyte activity were negatively impacted by high environmental conditions in laying hens (Mashaly et al., 2004). Immunoglobulin M (IgM) and Immunoglobulin G (IgG) antibody titer for primary and secondary immune responses, and the ability of macrophages to perform phagocytosis were also reported to be reduced under heat-stress conditions with broilers (Bartlett and Smith, 2003; Niu et al., 2009). The reduced ability of macrophages along with a reduction in macrophage basal and induced oxidative burst, have also been observed in heat-stressed broilers (Quinteiro-Filho et al., 2010; Quinteiro-Filho et al., 2012).

Even though poultry are under high ambient temperature stress conditions, levels of reactive oxygen species (ROS) have been shown to increase, which causes the bird to enter a state of oxidative stress. In this state, the bird will recruit heat-shock proteins (HSP) to protect itself from the detrimental effects of ROS and oxidative stress (Droge, 2002). In studies done in broilers and laying hens, HSP70 expression, a major protein in the stress response serving for protection and adaptation to stress, was upregulated in birds exposed to heat stress compared to birds under normal temperature conditions (Felver-Gant et al., 2012; Gu et al., 2012). On the cellular level, the structure and physiology of the cell, transcription impairment, RNA processing, translation, oxidative metabolism, and the cellular membrane structure and function are negatively impacted under heat stress conditions (Iwagami, 1996; Mage and de Kruijff, 1995). Elstner (1991) and Halliwell and colleagues (1992) have shown that heat stress conditions increase the amount of ROS which can be detrimental to lipids, proteins, carbohydrates, and DNA (Mates et al., 1999).

1.3 NEGATIVE IMPACTS OF HEAT STRESS ON POULTRY REPRODUCTIVE FUNCTION AND PRODUCTION

There have been many studies conducted to determine the effects of heat stress on broiler growth performance as well as on laying hen egg production. In 42-day-old broilers subjected to chronic heat stress, feed intake and body weight were decreased 16.4% and 32.6%, respectively; thereby negatively impacting feed-conversion ratio with an increase of 25.6% (Sohail et al., 2012). When broilers were subjected to two different heat stressors (31°C and 36°C), Quinteiro-Filho et al. (2010) reported that heat stress decreased feed consumption and body weight gain, with a more pronounced effect observed with the higher temperature degree (25 vs. 41% decrease in BWG at 31 and 36°C, respectively). Interestingly, the higher temperature decreased feed-conversion ratio and mortality, whereas the lower temperature had no impact on growth performance (Quinteiro-Filho et al., 2010). Mortality has been shown to increase in layer hens subjected to constant high temperature and humidity conditions over 5 wk of life (Mashaly et al., 2004), whereas broilers prior to marketing also had an increase in mortality when exposed to heat stress later in their life (Arjona et al., 1988). Additional studies reported a consistent, negative impact on growth performance in broilers subjected to heat stress conditions (Bartlett and Smith, 2003; Deeb and Cahaner, 2002; Ghazi et al., 2012; Niu et al., 2009); however, stocking density of broilers should also be considered as it may be a potential confounding factor for both broiler productivity, as well as the overall welfare of the bird (Estevez, 2007; Lara and Rostagno, 2013). It has also been reported that heat stress can have a negative effect on carcass composition by an increasing fat deposition in broiler carcasses (Baziz et al., 1996; Lu et al., 2007).

Consistent with broilers, heat stress has been reported to decrease the feed intake and body weight gain, and increase feed-conversion ratio were reported in heat-stressed layers; however, egg production and quality were also negatively impacted by heat stress conditions (Deng et al., 2012; Lin et al., 2004; Mashaly et al., 2004; Star et al., 2009). In particular, Deng et al. (2012) showed a decrease in feed intake, resulting in a decrease in egg production by about 28%, whereas Star and colleagues (2009) demonstrated similar results with the added decreases in average eggs weights by about 3%. Lin et al. (2004) reported a decrease in egg shell thickness and an increase in egg breakage in heat stress conditions, whereas both Ebeid et al. (2012) and Mack et al. (2013) reductions in egg weights, egg shell thickness, eggshell weight, and percentage of eggshell on the egg.

Reproductive function of poultry has also been found to be negatively impacted by heat stress conditions with sex-specific effects. When male White Leghorns and broiler breeders were subjected to heat stress, semen volume, concentration and proportion of live sperm cells to dead sperm cells, and the motility score of live sperm cells decreased with the color of the semen also being affected (Joshi et al., 1980; McDaniel et al., 1995; McDaniel et al., 2004). When observing the reproductive hormonal levels in the hypothalamus in female broiler breeders and laying hens, heat stress reduced systemic levels of luteinizing hormone, progesterone and functionality of the ovaries (Donoghue et al., 1989; Elnagar et al., 2010; Novero et al., 1991; Rozenboim et al., 2007).

In summary, the negative effects of heat stress on poultry behavior, immunological and physiological responses have been well studied and reported. These factors can pose detrimental effects on overall poultry production, such as broiler growth performance and laying hen egg production. Heat stress costs the poultry industry alone about \$128 to \$165 million annually (St-

Pierre et al., 2003). Therefore, much research effort has been directed to finding methods to prevent the negative impact of heat stress on poultry production to improve poultry sustainability.

1.4 METHODS IN PREVENTING HEAT STRESS IN POULTRY

Strategies for preventing heat stress in poultry have been well reviewed (Lin et al., 2006). These strategies can be summed up into three categories, including genetics, management, and nutrition. Feather mass can be reduced by 20 to 40% relative to body weight (Mérat, 1986) with the naked-neck gene being expressed as heterozygous or homozygous. The advantageous effect of this gene on broiler growth rate, meat yield, body weight, and feed efficiency in high environmental temperatures has been well documented (Cahaner et al., 1993; Hanzl and Somes, 1983; Patra et al., 2002), and it is speculated that the lower feather mass increases the effective surface of heat dissipation and increases the sensible heat loss from the neck under high environmental conditions (Yahaz et al., 1998). Selective breeding of breeders to produce heat tolerable offspring may be more applicable rather than utilizing a specific gene. With the selection of high growth rate and improved feed efficiency being top priorities in the modern poultry industry, poor heat tolerance is a consequence to this selection. Gonet et al. (2000) reported that three different female breeder lines with similar growth performance levels showed different thermal responses under a hot environment which, in turn, influenced the heat tolerance of the offspring, with offspring from one line having a lower body temperature.

Use of intermittent light and early heat conditioning are management strategies that can be used to assist in combating heat stress in poultry. Buyse and colleagues (1994) showed that broilers under a 1:3 h light-to-dark intermittent light program produced less heat early and later

in age compared to broilers under a continuous lighting program. Early heat conditioning of broiler chicks by exposing them to heat stress conditions for 24 hours between 3 and 5 days of age is stated to be one of the best methods of improving heat tolerance in broilers (Lin et al., 2006). Early heat conditioning in broiler chicks appeared to induce heat tolerance later in their lives prior to marketing without negatively impacting mortality unlike heat stress later in the life of the broilers (Arjona et al., 1988).

Finally, the use of nutrition (feed or water additives) can be used to aid poultry in coping with heat stress. Lin et al. (2006) reviewed the supplementation of amino acids and vitamins on ameliorating the negative effects of heat stress. Interestingly, the supplementation of amino acids presents an inconsistent picture. Mendes et al. (1997) demonstrated that an increase in lysine and arginine: lysine ratios failed to alter broiler growth performance under heat stress; yet, Brake et al. (1998) reported that increasing the arginine: lysine ratio improved growth performance under heat stress conditions. Supplementation of vitamins A and E have been shown to increase the egg production of laying hens under heat stress conditions (Lin et al., 2002; Kirunda et al., 2001). However, little, if any, published information exists concerning the use of phytogetic additives to mitigate the negative effects of heat stress on poultry.

1.5 OVERVIEW OF PHYTOGENIC ADDITIVES

Phytogetic feed additives are organic, plant-derived feed products (herbs, spices, essential oils) or compounds that have gained interest as alternative feeding strategies in animal agriculture production and scientific research. Phytogetic products have been used for their medicinal purposes for centuries throughout the world. The compositions of active ingredients in these additives vary widely depending on what part of the plant being used (seeds, leaf, stem,

root, and fruits), time of harvest, geographical origin, and, finally, the processing technique to get the finished product (Hashemi and Davoodi, 2010). The reason this class of feed additives gained such interest in animal agriculture is due to the ban on antibiotic growth promoters in the European Union and the United States, but also due to the positive effects they have on animal production, including improved growth performance, and increased palatability of feed (Hashemi and Davoodi, 2010). Certain additives have also shown improvements to animal health and wellbeing from their antimicrobial, antioxidant, anti-stress, gut microflora manipulation, and immune enhancement properties (Hashemi and Davoodi, 2010).

1.6 EFFECTS OF PHYTOGENICS ADDITIVES ON POULTRY PRODUCTION

The use of phytogetic feed additives to improve poultry production has been well reviewed (Hashemi and Davoodi, 2010; Windisch et al., 2008). In studies done with broilers, researchers have found that different phytogetic products and compounds increase BWG (Alçiçek et al., 2004; Guo et al., 2004; Hernández et al., 2004), decrease feed intake (Alçiçek et al., 2004; Cabuk et al., 2006), and improve feed conversion efficiency (Amad et al., 2001). Hernández et al. (2004) reported that dietary inclusion of extracts from several herbs, such as sage, thyme, and rosemary also improved feed digestibility, which led to improved BWG in their study. The results suggest these products increase the amount of nutrients being available to the bird from an increase in feed digestion along with additional nutrients from the additives which led to the decrease in feed intake (FI) as nutrient requirements were met with less feed.

Phytogenics have also been reported to have other beneficial effects, including antimicrobial and antioxidant activities that can improve gut health in broilers (Aksit et al., 2006; Bazargani et al., 2014; Botsoglou et al., 2002a, b; Olnood et al., 2015). The improvements in gut health is

stated to be the primary mode of action of phytogenic feed additives from beneficially affecting the ecosystem of gastrointestinal microbiota through controlling potential pathogens (Hashemi and Davoodi, 2010). Consequently, phytogenics relieve the poultry from immune defense stress during stressful situations and increase the intestinal availability of essential nutrients for absorption, thereby helping birds to grow better within the framework of their genetic potential (Hashemi and Davoodi, 2010).

1.7 CONNECTION BETWEEN HEAT STRESS, PHYTOGENICS, AND LIPID METABOLISM

“The liver is a highly metabolic tissue that plays a vital role in digestion (bile production), metabolism, immunity, and storage of nutrients, as well as detoxification. The chicken liver also serves as the main site for *de novo* fatty acid synthesis (lipogenesis) and fat storage. It has been shown that the liver is responsive and susceptible to heat stress (HS) (Lin et al., 2006; Tang et al., 2015), and that HS alters the hepatic lipid metabolism (Faylon et al., 2015; Dalvi et al., 2017; Jastrebski et al., 2017) and increases fat deposition in broiler chicken carcasses (Baziz et al., 1996; Lu et al., 2007).”

“Drinking *Morinda citrifolia* (Noni) juice has been shown to enhance hepatic antioxidant capacity, improve lipid homeostasis, and protect the liver from environmental and chemical stressors (Wang et al., 2008a, b; Chang et al., 2013; Lin et al., 2017). This medicinal plant has been widely used in human nutrition and health (Issell et al., 2009). Due to the recent concerns in the sub-therapeutic use of antibiotics in food animal industries, Noni fruits and leaves have also gained

considerable popularity in animal nutrition (Brooks et al., 2009; Sunder and Kundu, 2015; Sunder et al., 2015).”

