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The Effects Of Heat Stress On Immunity In Laying Hens And Dairy Cattle

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THE EFFECTS OF HEAT STRESS ON IMMUNITY IN LAYING HENS AND DAIRY CATTLE

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To my parents and Jonathan for all their love and support

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LIST OF ABBREVIATIONS

µl	Microliter
µM	Micrometer
ACD	Acid Citrate Dextrose
ACTH	Adrenocorticotropic hormone
AP	Air Perch
BA	Basophils
BW	Body Weight
CD	Cluster of differentiation
cDNA	Complementary DNA
CP	Cool Perch
CT	Control
d	Day
db	Dry bulb
DBT	Dry bulb temperature
DCFDA	Dichlorofluorescin diacetate
Dec205	Cluster of differentiation 205
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide

EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EO	Eosinophils
FITC	Fluorescein isothiocyanate
FPTI	Failure passive transfer immunity
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
H	Humidity
H/L	Heterophil to lymphocyte ratio
HBSS	Hank's Balanced Salt Solution
HCT	Hematocrit
HPA	Hypothalamic-pituitary-adrenal axis
HS	Heat stress
HSP	Heat shock protein
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kcal	Kilocalorie
kg	Kilogram
L	Liter
L: D	Light to dark
LPS	lipopolysaccharide

LY	Lymphocytes
Max	Maximum
mg	Milligrams
Min	Minimum
mL	Milliliter
mM	Millimolar
MO	Monocytes
mRNA	Messenger ribonucleic acid
N/L	Neutrophil to Lymphocyte ratio
NE	Neutrophil
ng	Nanogram
nm	Nanometers
NP	No Perch
PCR	Polymerase chain reaction
pg	Picogram
PP	Plasma protein
RA	Receptor antagonist
ROS	Reactive oxygen species
RR	Respiration rates
RT	Rectal temperatures
SW	Spleen weight
T	Temperature
THI	Temperature- humidity index

THVI	temperatures-humidity-velocity index
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNZ	Thermal neutral zone
V	Velocity
WBC	White blood cells
WBT	Wet bulb temperature
wk	Week

ABSTRACT

Strong, Rebecca A. M.S., Purdue University, December 2014. The effects of heat stress on immunity in laying hens and dairy cattle. Major Professors: Drs. Heng-wei Cheng and Susan Eicher.

With the increase in global climate change and the population growth driving the high demand for additional food production, heat stress (HS) is a major concern in the livestock industry across all species. Animals experience HS when exposed to high environmental temperatures outside their thermal neutral zone. The level of the effects can vary due to the length and intensity of HS to which the animal is exposed to. In experiment one, laying hens with access to cooled perches during HS had a lower heterophil to lymphocyte ratio compared to the control hens after 4 h of acute heat stress, indicating cooled perches as a method to alleviate the effects of HS on laying hen immunity. In the second experiment, HS on dams during late gestation had detrimental effects on biomarkers of the calf's innate immunity, including an increase in neutrophils, lower plasma proteins, and greater toll-like receptor 4 in calves born to HS dams. In conclusion, HS greatly impacts many different species and poses a wide threat on the health and wellbeing of animals due to the global climate changes and increased demands on the livestock industry. Thermally cooled perches, as a method to improve hen immunity during HS, has allowed additional knowledge for creating a long-term strategy

to alleviate HS in laying hens. The changes found in neonatal immunity after exposure to late gestational prenatal HS has potentially opened other avenues of research to better understand the effects of prenatal HS on the offspring of livestock.

CHAPTER I

LITERATURE REVIEW

Definition of Heat Stress

Heat stress (HS) occurs after an exposure to high ambient temperatures beyond the thermal neutral zone (TNZ) for a given species. Both acute, a brief intense HS episode, and pro-longed exposure to high temperatures, or chronic HS, can cause adverse effects on the animal's well-being. The TNZ refers to the range of temperatures at which the animal does not have to actively regulate their biological system to maintain body temperature. The TNZ is a physiological range with limited variability of biological function (Scholander et al., 1950). Individual animals can exhibit a wide range of reactions to HS, showing from little effect on their health to experiencing mortality. Thus, a better method to finding the effects of HS on an animal is by determining its biological optimum temperature (Nichelmann, 1983). However, even with the determined optimal temperature per species, different biological functions and activities play a role for different individuals. For example, the optimum temperature for hen egg production is estimated to be between 19°C and 22°C, but between 18°C and 30°C for meat producing birds (Charles, 2002). Farm animals have various zones of thermal comfort predominantly dependent on the species of animal and their physiological status (internal factors) and relative humidity, velocity of ambient air, and the degree of solar radiation (external factors) also contribute to their TNZ (NRC, 1981). By including these additional factors, a more useful index can be created in determining the optimal temperature and potential for HS (Thom, 1959; Hahn et al., 2009).

As guidelines, the theory of Livestock and Poultry Heat Stress indices (LPHSI, 1990) was developed in order to determine the magnitude of HS of an animal.

Various indexes derived from meteorological measurements have been developed and recently reviewed by Hahn et al. (2009). The most common one is the temperature-humidity index (THI) (Thom, 1959). The following formula for THI takes into account the ambient temperature and the humidity to estimate the magnitude of HS:

THI = db°F-{(0.55-0.55RH) (db°F-58)}

In the formula, db is defined as the dry bulb temperature in °F and RH is the relative humidity (RH%/100). The obtained values are used to establish the severity of HS: <82 indicate an absence of HS; 82-84 indicates moderate HS, 84-86 indicates severe HS and >86 indicates extreme severe HS (LPHSI, 1990).

THI, however, does not account for important climatic variables such as air movement or solar radiation. In addition, THI does not include management or animal factors, such as the effect of shade or the genotype and phenotypic differences. Several other formulas have been developed for determining the HS potential to account for the additional factors. Tao and Xin (2003), for example, made improvements on the THI by creating the temperature-humidity-velocity index (THVI), which includes wind speed to determine the HS level in poultry. Wind speed has been shown to be favorable to birds exposed to HS conditions (Ruzal et al., 2011). Therefore, the following formula of THVI may be advantageous to improving the method in determining the severity of HS.

$$THVI = (0.85 \times DBT + 0.15 \times WBT) \times V^{-0.058}$$

In the formula, DBT is defined as the dry bulb temperature in °C, WBT is the wet bulb temperature in °C, and V represents the air velocity. Similarly to THI, the obtained numerical values are used to establish the negative effects of HS: ≤70 being

normal, 70-75 indicates alert, 76-81 being in danger, and ≥ 82 indicating emergency (Tao and Xin, 2003). Additional indices have incorporated the previously stated variables to overcome the limitations of different THI (Mader et al., 2006; Gaughan et al., 2008). For example, Mader et al. (2010) developed a comprehensive climate index applicable under a wide range of environmental conditions by providing an adjustment to ambient temperature, relative humidity, wind speed, and radiation. However, to simplify those methods, Dikmen and Hansen (2009) indicated that dry bulb temperature is a good predictor as THI of rectal temperatures of lactating Holsteins in a subtropical environment.

Impact of Heat Stress on Poultry and Livestock

The earth's climate has warmed in the last century with current climate models indicating a 0.28°C increase per decade for the next two decades and predicted the increase in global average surface temperature by 2100 to be between 1.88°C and 4.08°C (IPCC, 2007). Therefore, the increasing concern on the thermal comfort of agricultural animals is not only justifiable for countries in tropical areas, but also for nations with high ambient temperatures (Nardone et al., 2010). Additionally, it has been estimated that more than 50% of the total world meat and 60% of milk originates from tropical and subtropical areas (FAO statistics, 2010). Given the increase in climatic temperature and the demand for great production in livestock and poultry, there is a growing concern for welfare of farm animals. In 1995, for example, the summer heat wave in the mid-central U.S. caused a $28 million loss in the cattle industry by animal death and reduced performance (Hahn, 1999). In 2006, a major

heat wave moving across the U.S. resulted in the death of 25,000 cattle and 700,000 birds in California (Nienaber and Hahn, 2007). Heat stress is a critical stressor, especially in regions with hot climates and farms unable to control the ambient temperature (Kadim et al., 2008). Possible interventions need to be further investigated to avoid or reduce HS associated mortality reducing farm animal production.

Regardless of species, economic losses are experienced by both the poultry and livestock industries, due to animals being raised in locations and seasons where efficient temperature conditions could be outside their TNZ. Heat stress caused an estimated total $2.4 billion annual loss to the U.S. livestock production industry in the absence of heat abatement. From this total, $128 to $165 million occurred in the poultry industry and $897 to $1500 million occurred in the dairy industry. Under optimum heat abatement, the annual economic loss was reduced to $1.7 billion over all farm animal production species. Optimum heat abatement reduced annual total economic losses from $98.1 to $61.4 million in laying hens and went from $1507 to $897 million per year in the dairy industry (St-Pierre et al., 2003).