In conclusion, HS has been shown to alter poultry behavior, and have many negative impacts on immune function, endocrine function, productivity, and fat deposition in carcasses. There are strategies, such as management, genetics, and nutrition, to ameliorate the negative effects of heat stress. Furthermore, phytogetic feed additives are a potentially-beneficial class of organic growth promotors in poultry production. The use of Noni products may be another possible strategy to use in poultry production to ameliorate the negative impact of HS on fat deposition in broiler carcasses but its mechanisms are not fully understood and require investigation.

1.8 OBJECTIVES

The objectives of my research during my master degree are to: 1) determine the effect of Noni supplementation on growth performance in heat-stressed broiler chickens; and 2) assess whether Noni supplementation modulate the lipogenic program in heat-stressed broilers.

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CHAPTER 2

Effect of *Morinda citrifolia* (Noni)-Enriched Diet on Hepatic Heat Shock Protein and Lipid Metabolism-Related Genes in Heat Stressed Broiler Chickens

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2.1 ABSTRACT

Heat stress (HS) has been reported to alter fat deposition in broilers, however the underlying molecular mechanisms are not well-defined; therefore, the objectives of the current study were to: (1) determine the effects of acute (2 h) and chronic (3 wk) HS on the expression of key molecular signatures involved in hepatic lipogenic and lipolytic programs; and (2) assess if diet supplementation with dried Noni medicinal plant (0.2% of the diet) modulates these effects. Broilers (n=480 males, 1 d) were randomly assigned to 12 environmental chambers, subjected to two environmental conditions (HS at 35°C vs. thermoneutral condition [TN] at 24°C) and fed two diets (control vs. Noni) in a 2 × 2 factorial design. Feed intake and body weights were recorded, and blood and liver samples were collected at 2 h and 3 wk post-heat exposure. HS depressed feed intake, reduced body weight, and up-regulated the hepatic expression of heat shock protein HSP60, HSP70, HSP90, as well as key lipogenic proteins fatty acid synthase (FASN), acetyl co-A carboxylase alpha (ACC α), and ATP citrate lyase (ACLY). HS down-regulated the hepatic expression of lipoprotein lipase (LPL) and hepatic triacylglycerol lipase (LIPC), but up-regulated adipose triglyceride lipase (ATGL). Although it did not affect growth performance, Noni supplementation regulated the hepatic expression of lipogenic proteins in a time- and gene-specific manner. Prior to HS, Noni increased ACLY and FASN in the acute and chronic experimental conditions, respectively. During acute HS, Noni increased ACC α , but reduced FASN and ACLY expression. Under chronic HS, Noni up-regulated ACC α and FASN but it down-regulated ACLY. In chicken hepatocyte cells, HS exposure of cells to 45°C for 2 hours down-regulated ACC α , FASN, and ACLY compared to cells maintained at 37°C. Treatment with quercetin, one bioactive ingredient in Noni, up-regulated the expression of ACC α , FASN, and ACLY under TN conditions, but it appeared to down-regulate ACC α and

increase ACLY levels under HS exposure. In conclusion, our findings indicate that HS induces hepatic lipogenesis in chickens and this effect is probably mediated via HSPs. The modulation of hepatic heat-shock protein HSP expression suggests that Noni might also be involved in modulating the stress response in chicken liver.

Keywords: heat stress, lipogenesis, lipolysis, noni, quercetin, chicken, liver

2.2 INTRODUCTION

Driven by economic demands and consumer preference for low-fat and high-protein sources, commercial broiler chickens have been intensively selected for high growth rate and high breast yield (Griffin and Goddard, 1994). Poultry meat and egg production has seen the largest increase during past decades and supports the livelihoods and food security of billions of people worldwide. However, this spectacular progress has also been accompanied by several undesirable changes, including hyperphagia (Denbow, 1994), metabolic disorders (Julian, 1998; Velleman, 2015), and hypersensitivity to high environmental temperature due to high metabolic activity and lack of sweat glands (Settar et al., 1999; Huang et al., 2015; Velleman, 2015).

Heat stress (HS) is detrimental to poultry production from its strong adverse effects on feed intake, growth, meat yield, welfare, and mortality (Dale and Fuller, 1980; Cahaner and Leenstra, 1992; Deeb and Cahaner, 2002; Deeb et al., 2002). Heat stress results in an estimated total annual economic loss to the U.S. poultry industry of more than \$100 million and, therefore, represents a serious financial burden (St-Pierre et al., 2003). At the animal level, its adverse effects can range from discomfort (mild stress) to multiple organ damage and, under extreme conditions (sever stress), leads to mortality following spiraling

hyperthermia. At the cellular level, HS induces many alterations, including, protein misfolding and aggregation (Ruan et al., 2017), cell cycle arrest (Trotter et al., 2001), redox state (Keyse and Emslie, 1992), and transcription modulation (Sanchez et al., 1994). Depending on the type, severity, and duration of the stress, cells can develop highly efficient stress response and protein quality control systems to ensure their survival or activate stress signaling cascades that proceed into cell-death pathways. These responses are controlled by a complex molecular network that is still not completely defined particularly in avian species. At the molecular level, a general rapid response to HS is increased synthesis of heat shock proteins (HSPs). Based on their monomeric molecular size, these ubiquitously expressed chaperones are classified into about six families, with HSP70 and HSP90 being the most extensively studied. Besides their classical roles as molecular chaperones and housekeepers (folding/unfolding, assembly/disassembly), HSPs are now understood to play a pivotal role in many cellular processes including transport and trafficking, protein degradation, and cell signaling (Drew et al., 2014). In turn, these cellular alterations induced by HS can lead to various neuroendocrine, physiological, and immunological adaptations including modulation of lipid and glucose metabolism (Sanz Fernandez et al., 2015; Victoria Sanz Fernandez et al., 2015).

The liver is a highly metabolic tissue that plays a vital role in digestion (bile production), metabolism, immunity, and storage of nutrients as well as detoxification. It has been shown that the liver is responsive and susceptible to HS (Lin et al., 2006; Tang et al., 2015) and that HS alters the hepatic lipid metabolism (Faylon et al., 2015; Dalvi et al., 2017; Jastrebski et al., 2017). Drinking *Morinda citrifolia* (Noni) juice has been shown to enhance hepatic antioxidant capacity, improve lipid homeostasis, and protect the liver from

environmental and chemical stressors (Wang et al., 2008a, b; Chang et al., 2013; Lin et al., 2017). The botanical name of this *Rubiaceae* plant is originally derived from the two Latin words “morus” ascribing to mulberry, and “indicus” imputing to Indian. It is known as Noni in Hawaii, *mulberry* in India, *mengkudu* in Malaysia, *nhaut* in Southeast Asia, *painkiller bush* or *cheese fruit* in Caribbean, *Canary Wood* in Australia, and many other names in different countries (for review see Potterat and Hamburger, 2007). This medicinal plant has been widely used in human nutrition and health (Issell et al., 2009). Due to the recent concerns in the use of sub-therapeutic doses of antibiotics in food animal industries, Noni fruits and leaves have gained considerable popularity in animal nutrition (Brooks et al., 2009; Sunder and Kundu, 2015; Sunder et al., 2015). However, the effect of Noni on chicken liver metabolism under control and HS conditions is not known. As chicken liver is the main site for *de novo* fatty acid synthesis (lipogenesis) and fat storage, and as there is a subtle balance between hepatic lipogenesis and lipolysis, we aimed to determine in the present study the effects of Noni-enriched diet on growth performance, circulating metabolite and hormone levels, as well as on the expression of hepatic lipogenesis- and lipolysis-related genes, in broiler chickens exposed to acute and chronic HS.

2.3 MATERIALS AND METHODS

2.3.1 Animals

One-day-old Cobb 500 by-product male chicks (480 total) were randomly assigned into 12 environmentally controlled chambers for rearing. Each chamber was composed of 2 pens (20 bird/pen) with their own feeder and water line. Each pen was given one of two diets: starter corn-soy based diet (Control; 3.9 Mcal ME Kg⁻¹ and 21% Crude Protein), or

control diet supplemented with 0.2% dried Noni plant (Noni; 3.9 Mcal ME Kg⁻¹, 19.8% Crude Protein), a commonly incorporated levels by poultry producers in tropical regions (Sunder and Kundu, 2016). Chicks were given *ad libitum* access to feed and clean water. The ambient temperature was reduced gradually from 32 to 24°C with relative humidity at 55 ± 5% for 21 days. On d 22, birds were allocated into two environmental conditions (thermoneutral [TN] at 24°C vs. heat stress [HS] at 35°C for 2 h or 3 wk) and received two finisher diets (Control, 3.9 Mcal ME kg⁻¹ and 16.4% CP vs. Noni, 3.9 Mcal ME kg⁻¹ and 16.8% CP) in a 2 × 2 factorial design. The day before the experiment, the chickens were equipped with a Thermochron temperature logger (iButton, DS1922L, Maxim, CA) by the device being placed into the mouth and massaged past the crop for continuous monitoring of core body temperature. The environmental temperature and relative humidity (RH) were also continuously recorded in each chamber. Birds were humanely euthanized by cervical dislocation after acute (2 h) and chronic (3 wk) HS. Blood samples were collected aseptically from wing veins using vacutainers with PST gel and lithium heparin (BD, NJ) at each time point and plasma was separated after centrifugation (1,500 x g for 10 min at 4°C) and stored at -20°C for later analyses of circulating metabolites. Liver samples (*n* = 8/group per time point) were quickly isolated, snap frozen in liquid nitrogen, and stored at -80°C for subsequent gene and protein expression analyses. All experimental procedures involving animals used in this study were conducted in accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health and the protocol was reviewed and approved by the University of Arkansas Animal Care and Use Committee under protocol 3 16084.

2.3.2 Cell Culture

Spontaneous immortalized chicken embryo liver cells (sim- CEL, Piekarski et al., 2014) were grown in Waymouth's media (Life Technology, Waltham, MA) supplemented with 10% fetal bovine serum (Life Technologies, Waltham, MA), and 1% penicillin/streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. At 80–90% confluency, cells were exposed to two environmental conditions (45°C vs. 37°C) and two treatments (50 µM quercetin, QCT vs. control) for 4 h. The dose of QCT was selected based on pilot and previous studies (Dok-Go et al., 2003; Martirosyan et al., 2016; Wang et al., 2017)

2.3.3 RNA Isolation, Reverse Transcription (RT), and Quantitative Real-Time PCR

Total RNA isolation from liver samples and sim-CEL cells was performed using Trizol reagent (ThermoFisher Scientific, Rockford, IL) according to manufacturer's recommendations. RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 Micro-Volume Plate using Synergy HT multi-mode micro plate reader (BioTek, Winooski, VT). The RNA samples were DNase treated and 1 µg RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) as we previously described (Rajaei-Sharifabadi H. et al., 2017). Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) was performed as described previously (Rajaei-Sharifabadi H. et al., 2017) using 5 µL of 10X diluted cDNA, 0.5 µM of each forward and reverse specific primer, and SYBR Green Master Mix (ThermoFisher Scientific, Rockford, IL) in a total 20 µL reaction. Oligonucleotide primers used for

chicken ATP-citrate lyase (ACLY), acetyl-CoA carboxylase alpha (ACC α), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD-1), malic enzyme (ME), sterol regulatory element-binding protein 1 and 2 (SREBP-1/2), SREBP Cleavage-Activating Protein (SCAP), insulin induced gene 2 (INSIG2), lipoprotein lipase (LPL), hepatic triglyceride lipase (LIPC), adipose triglyceride lipase (ATGL), fatty acid translocase (CD36), peroxisome proliferator- activated receptor alpha and gamma (PPAR α/γ), and ribosomal 18S as housekeeping gene were summarized in Table 1. The qPCR cycling conditions were the same as described in Lassiter et al. (2015). Relative expression of target genes was determined by the $2^{-L'L'Ct}$ method (Schmittgen and Livak, 2008) and the group with control diet under thermoneutral condition was used as calibrator.