Although the mechanisms by which HS consequences arise are continually under analysis, HS has been consistently found to hinder the production in livestock and poultry. In poultry, it includes a decrease in feed intake (Sohail et al., 2012; Quinteiro-Filho et al., 2012; Habibian et al., 2013), impaired growth performance (Attia et al., 2011; Ghazi et al., 2012; Imik et al., 2012), decrease in egg production (Mashaly et al., 2004; Star et al., 2009; Deng et al., 2012; Mack et al.,2013), impaired egg qualities (Lin et al., 2004b; Ajakaiye et al.,2011; Ebeid et al., 2012; Mack et

al.,2013), and an increase in mortality (Mashaly et al., 2004; Quinteiro-Filho et al., 2010). In cattle, HS causes a decrease in feed intake and BW gain (do Amaral et al., 2009). Other effects in cattle due to HS include a reduction in milk production (Bohmanova et al., 2007; Boonkum et al., 2011) and an impaired reproductive performance (Thompson and Dahl, 2012).

Poultry

Physiological and Behavioral Responses

Birds are homeotherms, having the ability to maintain their body temperature within a narrow range. The optimum temperature for laying hen performance is likely to be between 19 and 22°C (Charles, 2002). Birds produce and dissipate heat to maintain a relatively constant body temperature. When the temperature and relative humidity exceed the comfort level, birds lose their ability to efficiently dissipate heat, which then leads to behavioral and physiological changes (Toyomizu et al., 2005; Gonzalez-Esquerra and Leeson, 2006). Birds become heat stressed without the balance between heat production and heat dissipation, or thermoregulation. An increase in air circulation, through ventilation, has been a proven factor in maintaining egg production in hens exposed to HS (Ruzal et al., 2011).

Birds do not have sweat glands. Therefore, the primary methods of heat loss in birds include respiratory evaporative cooling, or panting and conductive heat transfer through their feet and wattles. When birds are challenged by HS, their body temperatures increase causing an increase in respiratory rate (Borges et al., 2004). Consequently, panting relies on water loss which may lead to dehydration (Belay and

Teeter, 1993) or respiratory alkalosis (Yahav et al., 1995). Birds have air sacs as an additional mechanism to promote heat exchange between their body and the environment. Air sacs are useful during panting, as they promote air circulation on surfaces, increasing heat loss via evaporation along with gas exchanges (Fedde, 1998). A recent study (Mack et al., 2013) showed that birds subjected to HS conditions spent more time with their wings elevated, drinking, panting, and resting, but spent less time feeding, moving, and walking. Sensible heat is used by birds to release heat, including radiation, convection, and conduction (Yahav et al., 2005). However, some methods of heat loss are challenging to the avian species due to their feather coverage trapping heat to prevent hypothermia. During HS, capillary blood flow is redistributed throughout the body to improve sensible heat loss (Wolfenson et al., 1981).

Mujahid et al. (2009) presented evidence that broiler chickens exposed to different durations of acute HS resulted in distinct time-dependent physiological responses. Increased mitochondrial reactive oxygen species (ROS) production and decreased avian uncoupling protein was confirmed in HS birds (Mujahid et al. 2009). The balance between ROS and antioxidant is disrupted in broilers exposed to HS (Wang et al., 2008), leading to oxidative stress during acute HS (Lin et al., 2006; Mujahid et al., 2006). Malondialdehyde is a biomarker used to measure oxidative stress in chickens by indirectly measuring the level of peroxidation due to ROS (Mujahid et al., 2009; Azad et al., 2010). In order to establish this balance during HS, the strategy for increasing antioxidant capability and activity in birds is common (Lin et al., 2006).

High environmental temperatures activate of the hypothalamic-pituitary-adrenal (HPA) axis. The stress-activated HPA axis was found to be responsible for the negative effects of HS on broiler performance and immune function (Quinteiro-Filho et al., 2012). The HPA axis regulates corticotrophin releasing hormone and adrenocorticotropic hormone are released from hypothalamic and pituitary cells (Bakshi and Kalin, 2000). In response to the physiological disruptions, more glucocorticoids are released. Glucocorticoids participate in the control of body homeostasis and stress response of various organisms (Lin et al., 2004a). In poultry, HS has been shown to cause elevated corticosterone concentrations (Garrig et al., 2006; Star et al., 2008; Quinteiro-Filho et al., 2010). Changes in corticosterone levels also occur due to environmental stimuli (Korte et al., 2005). Increased corticosterone, as the final product of the HPA axis, causes numerous effects on behavior, metabolic pathways, and immune functions (Haller et al., 2000). Lara and Rostagno (2013) reviewed the literature and concluded further research is still needed to improve the knowledge of the basic mechanisms associated with the negative effects of HS in poultry.

Production Responses

In recent studies, broilers subjected to chronic HS significantly reduced feed intake, and caused a lower BW and a higher feed conversion ratio (Niu et al., 2009b**;** Sohail et al., 2012). Similarly, broilers subjected to acute HS had a lower feed intake, BW gain, but a lower feed conversion (Quinteiro-Filho et al., 2010; Quinteiro-Filho et al., 2012). Habibian et al. (2013) also reported a significant reduction in BW and

feed intake in chronic HS broilers, however, the feed conversion ratio was increased. Naseem et al. (2005) also found an increase in feed conversion in broiler chickens exposed to HS. Taken together, HS (acute or chronic) impairs growth performance in broilers (Attia et al., 2011; Ghazi et al., 2012; Imik et al., 2012). In laying hens, similar to its effects on broilers, HS resulted in decreased BW, feed efficiency, egg production and egg quality (Mashaly et al., 2004; Deng et al., 2012). Lin et al. (2004b) further reported HS decreased production performance, as well as reduced eggshell thickness, and increased egg breakage. Additionally, a reduction of feed conversion, egg production, and egg weight were identified in laying hens subjected to HS (Star et al., 2009). Ebeid et al. (2012) reported HS caused 1% reduction in egg weight, egg shell thickness, eggshell weight, and eggshell percent. Ajakaiye et al. (2011) also showed a reduction in egg shell weight and thickness. Corroborating these results, Mack et al. (2013) observed decreased egg production, egg weight, and egg shell thickness in laying hens exposed to HS.

The egg production is dependent on gonadal steroids, such as progesterone, testosterone, and estradiol, which are all inhibited by HS (Rozenboim et al., 2007). The cellular mechanism of how HS negatively affects the bioactivity of gonadotropin is less understood. Most likely, it is due to multiple factors affecting animals' physiological homeostasis under HS, such as a reduction in feed intake (Ajakaiye et al., 2011) and an increase in oxidative stress (Lin et al., 2006). The correlation between decreased feed intake and increased mortality could also be responsible for the reduced egg production (Ortiz et al., 2006). An increase in mortality to laying hens (Mashaly et al., 2004) and broilers (Quinteiro-Filho et al., 2010) were reported

after heat exposure. Warriss et al. (2005) demonstrated a seasonal impact with a peak mortality occurring in the summer months.

Chickens' reaction to HS is affected by multiple factors. The reported variability of the effects of HS on chicken health may be explained by the examined birds, such as health and production status, and or genetic background. The variation may also be due to the variety of intensity and duration of HS applied to the chickens. Stocking density is also a major factor affecting chicken productivity and physiological homeostasis, as well (Estevez, 2007).

Immunocompetence

Decreased immune function can result in the chickens' inability to respond to inflammation and or infection appropriately. Gram-negative bacteria is a common pathogen found in the current poultry housing systems (Zucker et al., 2000), leading to welfare concern, especially under HS. Heat-stressed hens had higher mortality after being exposed to a vaccination of *Escherichia coli* than hens in controlled environmental temperatures (Compean et al., 2011). It has been concluded that high environmental temperatures affect the immune response in chickens (Naseem et al., 2005) by altering specific immunological biomarkers such as a greater H/L ratio, (Felver-Gant et al., 2012). Heat stress has been shown to limit immunocompetence through decreasing antibody production in hens (Mashaly et al., 2004) and broilers (Niu et al., 200b; Habibian et al., 2013). Broilers subjected to HS also had lower levels of total circulating antibodies (Bartlett and Smith, 2003).

Invading pathogens are controlled by either the innate, adaptive or both immune responses. Adaptive immunity is mediated by B, T or both lymphocytes and often not rapid enough to destroy the invaded microorganism since its response involves cell proliferation, gene activation, and protein synthesis. Depending on prior exposure, a more rapid response (immediate response) to defend against the pathogen can be provided by the innate immune system, which recognizes pathogens by germ-line-encoded pattern recognition receptors. However, as Regnier et al. (1980) suggested that heat-induced immunosuppression may depend on the breed of the bird. The effects on immune responses may also depend on the length and intensity of the heat exposure (Henken et al., 1982; Kelley, 1983).

The recognition of pathogen-associated molecular patterns, which refer to the constituents found on the pathogen that are not normally found in the host, is mainly due to the family of toll-like receptors (TLR) located primarily on the cell surface of macrophages (Werling and Jungi, 2002). Following this innate response, TLR initiate the adaptive immune response by activating antigen-presenting cells through inducing the production of cytokines. TLR-4, for example, detects lipopolysaccharide (LPS) from gram-negative bacteria and is thus important in the activation of the innate immune system. Sahin et al. (2010) also found an increase in both serum tumor necrosis factor α (TNF- α) and interleukin (IL)-6 in heat-stressed quails. Deng et al. (2012) demonstrated that levels of serum TNF- α, IL-1, and corticosterone in hens were elevated during HS, but there was no change in IL-6.