2.3.4 Protein Isolation and Western Blot Analysis

Liver tissues and sim-CEL cells were homogenized in lysis buffer as previously described by Lassiter et al. (2015). Total protein concentrations were determined using Synergy HT multi-mode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Protein samples (30 μ g for cells, 80 μ g for tissues) were run on NuPAGE 4–12% Bis-Tris Gels (Life Technologies, Waltham, MA). The transferred membranes were blocked for 1 h at room temperature, and incubated with primary antibodies (diluted 1:1000) overnight at 4°C. The following polyclonal antibodies were used: rabbit anti-FASN (Novus Biologicals, Littleton, CO), rabbit anti-ACC, rabbit anti phospho-ACC (Cell Signaling Technologies, Danvers, MA), rabbit anti- ACLY (LSBio, Seattle, WA), mouse anti-HSP70 (ThermoFisher Scientific, Waltham, MA), rabbit anti-HSP90 (Thermo Fisher Scientific, Waltham, MA), goat

polyclonal anti- HSP60 (Santa Cruz Biotechnology, Dallas, TX), and rabbit anti- GAPDH as housekeeping protein (Santa Cruz Biotechnology, Dallas, TX). The secondary HRP conjugated antibody, at a dilution of 1:5000, was used for 1 h at room temperature. Pre-stained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (BioRad, Hercules, CA). The signal was visualized by enhanced chemiluminescence (ECL plus; GE Healthcare Bio-Sciences, Buckinghamshire, UK) and captured by FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0, 1993–2011, Proteinsimple, Santa Clara, CA).

2.3.5 Immunofluorescence Staining

Immunofluorescence staining was performed as previously described by Dridi et al. (2012). Briefly, cells were grown in chamber slides and fixed with methanol for 20 min at -20°C . Cells were blocked with serum-free protein block (Dako, Carpinteria, CA) for 1 h at room temperature, and then incubated with primary antibodies (1:200, in Antibody Diluent, Dako, Carpinteria, CA) overnight at 4°C . The signal was visualized with DyLight 488- or 590-conjugated secondary antibody (Thermo Fisher Scientific, Grand Island, NY). Slides were cover slipped with a Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and images were obtained and analyzed using Zeiss Imager M2 and AxioVision software (Carl Zeiss Microscopy).

2.3.6 Plasma Metabolite and Hormone Measurement

Plasma glucose, triglyceride, cholesterol, lactate dehydrogenase (LDH), and uric acid levels were measured using an automated spectrophotometer as described by Nguyen et al.

(2015). Plasma levels of total 3, 5, 3'-triiodothyronine (T₃) and thyroxine (T₄) were determined using coated tube radioimmunoassay kits (MP Biomedicals, Solon, OH) as described by Rajaei-Sharifabadi et al. (2017).

2.3.7 Statistical Analysis

Growth performance (feed intake and body weight) data were analyzed by two-way repeated measures ANOVA. The rest of the data from both *in vivo* and *in vitro* experiments were analyzed by two-way ANOVA with diet (Control vs. Noni or quercetin [QCT]) and ambient temperature (TN vs. HS) as the main effects. If ANOVA revealed significant effects the means were compared by Tukey's multiple comparison test using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, CA). Differences were considered significant at $P < 0.05$.

2.4 RESULTS

2.4.1 Growth Performance

As depicted in Figure 1, there was no difference among all groups in daily individual or cumulative feed intake (Figures 1A, B), as well as in weekly BW and BW gain during the first 3 wk of the experiment (Figures 1C, D). However, shortly after HS exposure (2 h), core body temperature was significantly increased by $\sim 1^{\circ}\text{C}$ in Control group but Noni supplementation not only delayed this increase but also attenuated it (Rajaei-Sharifabadi et al., 2017). During the acute HS exposure, feed intake was significantly reduced by 10% in Control-fed diet and 13% in Noni-fed broilers compared to their counterparts maintained under TN conditions (Rajaei-Sharifabadi L. E. H. et al., 2017). This reduction in feed intake

was increased over the extended period of HS to reach 28 and 29% after 3 wk of HS in Control diet- and Noni-fed groups, respectively, compared to their counterparts maintained under TN conditions (Figures 1A, B). This, in turn, resulted in a significant reduction in BW by 38% in control diet and 40% in Noni-fed heat stressed groups compared to their TN counterparts (Figures 1C, D).

2.4.2 Metabolites and Thyroid Hormone

Acute HS did not affect the plasma levels of all measured parameters (Rajaei-Sharifabadi et al., 2017); however, chronic HS significantly increased plasma glucose and uric acid levels (Table 2), and significantly decreased plasma T₃ and T₄ concentrations (Table 2). Plasma triglyceride, cholesterol, and LDH levels were not affected by HS, (Table 2). Noni supplementation did not elicit any changes to these parameters (Table 2).

2.4.3 Heat Stress and Noni Modulate Hepatic HSP Expressions in a Time-Specific Manner

As shown in Figure 2, the hepatic expression of HSP60, HSP70, and HSP90 was significantly up-regulated after acute, but not after chronic, heat stress (Figures 2A–H). During the acute experimental conditions and in comparison with the Control diet, Noni supplementation did not affect the expression of all tested HSPs in the liver of TN group (Figures 2A–D), but Noni it did down-regulate that of HSP90 in the HS group (Figure 2D). Under the chronic experimental conditions, Noni supplementation increased the hepatic expression of HSP60 in both TN and HS groups (Figures 2E, F) and that of HSP90 in the TN group only (Figure 2H). The hepatic expression of HSP70 remained unchanged between the control and Noni groups under both environmental (TN and HS)

conditions (Figures 2E, G).

2.4.4 Heat Stress and Noni Supplementation Differentially Regulates the Hepatic Expression of Lipogenic Genes and Proteins

Under TN conditions and prior to HS, Noni supplementation significantly increased the protein levels of hepatic ACLY, but not that of ACC α and FASN compared to the Control diet-fed group (Figures 3A–D). However, the mRNA abundances of these genes remained unchanged (Figures 3E–I). During acute HS, Noni supplementation significantly increased the protein levels of hepatic ACC α , but reduced that of FASN, and ACLY, as well as the gene expression of hepatic ME and SCD-1, compared to the Control diet-fed group (Figures 3A–I). Interestingly, when all groups were pooled together, acute HS significantly up-regulated the hepatic expression of ACC α , ACLY, and FASN, but it down-regulated the hepatic expression of all tested genes compared to TN group (Figures 3E–I). The induction of FASN and ACLY protein was more noticeable in the heat-stressed birds fed with Control diet, resulting in a significant interaction ($P = 0.02$ for FASN, $P = 0.003$ for ACLY) between environment and diet (Figures 3B–D).

Similarly, during the chronic experimental conditions and prior to HS, Noni supplementation did not affect the mRNA abundance of all tested genes (Figures 4E–I) as well as that of hepatic ACC α , and ACLY protein (Figures 4A, B, D). However, the expression of FASN protein was significantly up-regulated in Noni-fed compared to Control-fed broilers (Figure 4C). During chronic HS, Noni supplementation significantly increased the protein levels of hepatic ACC α and FASN, but decreased that of ACLY

compared to Control-fed group (Figures 4B–D). The mRNA levels of all tested genes did not differ between Noni- and control diet-fed groups reared under chronic HS (Figures 4E–I). Interestingly, when all groups were pooled together, chronic HS significantly down-regulated the hepatic expression of all tested genes (Figures 4A–I).

2.4.5 Heat Stress Modulates the Hepatic Expression of Lipogenesis-Related Transcription Factors

As shown in Figure 5, both acute and chronic HS significantly down-regulated the hepatic expression of SREBP-1 and SCAP gene (Figures 5A, C, D, F); however, it did not affect that of SREBP-2 (Figures 5B, E), INSIG-2, PPAR α , or PPAR γ (Table 3). Noni supplementation did not elicit any change to the expression of these transcription factors under either environmental (TN and HS) conditions (Figures 5A–F).

2.4.6 Heat Stress Modulates the Hepatic Expression of Lipolytic Genes

In comparison with the Control diet, Noni supplementation did not affect the hepatic expression of LPL, ATGL, LIPC, and CD36 genes under the TN environment for both acute and chronic experimental conditions (Figures 6A–H). Under HS exposure, however, Noni significantly decreased the mRNA levels of hepatic ATGL in both acute and chronic experimental conditions (Figures 6B, F). When all groups were pooled together, both acute and chronic HS down-regulated the hepatic expression of LIPC and CD36 gene compared to TN conditions (Figures 6C, D, G, H). However, the hepatic LPL mRNA levels were significantly decreased only after chronic but not acute HS (Figures 6A, E). Interestingly, both acute and chronic HS up-regulated the hepatic expression of ATGL

gene compared to TN condition (Figures 6B, F) and this increase was more pronounced in the Control-fed group.

2.4.7 QCT, the Active Ingredient of Noni, Modulates Lipogenic Gene Expression in Sim-CEL Cells

Heat stress significantly down-regulated the expression of lipogenic genes at mRNA and protein levels (Figures 7A–E, Table 4), as well as their related transcription factors SREBP-1, SREBP- 2, and SCAP (Figure 7F, Table 4). However, HS did not alter the expression of LPL, ATGL, and LIPC (Table 4). The up-regulation of both HSP60 and HSP70, demonstrated by immunofluorescence staining (Figure 7E), indicated that sim-CEL cells were undergoing stress induced by heat load. Quercetin treatment significantly increased ACC α , FASN, and ACLY protein levels in control (37°C) environment only, however it did significantly reduced the phosphorylated levels of ACC α at Serine 79 site and increased that of ACLY protein levels under HS conditions (Figures 7B,D).

2.5 DISCUSSION

Heat stress adversely affects poultry production through depressed feed intake and growth as shown in many previous reports (Dale and Fuller, 1980; Cahaner and Leenstra, 1992; Leenstra and Cahaner, 1992; Deeb et al., 2002) and confirmed by the present study. These effects are mediated by numerous molecular, neuroendocrine, and physiological processes including nutrient partitioning and lipid metabolism that are not completely defined, at least, in avian species.

As commercial broilers are fed lipid-poor diets (<10%), the majority of the

accumulated fat is derived from the liver which is the main site for lipogenesis (Goodridge and Ball, 1967; Leveille et al., 1968; Yeh and Leveille, 1971). Indeed, the avian liver is responsible for more than 90% of *de novo* fatty acid synthesis and is also a site of lipid storage in birds (Goodridge and Ball, 1967; Leveille et al., 1968; Yeh and Leveille, 1971). The increased levels of hepatic FASN and ACLY proteins, rate-limiting enzymes in *de novo* fatty acid synthesis, indicate that HS-induce hepatic lipogenesis in chickens which corroborates a previous study reported by Geraert et al. (1996) and Jastrebski et al. (2017). However, the upregulation of p-ACC α ^{Ser79} by HS, which indicates that the enzyme is less active, is intriguing. Although it is not clear at this time-point why ACC α is down-regulated, it is possible that a compensatory increase in another ACC isoform, such as ACC β (ACC2), is involved. Furthermore, as its regulation is complex, it is possible that the down-regulation of ACC α might be due to a negative feedback loop. For instance, the ACC α decrease could result from an increase in glucagon and/or catecholamine levels, which has been reported to be induced by stressful stimuli including HS and feed deprivation (Bloom et al., 1973; Iguchi et al., 2012). Although we didn't determine the plasma glucagon levels in the present study, the significant increase in circulating glucose concentration (23%) by HS, despite the significant reduction in feed intake, supports the above mentioned hypothesis. In fact, during HS along with reduced energy intake, glucagon plays a key role in maintaining glucose homeostasis via stimulating hepatic glycogenolysis and gluconeogenesis (Exton and Park, 1968; Garrison and Haynes, 1973; Watford, 1985), which is evidenced in our study by the upregulation of hepatic phosphoenolpyruvate carboxykinase (PEPCK), glycogene phosphorylase (GLGP), and fructose 2,6- biphosphatase 4 (PFKFB4) (data not shown). An additional possibility is that HS activates the energy sensor AMPK α 1/2 (data

not shown) and AMPK has been shown to phosphorylate ACC α ^{Ser79} (Park et al., 2002). We should note that we measured only one ACC phosphorylation site (Serine79); however, there are several other sites including Serine1200 and Serine1215 (Brownsey et al., 2006) that might be differently regulated and phosphorylated by HS.

Heat stress down-regulated the hepatic expression of all tested lipogenic genes, as well as their related transcription factors, which is different from previous integrated transcriptomic studies reported in rodents and chickens (Bhusari et al., 2007; Jastrebski et al., 2017). This discrepancy might due to the technique used and the choice of the normalization (housekeeping) control (real- time qPCR in our study vs. RNAseq or microarray in previous studies) and/or experimental conditions (species, age, duration, intensity, and/or severity of HS, diet composition, etc.). The absence of correlation between gene and protein expression in our study indicates that the transcription and translation of these target markers are differentially regulated by HS which is not surprising. It is plausible that under our experimental stress conditions, the pool of already synthesized lipogenic mRNA might be efficiently translated with a quick turnover or degradation; however its protein product accumulation increases and remain in the cellular pool due to its high half-life (Schwanhausser et al., 2011). The protein stability may also increase by post-translational modification like acetylation or glycosylation. An additional possibility is that mRNA translation can be upregulated by RNA-binding proteins and/or down- regulation of micro (mi) RNA targeting that mRNA.

Interestingly, our study showed that the lipolytic pathway is also expressed in the chicken liver which is a considerable site for lipid storage and secretion (Hermier, 1997). Heat stress seems to regulate the expression of hepatic lipolysis-related genes in a time-

specific manner. For instance, LPL mRNA abundance was not affected by acute HS, but it was decreased by chronic HS. Both acute and chronic HS up-regulate hepatic ATGL, but decreased LIPC expression which may explain the unchanged levels of plasma triglycerides. As LPL has other functions beside its hydrolytic activity, it is conceivable that HS might affect the maturation of plasma HDL (Strauss et al., 2001), uptake of lipoprotein-associated lipids and vitamins (Rinninger et al., 1998, 2001; Goti et al., 2002), and/or degradation of lipoprotein (Heeren et al., 2002) which warrant further investigations. Of particular interest, a novel function of LPL in the regulation of appetite and energy balance regulation has recently been postulated (Wang et al., 2011). Thus, it is possible that the reduced feed intake and body weight induced by HS in this study might be mediated via down-regulation of LPL expression and this merit further in depth studies.

As expected, and in agreement with previous studies (Xie et al., 2014; Wang et al., 2015), HSPs (HSP60, HSP70, and HSP90) were all up-regulated by acute HS, but remained unchanged after longer HS exposure compared to TN conditions. This later observation is not surprising and indicates that the birds become acclimated to chronic HS which, in turn, results in no further increase in cellular HSPs (Givisiez et al., 1999). Noni supplementation reduced the expression of HSP90, but not that of HSP60 and HSP70-induced by acute HS suggesting that the regulation of HSP by Noni is family (molecular weight)-dependent. However, contrary to our expectation, Noni-enriched diet up-regulated the hepatic expression of HSP60 and HSP90 in broilers maintained under TN conditions, as well as that of HSP60 in chronic heat-stressed broilers without affecting the expression of HSP70. The down regulation of hepatic HSP90 during acute HS supports the notion that Noni might alleviate stress induced by heat load and this has been substantiated by the reduction of core

body temperature-induced by HS (data not shown). However, the up-regulation of both HSP60 and HSP90 during the chronic but not acute TN conditions is intriguing. It has been reported that Noni juice induced stress and reduced body weight in mice (Muto et al., 2010). This is an unlikely scenario in our experimental conditions because birds fed Control or Noni diet showed similar feed intake, body weight, and body weight gain without any sign of discomfort or stress (physical activity and behavior). In addition, necropsy analysis of all major organs in both groups revealed no pathology. Although the physiological meaning and biological determinants are not known at this time point, it is foreseeable that age (3 vs. 6 weeks in acute vs. chronic conditions, respectively) might impact the effect of Noni on the hepatic HSP60 expression. Age-dependent differences in liver gene expression have been recently reported (Uno et al., 2017), and HSP60 levels have been found to progressively decline with increasing age in humans (Rea et al., 2001). Besides their role in stress response, HSPs also act as molecular chaperones under normal physiological conditions where they carry out such essential functions as protein translocation, folding, and assembly (Hendrick and Hartl, 1993). It is, therefore, possible that HSP might mediate the effect of Noni in regulating these cellular processes; however, owing to the gap in avian HSP biology, experimental evidence using mechanistic and functional studies supporting these possibilities needs to be provided.

The lipogenic proteins followed a similar hepatic expression pattern as HSPs across our experimental conditions. For instance, HSPs (HSP70 and HSP90), FASN, and ACLY showed similar expression patterns in the liver of acute heat-stressed broilers. Similarly, the hepatic expression of HSP60 and FASN follow the same trend under chronic HS conditions. As HSPs are now understood to be involved in many cellular processes

from transcription regulation to cell death signaling (Rutherford and Zuker, 1994), our data suggest that HS might modulate the expression of lipogenic protein through HSPs. Tang and co-workers (2016) have shown that HSP60 silencing directly altered the expression of FASN and ACC α in glioblastoma cells. Fatty acid synthase has also been recently identified as a bona fide HSP90 client (Fierro-Monti et al., 2013). Zhang et al. (2012) reported that HSP90 binds to AMPK and regulates ACC α expression.

To gain a better understanding of HS responses, we next evaluated the effect of HS alone or in combination with QCT on the same parameters by using chicken hepatocyte cell culture. We chose QCT because it is a highly bioactive ingredient of Noni, with an average concentration of 7.4 mg/g (CP Foods, personal communication; Kampkotter et al., 2007). Although the optimal temperature of cell culture (37°C) and chicken body temperature (~41°C) are quite different, HS induced the expression of HSP60 and HSP70 in sim-CEL cells comparably to chicken liver tissues. However, it down-regulated the expression of lipogenic (ACC α , FASN, and ACLY) proteins *in vitro* which is opposite to that in animals. This indicates, firstly, that HS may directly alter the expression of hepatic lipogenic genes independent of the reduction of feed intake. Secondly, the differential expression profile of hepatic lipogenic proteins between the *in vivo* and *in vitro* HS studies might be due to the involvement of other organs, such as the brain (central neural pathways for thermoregulation; Morrison and Nakamura, 2011) and the hormonal milieu. In fact, we have previously shown that HS altered the hepatic expression of leptin (Dridi et al., 2008) which is a key regulator of lipogenic genes (Dridi et al., 2005). Additionally, HS has been shown to dysregulate several other appetite-related hormones, including ghrelin, thyroid hormone, and adipocytokines (i.e.,

adiponectin), and these hormones orchestrate the regulation of hepatic lipogenic genes (Yin et al., 2015; Tao et al., 2006; Buyse et al., 2009). The QCT treatment induces the expression of lipogenic protein in sim-CEL cells maintained under both control and short (4 h) HS exposure, indicating that a short-term control mechanism of lipogenesis by QCT is effective.

Moreover, the prompt responsiveness of cultured hepatocytes to QCT suggests that the QCT effect may be direct rather than secondary. The discord of QCT effects between chicken and mammalian hepatocytes is striking. It has been reported that QCT administration inhibits FASN expression in human HepG2 (Li et al., 2013; Zhao et al., 2014), rat H4IIE (Zhou et al., 2014), and rat BRL-3A cells (Wang et al., 2013). This discrepancy might be due to dose and/or species-specific regulation of lipogenic genes by QCT (Dridi et al., 2006). In fact, QCT has been shown to have a dose-dependent biphasic effect on mammalian cell proliferation (van der Woude et al., 2003) and on CYP1A2 expression (Kang et al., 2004).

In conclusion (Figure 8), HS induces the hepatic expression of HSPs and stimulates *de novo* fatty acid synthesis in broilers which may explain the increased fat deposition observed during hot season (Geraert et al., 1996). In this study, Noni supplementation did not ameliorate feed intake and growth depression under HS conditions, but it did modulate the hepatic expression of HSPs in a time-specific manner suggesting a potential role in stress response that merit further investigations. Possibly, the lack of effect of Noni in the present study could be attributed to clean litter and low relative humidity (20%) in the experimental environmental chambers. A less pristine

environment such as that found in commercial poultry houses may be required to demonstrate potential benefits of Noni.