Heat shock proteins (HSPs) are classified as either constitutive, which are always present, or inducible, which are only observed after heat stess. The most prevalent

inducible HSP is HSP70 and is known to be an important indicator of the thermotolerance response in birds (Yahav et al., 1997; Mazzi et al., 2003). The expression of HSP70 in response to stress serves to protect against the initial insult and produces a state of resistance to later stress (Kregel, 2002). Following HS, an increase in the circulating HSP70 levels were reported in chickens (Cahaner et al., 2008; Soleimani et al., 2011; Gu et al., 2012).

There are several types of immune cells in birds. Heterophils, the avian neutrophil, are granulated leukocytes formed in the bone marrow. They are phagocytic cells essential in performing the innate immunity by defending the organism against bacteria, viruses, or foreign particles. Lymphocytes are non-granulated leukocytes formed in lymphoid tissues, such as the thymus and the bursa of Fabricus in birds. They play an important physiological role in adaptive immunity, specifically for the production of antibodies. The heterophil/lymphocyte (H/L) ratio has been used as a stress indicator in birds, demonstrating its changes through the interaction with circulating levels of corticosterone (Gross and Siegel, 1983; Puvadolpirod and Thaxton, 2000). The changes of the H/L ratio (Gross and Siegel, 1983) caused by a decrease in the number of circulating lymphocytes (Aengwanich, 2008), an increase in the number of heterophils (Mogenet and Youbicier-Simo, 1998; Borges et al., 2004) or both. Recent studies have demonstrated that HS can alter levels of circulating cells resulting in an increase in the H/L ratio (Prieto and Campo, 2010; Soleimani et al., 2011; Felver-Gant et al., 2012; Habibian et al., 2013).

With a restricted lymphatic system, hens rely on the spleen as an important immune organ and the primary source of antigen-presentation (Jeurissen, 1993).

Additionally, the spleen also helps the bird in blood filtration and storage of multiple cell-types involved with the hen's immune system. Cheng et al. (2004) found that hens with greater spleen weight have higher ability to produce a normal immune response. Spleen weight decreased after exposure to high temperatures in laying hens (Felver-Gant et al., 2012; Ghazi et al., 2012) and broiler chickens (Bartlett and Smith, 2003; Niu et al., 2009b; Quinteiro-Filho et al., 2010). A reduction in liver weight was also observed in chickens subjected to HS conditions (Bartlett and Smith, 2003; Felver-Gant et al., 2012). The liver is another important organ in regulating immunity due to its abundance of innate cells responsible for defending foreign antigens (Holz et al., 2008). An increase in corticosterone has been shown to negatively affect lymphoid organ proliferation and function (Post et al., 2003; Shini et al., 2008).

Supplementation

Numerous nutritional supplements have been implemented in poultry diets to alleviate HS. Daghir (2009) reviewed the literature on nutritional strategies to reduce HS in broilers and concluded that nutritional manipulation can be useful in reducing negative effects of high ambient temperatures. However, a reduction in feed intake challenges the concentration of nutrients required to maintain health and productivity in poultry subjected to HS. Therefore, supplementing drinking water may be a more suitable method for poultry during high ambient temperatures. For heat-stressed broilers, supplementation of vitamins, such as A, D, E, and B complex, through drinking water have been reported to be beneficial for the performance and immune function in broilers (Ferket and Qureshi, 1992). The thermotolerance of chickens

exposed to severe HS could also be improved by supplementing drinking water with potassium chloride (Ahmad et al., 2008). Additionally, an improvement in the average feed consumption, egg production, egg weight, and egg shell thickness were observed with supplementation of vitamin C in drinking water in laying hens (Khan and Sardar, 2005).

The immune response of broilers under HS conditions could be improved by dietary vitamin E (Niu et al., 2009a), by decreasing the H/L ratio, i.e., increases in lymphocytes number (Habibian et al., 2013), improving activity of macrophages (Konjufca et al., 2004) and increasing antibody production and function (Singh et al., 2006). Likewise, vitamin A (Lin et al., 2002), selenium (Niu et al., 2009a; Habibian et al., 2013) and chromium supplementation (Bahrami et al., 2009) have been linked to improving the immune response in broilers subjected to HS. Organic chromium may also have some positive effects on serum glucose in broilers under high ambient temperatures (Moeini et al., 2011). Mahmoudnia and Madani (2012) reviewed the literature and concluded that dietary supplementation of betaine on broilers in warm weather has certain benefits on performance traits, such as BW gain and feed conversion ratio at early stages of growth. Betaine also improves respiration rate and humoral immune competence in slow-growing chicks exposed to HS (Attia et al., 2009). Betaine is further suggested to improve immunity and reduce mortality in broilers (Khattak et al., 2012). Different levels of dietary zinc have been investigated and suggested to be an important component of the poultry diet during HS, as well (Sahin et al., 2009).