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2.7 APPENDIX

Table 1. Oligonucleotide real-time qPCR primers

Gene	Accession number^a	Primer sequence (5' → 3')	Orientation	Product size (bp)
<i>ACCα</i>	NM_205505	CAGGTATCGCATCACTATAGGTAACAA	Forward	74
		GTGAGCGCAGAATAGAAGGATCA	Reverse	
<i>ACLY</i>	NM_001030540	CTTTTAAGGGCATTGTTAGAGCAAT	Forward	65
		CCTCACCTCGTGCTCTTTCAG	Reverse	
<i>FASN</i>	J03860	ACTGTGGGCTCCAAATCTTCA	Forward	70
		CAAGGAGCCATCGTGTAAGC	Reverse	
<i>SCD-1</i>	NM_204890	CAATGCCACCTGGCTAGTGA	Forward	52
		CGGCCGATTGCCAAAC	Reverse	
<i>ME</i>	AF408407	AGATGAAGCTGTCAAAGGATATGG	Forward	62
		CACGCCCTTCACTATCGA	Reverse	
<i>SREBP-1</i>	AY029224	CATCCATCAACGACAAGATCGT	Forward	82
		CTCAGGATCGCCGACTTGTT	Reverse	
<i>SREBP-2</i>	AJ414379	GCCTCTGATTCGGGATCACA	Forward	63
		GCTTCTGGCTCTGAATCAATG	Reverse	
<i>SCAP</i>	XM_001231539	TGGCCCAGAGACTCATCATG	Forward	67
		GCAGGATCCGTATAAACCAGGAT	Reverse	
<i>INSIG2</i>	NM_001030966	CAGCGCTAAAGTGGATTTTGC	Forward	65
		CAATTGACAGGGCTGCTAACG	Reverse	

Table 1. (Cont.) Oligonucleotide real-time qPCR primers

Gene	Accession number^a	Primer sequence (5' → 3')	Orientation	Product size (bp)
<i>PPARα</i>	AF163809	CAAACCAACCATCCTGACGAT	Forward	64
		GGAGGTCAGCCATTTTTTGA	Reverse	
<i>PPARγ</i>	NM_001001460	CACTGCAGGAACAGAACAAAGAA	Forward	67
		TCCACAGAGCGAAACTGACATC	Reverse	
<i>LPL</i>	NM_205282	GACAGCTTGGCACAGTGCAA	Forward	62
		CACCCATGGATCACCAAAA	Reverse	
<i>LIPC</i>	XM_425067	GGTCTCAGTGTTGGCATCAAAC	Forward	72
		AGGCTGAAAGGTGCCTCCAT	Reverse	
<i>ATGL</i>	EU240627	GCCTCTGCGTAGGCCATGT	Forward	60
		GCAGCCGGCGAAGGA	Reverse	
<i>CD36</i>	NM_001030731	ACTGCGCTTCTTCTCCTCTGA	Forward	68
		TCACGGTCTTACTGGTCTGGTAAA	Reverse	
<i>18S</i>	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	

^a Accession number refer to Genbank (NCBI). ACC α , acetyl-CoA carboxylase alpha; ACLY, ATP citrate lyase; ATGL, adipose triglyceride lipase; CD36, fatty acid translocase; FASN, fatty acid synthase; INSIG2, insulin-induced gene 2; LIPC, hepatic triacylglycerol lipase; LPL, lipoprotein lipase; ME, malic enzyme; PPAR α/δ , peroxisome proliferator activated receptor α/δ ; SCAP, sterol regulatory element-binding protein cleavage-activating protein; SCD-1, stearoyl-CoA desaturase 1; SREBP-1/2, sterol regulatory element-binding protein 1/2

Table 2. Effects of Noni supplementation and HS on circulating metabolite and hormone levels in broiler chickens.

Parameters ³	Experimental groups ¹				P value ²		
	TN-C	TN-N	HS-C	HS-N	D. effect	E. effect	Interaction
Chronic HS							
<i>Glc (mg/dL)</i>	272.75 ± 4.7 ^a	274.5 ± 10.2 ^a	330.37 ± 11 ^b	343.85 ± 10.7 ^b	0.52	<0.0001	0.62
<i>Chol (mg/dL)</i>	144.50 ± 11 ^a	145.5 ± 1.8 ^a	188.37 ± 16 ^a	192.85 ± 19 ^a	0.88	0.02	0.92
<i>TG (mg/dL)</i>	35.50 ± 2.7 ^a	51 ± 5.1 ^a	42.6 ± 7 ^a	52 ± 17 ^a	0.36	0.76	0.82
<i>LDH (U/L)</i>	1795 ± 196 ^a	4945 ± 1881 ^a	2622.5 ± 870 ^a	4562.8 ± 2530 ^a	0.22	0.91	0.76
<i>UA (U/L)</i>	5.02 ± 1.1 ^a	5.25 ± 0.4 ^a	9.96 ± 1.3 ^{ab}	14.8 ± 1.9 ^b	0.15	0.0004	0.19
<i>T₃ (ng/mL)</i>	1.65 ± 0.2 ^a	1.53 ± 0.06 ^a	0.88 ± 0.07 ^b	0.818 ± 0.1 ^b	0.39	<0.0001	0.81
<i>T₄ (ng/mL)</i>	14.6 ± 2 ^a	12.03 ± 0.5 ^{ab}	9.73 ± 0.8 ^c	9.60 ± 0.8 ^{bc}	0.23	0.003	0.27

¹TN-C, thermoneutral-control diet; TN-N, thermoneutral-Noni diet; HS-C, heat stress-control diet; HS-N, heat stress-Noni diet.

²D, diet; E, environment. Different letters indicate significant difference at $P < 0.05$

³Glc, glucose; Chol, cholesterol; TG, triglycerides; LDH, lactate dehydrogenase; UA, uric acid; T₃, triiodothyronine; T₄, thyroxine.

Table 3. Effects of Noni supplementation on hepatic expression of *INSIG2*, *PPAR α* and *PPAR γ* genes in heat stressed broiler chickens

Parameters ³	Experimental groups ¹				P value ²		
	TN-C	TN-N	HS-C	HS-N	D. effect	E. effect	Interaction
Acute HS							
<i>INSIG2</i>	1 ± 0.2	0.60 ± 0.06	0.62 ± 0.09	0.97 ± 0.2	0.91	0.99	0.10
<i>PPARα</i>	1 ± 0.3	0.57 ± 0.02	1.11 ± 0.1	1.21 ± 0.3	0.65	0.30	0.47
<i>PPARγ</i>	1 ± 0.3	0.74 ± 0.1	0.83 ± 0.2	1.24 ± 0.3	0.82	0.64	0.36
Chronic HS							
<i>INSIG2</i>	1 ± 0.1	0.63 ± 0.1	0.92 ± 0.1	0.90 ± 0.1	0.26	0.55	0.29
<i>PPARα</i>	1 ± 0.3	0.58 ± 0.1	0.97 ± 0.1	0.87 ± 0.2	0.32	0.61	0.54
<i>PPARγ</i>	1 ± 0.3	0.43 ± 0.1	1.51 ± 0.3	1.58 ± 0.8	0.73	0.26	0.66

¹TN-C, thermoneutral-control diet; TN-N, thermoneutral-Noni diet; HS-C, heat stress- control diet; HS-N, heat stress-Noni diet.

²D, diet; E, environment.

³*INSIG2*, insulin induced gene 2; *PPAR α / γ* , peroxisome proliferator activated receptor alpha/gamma.

Table 4. Effects of quercetin (QCT) supplementation on hepatic expression of lipogenic-lipolytic genes in heat stressed Celi cells.

Parameters ³	Experimental groups ¹				P value ²		
	C-37°C	QCT-37°C	C-45°C	QCT-45°C	D. effect	E. effect	Interaction
<i>ACCα</i>	1 ± 0.08 ^a	1.16 ± 0.08 ^a	0.61 ± 0.08 ^b	0.65 ± 0.1 ^b	0.36	0.0032	0.56
<i>ACLY</i>	1 ± 0.07 ^a	0.89 ± 0.04 ^a	0.37 ± 0.03 ^b	0.36 ± 0.05 ^b	0.30	<0.0001	0.39
<i>FASN</i>	1 ± 0.1 ^a	0.91 ± 0.1 ^a	0.19 ± 0.03 ^b	0.26 ± 0.1 ^b	0.94	<0.0001	0.49
<i>SCD-1</i>	1 ± 0.06 ^a	1.38 ± 0.1 ^a	0.15 ± 0.01 ^b	0.20 ± 0.03 ^b	0.02	<0.0001	0.06
<i>SREBP-2</i>	1 ± 0.05 ^a	1.11 ± 0.08 ^a	0.34 ± 0.02 ^b	0.45 ± 0.04 ^b	0.09	<0.0001	0.98
<i>SCAP</i>	1 ± 0.07 ^a	1.36 ± 0.1 ^a	0.24 ± 0.05 ^b	0.39 ± 0.05 ^b	<0.0001	0.01	0.21
<i>LPL</i>	1 ± 0.3 ^a	1.45 ± 0.4 ^a	1.08 ± 0.2 ^a	1.59 ± 0.5 ^a	0.32	0.8	0.95
<i>ATGL</i>	1 ± 0.1 ^a	1.91 ± 0.4 ^a	1.66 ± 0.2 ^a	1.80 ± 0.2 ^a	0.09	0.34	0.20
<i>LIPC</i>	1 ± 0.2 ^a	0.80 ± 0.2 ^a	0.45 ± 0.07 ^b	0.70 ± 0.1 ^a	0.90	0.16	0.32

¹C, Control; QCT, quercetin.

²D, diet; E, environment.

³*ACCα*, acetyl-CoA carboxylase alpha; *ACLY*, ATP citrate lyase; *FASN*, fatty acid synthase; *SCD-1*, stearoyl-CoA desaturase 1; *SREBP-1/2*, sterol regulatory element-binding protein 1/2; *SREBP* cleavage-activating protein; *LPL*, lipoprotein lipase; *ATGL*, adipose triglyceride lipase; *LIPC*, hepatic triacylglycerol lipase.

Figure 1

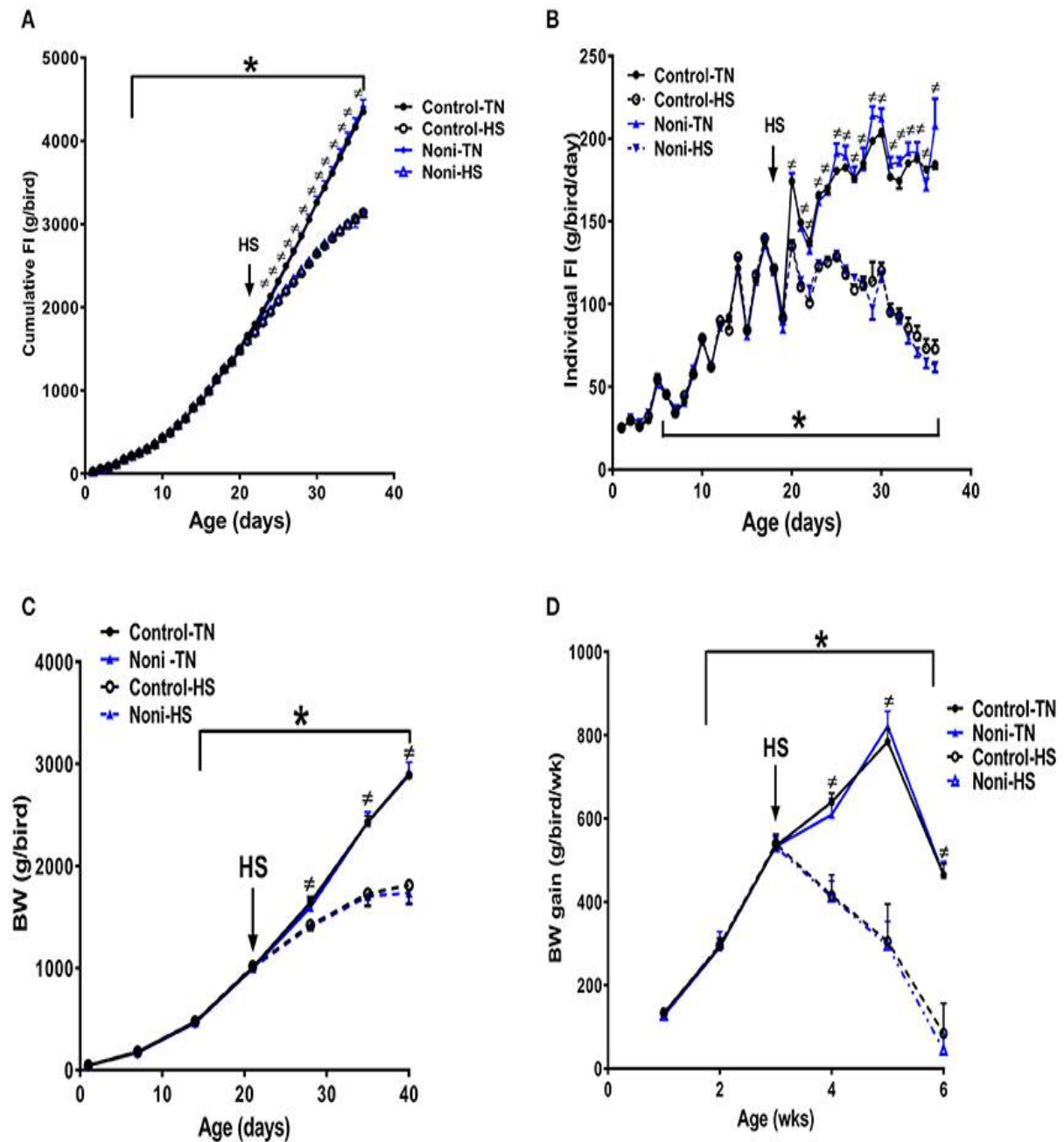


Figure 1. Effects of heat stress (HS) and Noni-enriched diet on feed intake and growth in broilers HS depressed cumulative (A) and individual (B) feed intake which in turn results in a reduction of body weight (BW, C), and BW gain (D). Data are presented as mean \pm SEM ($n = 160$). # denote significant difference between TN and HS conditions at $P < 0.05$. *Indicate significant effect of time at $P < 0.05$.

Figure 2

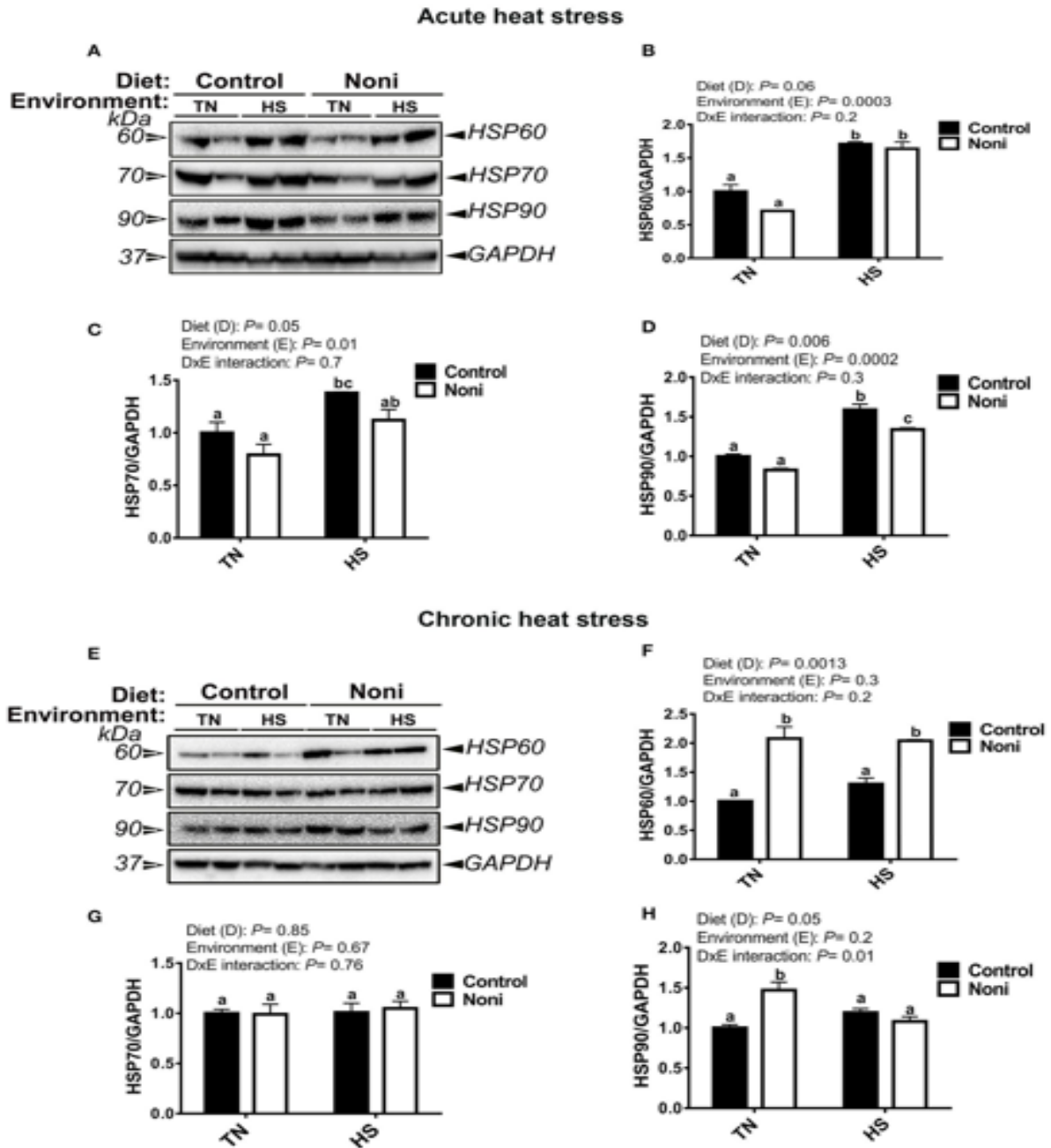


Figure 2. Effect of Noni supplementation on hepatic heat shock protein (HSP) expression in heat-stressed (HS) broilers and those maintained under thermoneutral (TN) conditions. (A–D) acute HS and (E–H) chronic HS. HSP levels were determined by Western blot and their relative expression was presented as a normalized ratio to housekeeping glyceraldehyde 3-phosphate dehydrogenase GAPDH protein. Data are presented as mean \pm SEM ($n = 8$ /group). Different letters indicate significant difference at $P < 0.05$.

Figure 3

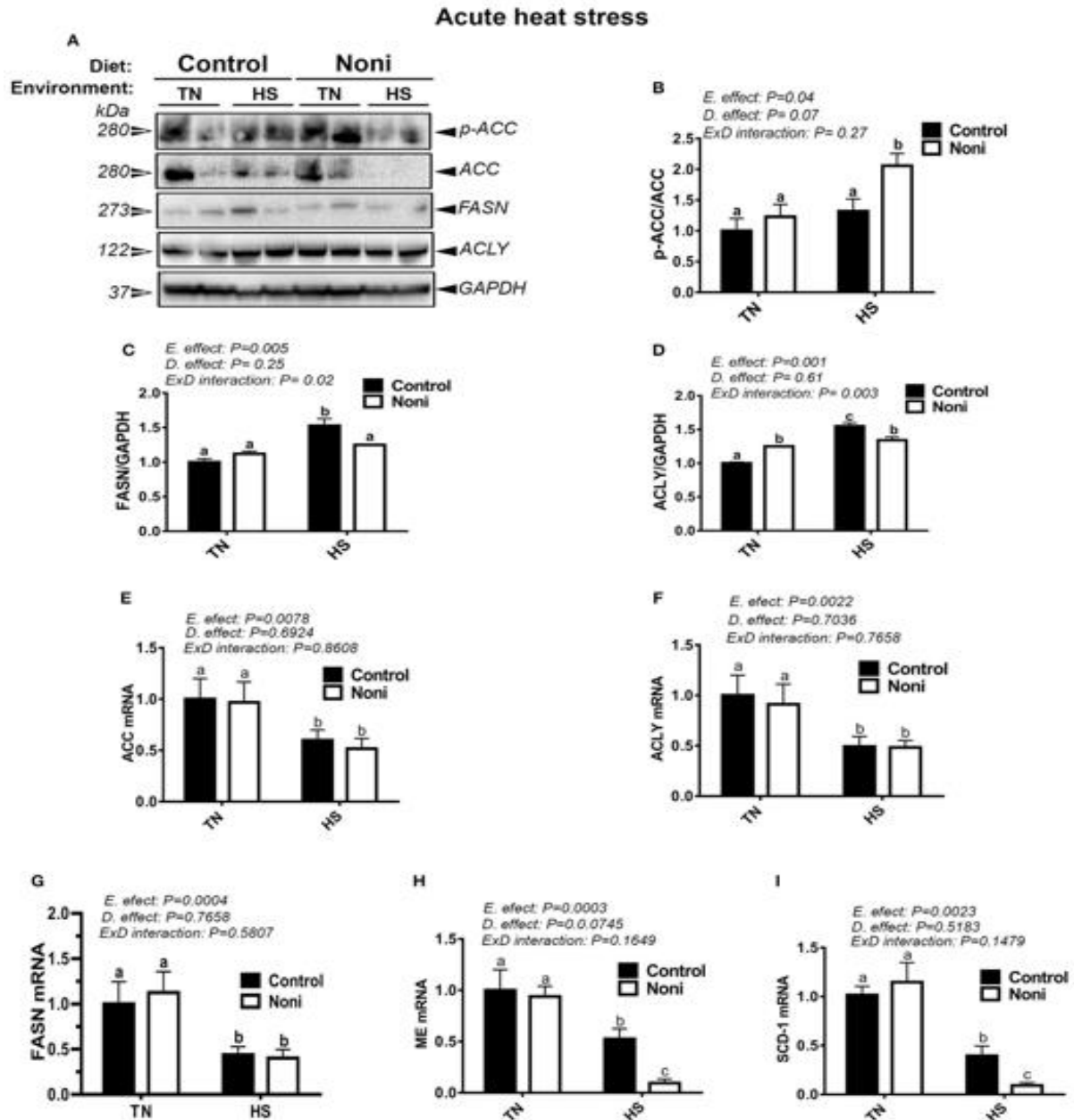


Figure 3. Effect of Noni supplementation on hepatic expression of lipogenic markers in acute heat-stressed (HS) broilers. Phosphorylated and pan levels of acetyl-CoA carboxylase alpha (ACC α) as well as fatty acid synthase (FASN) and ATP citrate lyase (ACLY) were determined using Western blot (a) and their relative expression was presented as normalized ratio of phosphorylated/total target protein (b-d). Relative abundance of ACC α (e), ACLY (f), FASN (g), malic enzyme (ME) (h), and stearoyl-CoA desaturase-1 (SCD-1) (i) mRNA was measured by qPCR. Data are presented as mean \pm SEM (n=8/group). Different letters indicate significant difference at $P<0.05$