Khan et al. (2012) reviewed the literature and determined that supplemental vitamin C can improve immune response and numerous production parameters. Under HS, vitamin C has been shown to enhance immune function in birds (Attia et al., 2009). In addition to immune function, vitamin C has also been shown to improve egg production in laying hens subjected to high temperatures (Sahin and Kucuk, 2003). Ajakaiye (2011) showed that vitamins E and C improve egg qualities in laying hens under HS. However, it was concluded that supplementing vitamin E to the diet during HS does not have any positive effect on the metabolism in laying hens (Yardibi et al., 2009) and did not improve growth performance of broilers (Niu et al., 2009b). In addition, the probiotic, *Bacillus licheniformis*, may also be useful for improving the adverse effects of heat on egg production and gut health in laying hens (Deng et al., 2012).

Genetic Selection

Alterations in management practices can improve the coping mechanism of laying hens to HS conditions, but these adjustments may be temporary and limited by costs. Genetic manipulation of breeders based on thermotolerance may be an alternative approach to improving egg production and survivability under HS conditions long-term. Genetic variations in response to HS have been observed between hens (Franco-Jimenez et al., 2007; Star et al., 2008; Soleimani et al., 2011) and broilers (Cahaner et al., 2008; Tirawattanawanich et al., 2011) among different breeds suggesting habitable differences in tolerance to HS.

The Hyline W-98 laying hens responded better to HS conditions with regard to egg production and egg quality measures than Hyline Brown or Hyline W-36 hens (Franco-Jimenez et al., 2007). It was reported that during recovery from HS, egg production in W-98 line returned to baseline faster than the other strains (Franco-Jimenez et al., 2007). During HS, Hyline W-98 hens had a lesser reduction in feed intake than Hyline W-36 and Hyline Brown laying hens, as well. In addition, the lowest mortality was observed in the Hyline W-98 hens, leading to the conclusion that this strain has mechanisms that improve its thermotolerance over Hyline W-36 and Brown hens (Franco-Jimenez et al., 2007). Star et al. (2008) reported that the White leghorn WB line (low survival rate and low natural humoral immune competence) was more sensitive to HS than the White leghorn WA (high survival rate and low natural humoral immune competence), WF (high survival rate and high natural humoral immune competence), and Rhode Island Red B1 (low survival rate and high natural humoral immune competence) lines. Lastly, Soleimani et al. (2011) observed that red jungle fowl, the non-selected strain of chicken, showed lower H/L ratio, higher plasma corticosterone concentrations, and higher HSP70 expression than commercial broilers after heat exposure. It was concluded that instead of improving coping mechanisms, the domestication and selective breeding are leading to individuals that are more susceptible to stress.

Two strains of White Leghorn hens: group-selected hens for high productivity and survivability, a kind gentler bird (KGB) line, and commercial hens individually selected for high egg production (DXL) have been widely studied when observing physiological homeostasis (Cheng and Muir, 2007) and immunological variables

(Fahey and Cheng, 2008). Production values significantly varied between the two lines under acute heat exposure, with the KGB line having lower mortality rates as well as greater egg production during HS (Hester et al., 1996). Felver-Gant et al. (2012) reported hen liver weight decreased with less of a response in the KGB line after heat exposure. Additionally, KGB hens had higher HSP70 concentrations following HS. Moreover, DXL hens showed an elevated H/L ratio under HS compared with their controls, whereas KGB hens showed a much lesser response.

In fast growing broilers under HS, feather coverage negatively affects thermoregulation because it hinders the dissipation of excessive internal heat (Yahav et al., 1998**;** Deeb and Cahaner, 1999). Therefore, it has thought to be advantageous to introduce genes such as the naked-neck gene or the featherless gene to alleviate HS. Cahaner et al. (2008) further investigated this hypothesis and reported feather coverage was found to significantly affect the thermoregulatory capacity of the broilers exposed to high environmental temperatures. In this study, broilers without feathers (featherless) and those with reduced feather coverage (naked-neck) were able to minimize the increase in body temperature. However, the naked-neck birds showed only a slight advantage over fully feathered birds indicating a reduction in feather coverage provided limited tolerance to HS. Additionally, featherless birds had greater breast meat yields than naked-neck and fully feathered commercial birds under hot conditions (Cahaner et al., 2008).

Furthermore, the effects of high-meat-yielding commercial broilers (B line), low-meat-yielding Thai indigenous (T line) chickens, and a Thai indigenous crossbred line selected as a candidate meat-type chickens to survive in the tropical environment (C

line) were evaluated on immune function and their capacity to tolerate the tropical climate (Tirawattanawanich et al., 2011). The H/L ratios, expressed in B-line chicks, were found to increase significantly than those present in the C- and T-line chicks in the summer season. In addition, the innate and humoral immunities of B-line chicks were significantly lower than T- and C-line chicks, especially during the summer season (Tirawattanawanich et al., 2011).