Figure 4

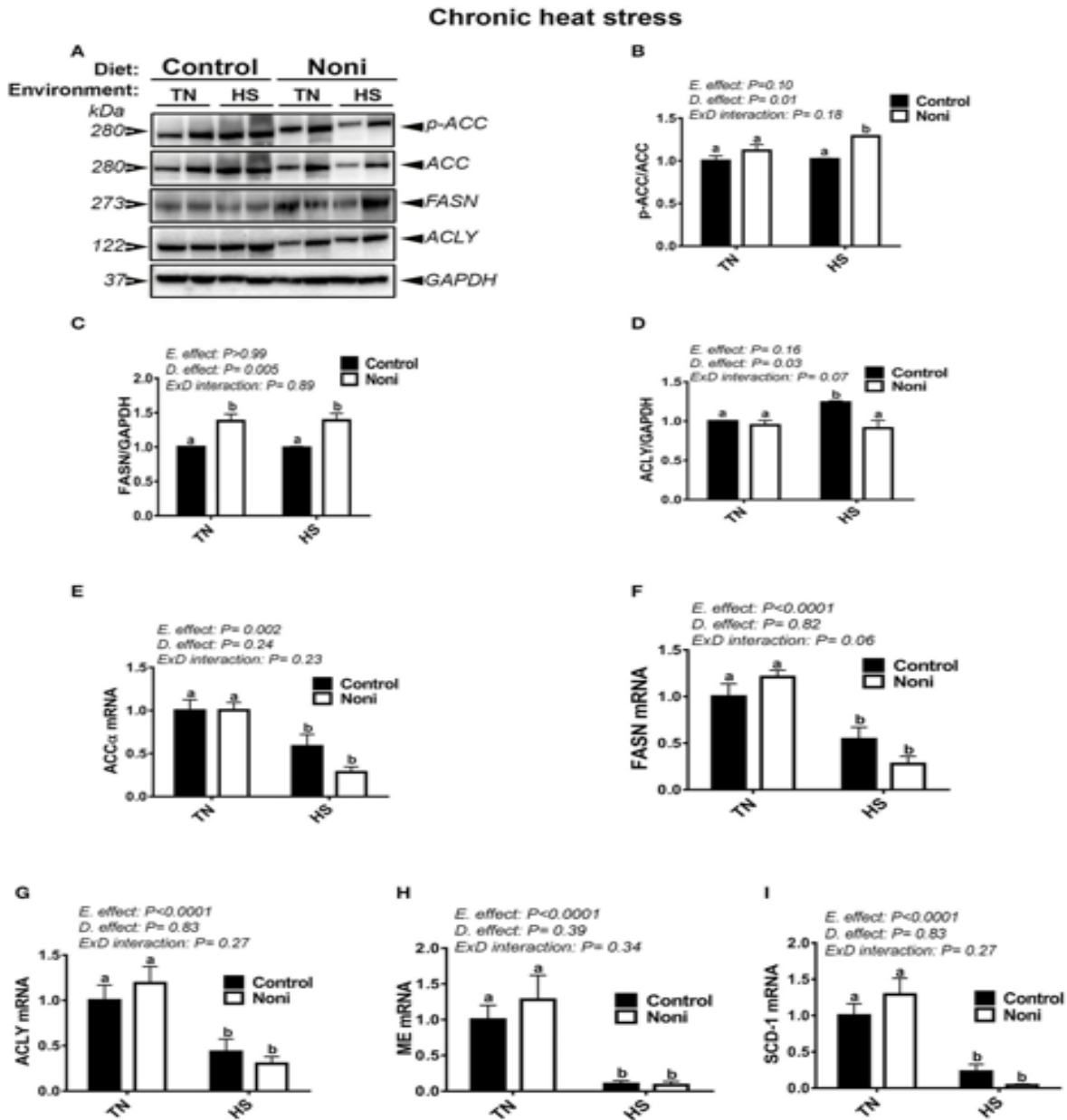


Figure 4. Effect of Noni supplementation on hepatic expression of lipogenic markers in chronic heat-stressed broilers. Phosphorylated and pan levels of acetyl-CoA carboxylase alpha (ACC α) as well as fatty acid synthase (FASN) and ATP citrate lyase (ACLY) were determined using Western blot (a) and their relative expression was presented as normalized ratio of phosphorylated/total target protein (b-d). Relative abundance of ACC α (e), ACLY (f), FASN (g), malic enzyme (ME) (h), and stearyl-CoA desaturase-1 (SCD-1) (i) mRNA was measured by qPCR. Data are presented as mean \pm SEM (n=8/group). Different letters indicate significant difference at $P<0.05$.

Figure 5

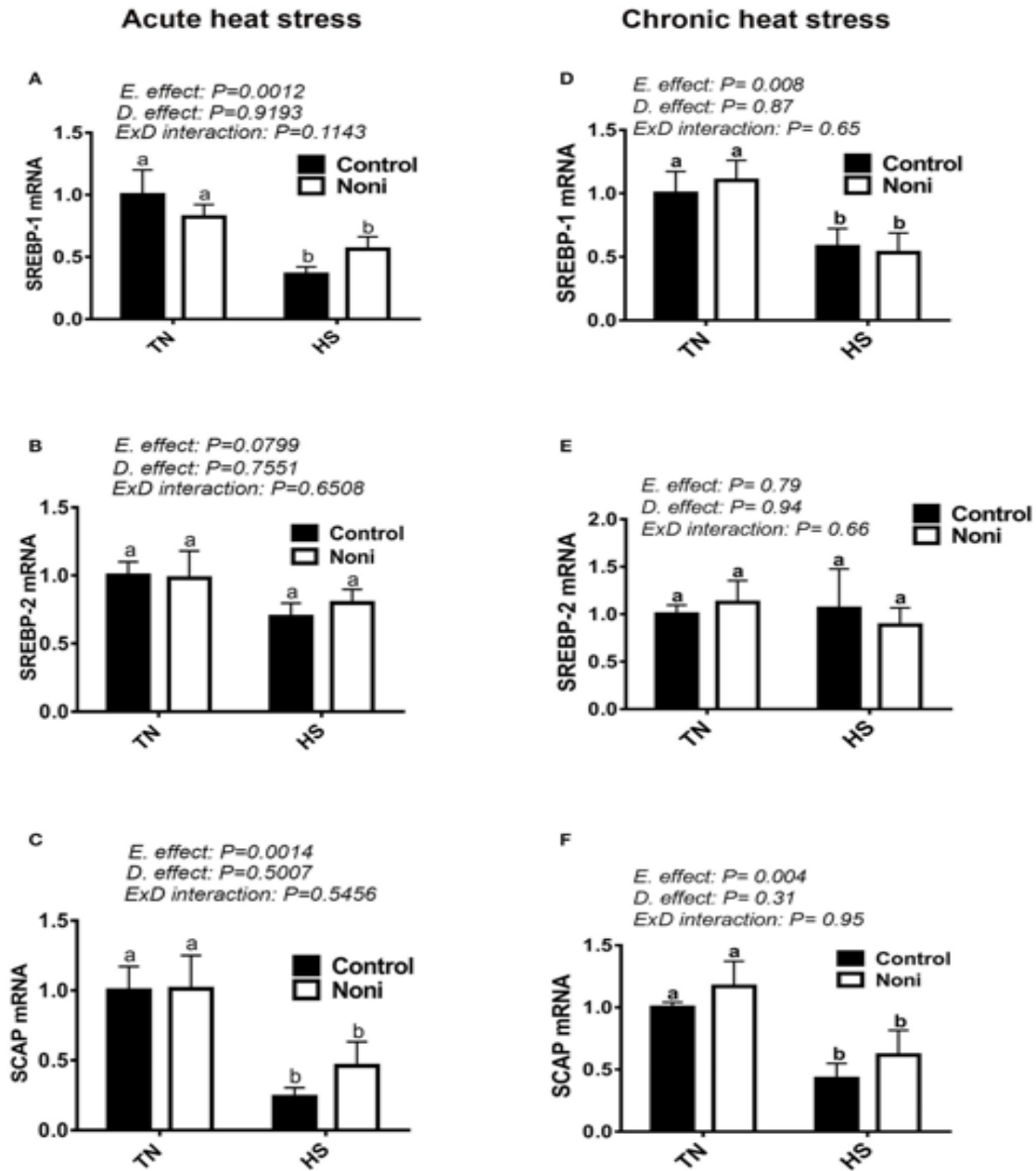


Figure 5. Effect of Noni on hepatic expression of lipogenesis-related transcription factors in heat stressed broilers. (A–C) acute HS and (D–F) chronic HS. Relative expression of sterol regulatory element-binding protein transcription factor 1 (SREBP-1) (A, D) sterol regulatory element-binding protein transcription factor 2 (SREBP-2) (B, E), and SREBP cleavage activating protein (SCAP) (C, F) was measured by real-time RT-PCR. Data are presented as mean \pm SEM. D, diet; E, environment; TN, thermoneutral; HS, heat stress. Different letters indicate significant difference at $p < 0.05$.

Figure 6

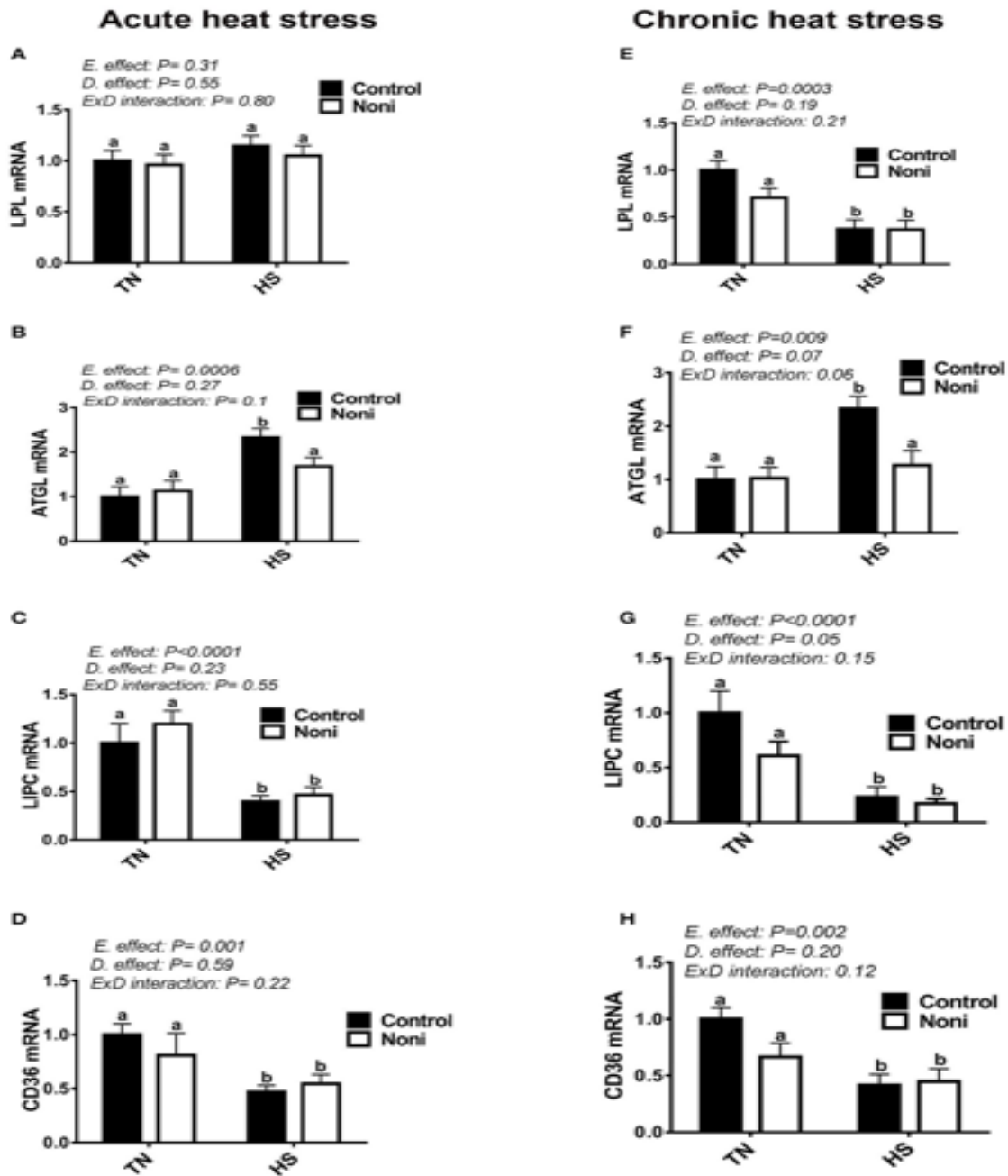


Figure 6. Effect of Noni supplementation on hepatic expression of lipolytic pathway in heat-stressed broilers. (A–D) acute HS, (E–H) chronic HS. Relative expression of lipoprotein lipase (LPL) (A, E), adipose triglyceride lipase (ATGL) (B, F), lipase C (LIPC) (C, G), and cluster of differentiation 36 (CD36) gene (D, H) was measured by real-time RT-PCR. Data are presented as mean \pm SEM ($n=8$ /group). Different letters indicate significant difference at $P < 0.05$.