Thermal Regulated Perches

Effective and economical techniques to minimize production losses that result from HS are important in the poultry industry. Broiler breeder hens (Muiruri et al., 1991) and broiler chickens (Reilly et al., 1991) had improved bird performance with access to cooled perches during high temperatures (Muiruri and Harrison, 1991; Reilly et al., 1991). In contrast, Estevez et al. (2002) did not find a significant difference in mean BW of broilers with access to cool perches. However, broiler chickens in a more recent study (Zhao et al., 2012) reported an increase in BW with a greater use of cool perches during the summer. Broilers with cooled roost access had greater live weight, breast meat weight and roost usage, lower mortality, and lower feed-to-gain ratios than ambient roost and floor birds (Okelo et al., 2003). Additionally, cooled perch access impacted broilers subjected to HS by decreasing panting frequency and reducing rectal temperatures (Zhao et al., 2012). Cooled perch availability increased BW gain and feed conversion efficiency of broilers in high ambient temperatures regardless of stocking density (Zhao et al., 2013). Zhao et al. (2013) also reported birds' use of cool perches increased with age, decreased with

high stocking densities, and changed behavioral patterns. Fewer studies have looked at the effect of cooled perches on well-being in laying hens exposed to HS although Reilly and Harrison (1984) found that conductive heat transfer from the feet of laying hens to a thermally controlled perch helped relieve HS. In laying hens, perch temperature strongly influenced the bird's resting postures. Birds with access to cold perches had a higher percent of resting with their heads tucked backwards allowing for more coverage of un-feathered areas (Pickel et al., 2011). Zone cooling, as opposed to whole-house cooling, during hot weather may be effective in relieving the negative effects of HS in chickens.

Cattle

Physiological and Behavioral Responses

The TNZ for dairy cows is between 5°C and 25°C (Roenfeldt, 1998). At ambient temperatures above 26°C, a cow reaches a point where she can no longer cool adequately and eventually enters heat stress. The body temperature of dairy cattle shows great susceptibility to high temperatures (Akari et al., 1984) as increasing rectal temperatures when THI is greater than 80 (Lemerle and Goddard 1986). The respiratory rate of cattle begins to increase when THI is 73 and more abruptly when THI is greater than 80 (Lemerle and Goddard 1986). Behavioral and physiological responses are initiated in order to reduce heat production and increase heat loss to maintain body temperature within normal ranges. There are two main mechanisms used by dairy cows to increase the amount of heat loss from the skin when HS causes an increase in internal heat production. The first is dilatation of the blood vessels in

the dermis so that more blood flows towards the skin's surface and heat can be lost to the environment. The second is by sweat production from the sweat glands. Cattle can tolerate higher temperatures at lower relative humidity than poultry, by dissipating excessive heat more effectively by sweating, whereas poultry do not have sweat glands. Homeostatic mechanisms are the initial responses and include increased sweating and respiration rates (McLean, 1963), as well as reduced heart rate and feed intake (Horowitz, 2002). Cows exposed to HS have higher rectal temperatures and elevated respiration rates compared with cooled cows (Ominski et al., 2002; do Amaral et al., 2011). Dairy cattle also spend less time lying when exposed to hot temperatures (Overton et al., 2002; Legrand et al., 2011). Additionally, cows would choose to stand in the shade instead of lying during warm conditions even when they have been deprived of lying for the previous 12 h (Schütz et al., 2008). Tucker et al. (2008) found that time spent standing increased by 10% when heat load increased by 15%, suggesting that cows spend more time standing to increase heat loss by increasing the surface amount of skin exposed to air flow or wind.

Cows under HS have an increase in insulin concentrations and glucose clearance (Wheelock et al., 2010). Prenatal stress has been shown to alter the HPA response to stress in calves (Lay et al., 1996). Upon activation of the HPA axis by HS, corticotrophin releasing hormone is produced and secreted, which then stimulates the endocrine cells in the anterior pituitary to synthesize and secret adrenocorticotropic hormone (ACTH) into the systemic circulation (Marketon and Glaser, 2008). Pituitary ACTH travels through the blood to the adrenal cortex, where it induces cells to secrete glucocorticoids (Fulford and Harbuz, 2005). Glucocorticoids have been

shown to suppress the synthesis and release of cytokines, such as IL-6 and TNF-α (Richards et al., 2001). A previous study showed that activation of the sympathetic nervous system also suppresses the function of dendritic cells and monocytes by inhibiting the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF (Benschop et al., 1994). The continued activity on the HPA axis may be related to the effects of prenatal stress on behavior and learning responses resulting in inhibited behavioral and anxiety (Braastad, 1998).