Figure 7

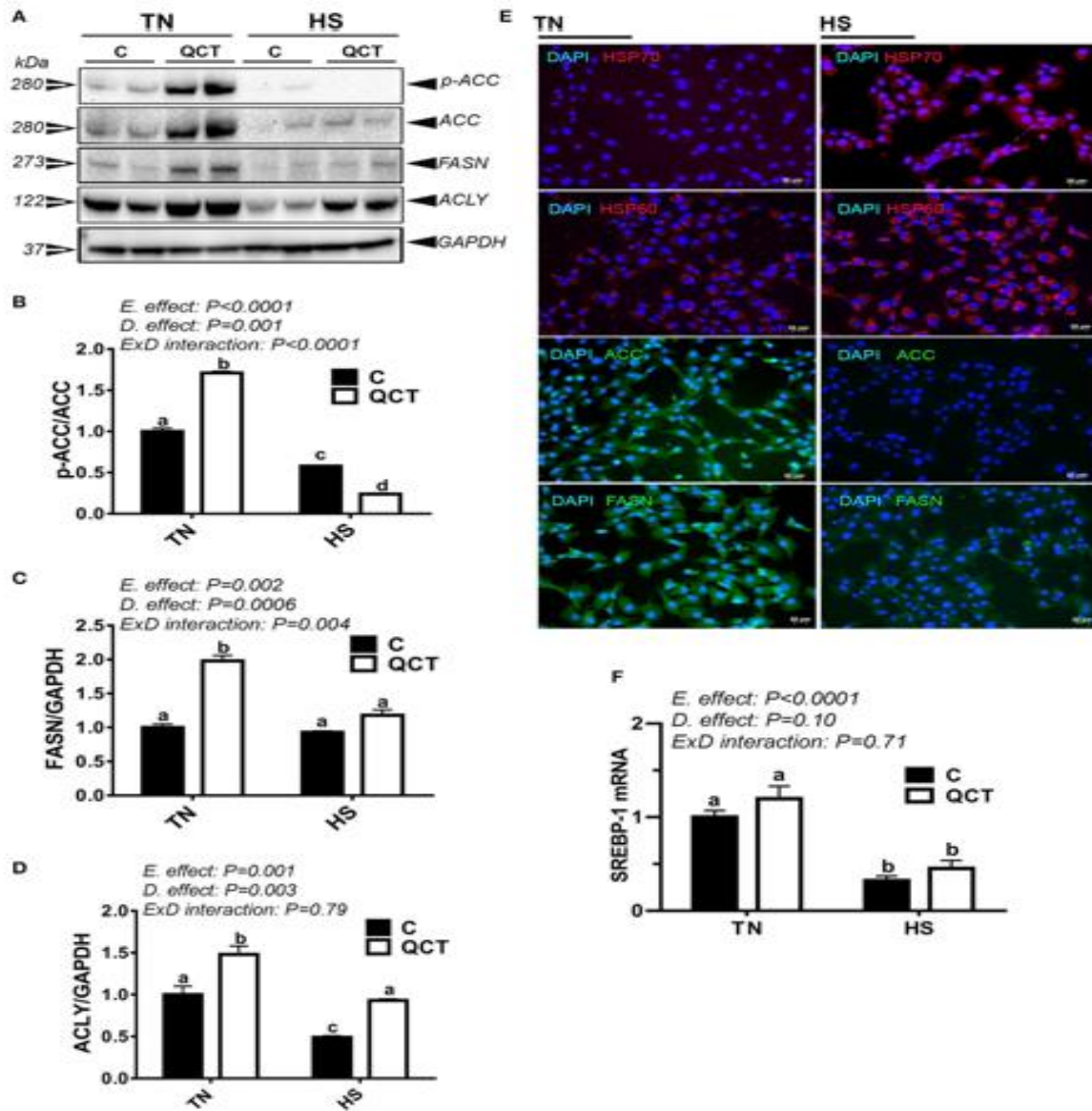


Figure 7. Effect of quercetin (QCT) on lipogenesis-related signatures and sterol regulatory element binding protein 1 (SREBP-1) gene expression in heat stressed sim-CEL cells. Phosphorylated and pan levels of acetyl-CoA carboxylase alpha ($ACC\alpha$) as well as fatty acid synthase (FASN) and ATP citrate lyase (ACLY) were determined using Western blot and their relative expression was presented as normalized ratio of phosphorylated/total target protein (A–D). Immunofluorescence staining (E) shows that heat stress (HS) induce heat shock protein (HSP) 60 and 70, and reduces $ACC\alpha$ and FASN protein levels in sim-CEL cells. SREBP-1 mRNA abundance was measured by real-time RT-PCR (F). Data are presented as mean \pm SEM ($n = 6$ /groups). Different letters indicate significant differences at $P < 0.05$. Western blotting figure is representative of 3 replicates.

Figure 8

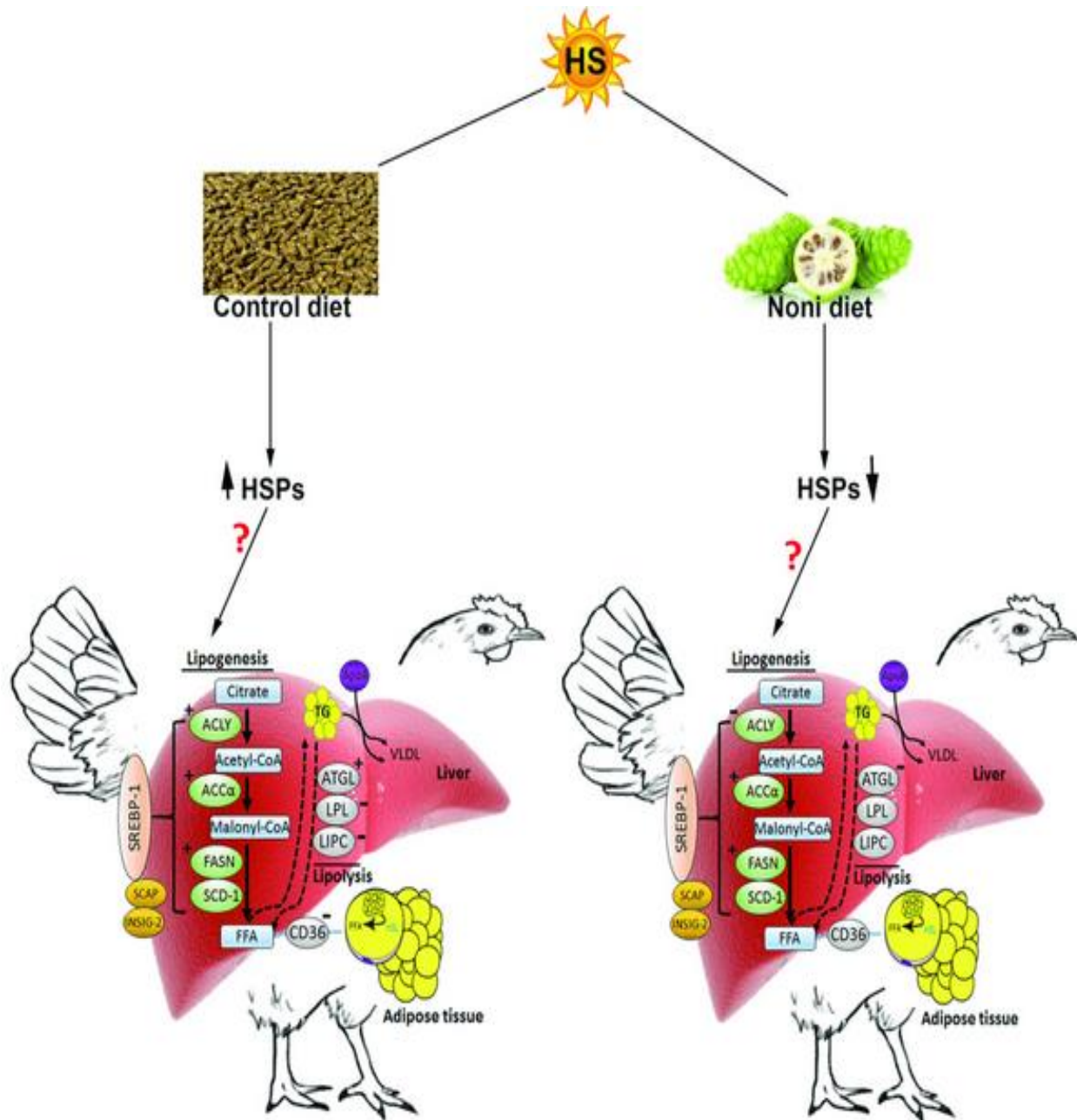


Figure 8. Schematic representation of the effect of heat stress and Noni supplementation on hepatic lipid metabolism in broilers (from protein expression analysis). Heat stress up-regulated the hepatic expression of heat shock proteins (HSP60, HSP70, and HSP90) as well as key lipogenic proteins (FASN, ACC α , and ACLY). HS down-regulated the hepatic expression of LPL and LIPC, but up-regulated ATGL. Noni supplementation down-regulated the hepatic expression of HSPs, ACC α , and FASN but it down regulated ACLY. \uparrow and “+” denote induction, \downarrow and “-“ denotes inhibition. HSL, hormone sensitive lipase; FFA, free fatty acids; VLDL, very low density lipoprotein.

CHAPTER 3: OVERALL CONCLUSIONS

In conclusion, HS has been shown to negatively impact behavior, endocrine function, immune function, reproduction, and growth performance in poultry along with a significant increase in fat deposition in broiler carcasses. These consequences of HS result in significant economic losses to the poultry industry which creates a need for strategies to prevent HS. Several strategies such as genetic selection, management, and nutrition have been utilized to ameliorate the negative effects of HS. The use of Noni has been shown to improve growth performance in broilers in a hot, humid climate when supplemented in the diet; however, this study did not show Noni supplementation to improve feed intake or growth performance under HS conditions. The data from this study also suggests that HS induces the hepatic expression of HSPs and stimulates *de novo* fatty acid synthesis in broilers which may explain the increased fat deposition seen in the carcasses. Furthermore, Noni supplementation appeared to have no significant effect on *de novo* fatty acid synthesis in broilers suggesting that this nutrition strategy cannot ameliorate the negative effects of HS on fat deposition in broilers. Perhaps, the lack of effect of Noni supplementation in these HS conditions could be credited to clean litter and low relative humidity (20%). This suggests that a less pristine environment such as that found in commercial poultry houses may be required to demonstrate potential benefits of Noni.

APPENDIX



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

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