Production Responses

The negative effects of HS in dairy cattle are well documented (West, 2003; Berman, 2005). HS reduces milk production, reproductive performance, and profit (St-Pierre et al., 2003; Bohmanova et al., 2007; Boonkum et al., 2011). HS dramatically decreased milk production during lactation (Collier et al., 2006). In the U.S, there was an annual loss of $5 to $6 billion attributed to HS in dairy cows (Ray et al., 1992). In dairy cattle, HS consistently results in reduced dry matter intake (DMI) (Adin et al., 2009). As a result of the reduced energy intake, cows under HS have lower BW gain in late gestation compared to those cows with heat abatement (do Amaral et al., 2009). In mid lactation, heat-stressed cows experience negative energy balance and use glucose as the main source of energy for milk synthesis (Wheelock et al., 2010). In contrast, heat-stressed cows under late gestation stay in positive energy balance (Kim et al., 2010) and do not have the same observed metabolic responses as cows under HS during mid-lactation (Tao et al., 2012b).

During HS, the body temperature of a cow increases and feed intake decreases thereby decreasing milk production (Coppock and West, 1986). Due to their high metabolic rate associated with milk production, modern high-producing dairy cows are more vulnerable to HS (Bianca, 1965). Compared to lactating cows, dry cows generate less metabolic heat (West, 2003) and have a higher upper critical temperature (Hahn, 1999). However, the effects of HS on the performance of cows during late gestation can be carried over to the next lactation (do Amaral et al., 2009). Cooling cows during the dry period increased milk production in the subsequent lactation (Adin et al., 2009). The milk yield response in the next lactation depends on the method and duration of cooling of the dry dairy cow, such as short-interval soaking in the middle of the day which only modestly increased milk production (Avendañ̃o-Reyes et al., 2006), or a more extensive method, including shade, fans, and sprinklers, which caused a more significant increase in milk yield (do Amaral et al., 2011). It is possible that the decrease in milk production in stressed cows could be due to a compromise in mammary growth induced by HS. Heat-stressed cows have decreases mammary cell proliferation relative to calving compared with cooled herd mates (Tao et al, 2011). Although the physiological mechanisms related to compromised gland development during the dry period under HS are not clear, it may be caused by hormone synthesis. Heat-stressed cows had greater concentrations of circulating prolactin relative to calving (do Amaral et. al., 2011), but a decrease in prolactin receptor gene expression in the liver (do Amaral et. al., 2010). Additionally, treatments did not affect circulating insulin, but cooled cows had decreased glucose and lower insulin concentrations in plasma postpartum compared with HS cows (Tao

et. al., 2012b). Thus, HS not only has effects on milk production, but also physiological parameters, as well.

Greater incidences of mastitis occur in heat stressed cows compared to cows in a thermal neutral environment (Gaughan et al., 2009). They theorized that heat exposure could cause the development of pathogens responsible for mastitis and may have negative effects on the animals' immune response. Hot environments also decrease the birth weights of calves (Collier et al., 1982). Another direct effect of HS includes thermal-related death during heat wave events. Overall, months with average daily temperatures greater than 24°C showed substantial increases in both calf and cow mortality with calf mortality being more sensitive to the thermal changes than cow mortality (Stull et al., 2008).

Hansen (2009) reviewed the literature and concluded HS had numerous detrimental effects on reproduction. Seasonal high ambient temperatures are associated with low reproductive performance in dairy and beef cows (Wolfenson, 2009). On average, conception rate drops by 24% during summertime (Bernabucci et al., 2010). This decrease in fertility is caused by an impaired ovarian function, lower expression of estrus, damaged oocyte health, and inhibition of embryonic development (Wolfenson, 2009). Garcı´a-Ispierto et al. (2006) indicated that HS can increase early fetal loss with each additional unit of THI from days 21 to 30 of gestation. Depending on the intensity of heat exposure, conception rates can drop to 10-20% in hotter months to 40-60% in cooler (Cavestany et. al., 1985). Oocyte competence for fertilization and subsequent development is reduced due to HS (Sartori et. al., 2002). HS can also alter follicular growth (Roth et al., 2000) and gene

expression (Argov et. al., 2005). Thompson and Dahl (2012) found that cows dried off in hot months had greater incidence of health disorders in early lactation and poorer reproductive performance compared with those dried in cool months.

Maternal Heat Stress Affects Offspring

Prenatal stress occurs when the offspring is affected by the stress of the dam before birth. Maternal HS during late gestation is a type of prenatal stress which affects the fetus and carryovers on to the offspring in postnatal life. There have been few studies in dairy cattle, but related research in other farm animals may be informative for dairy cattle. HS during late gestation decreases not only gestation length and birth weight of the offspring, but also placental weight (Bell et al., 1989). Lundborg et al. (2003) found associations between dam-related effects on heart girth at birth, morbidity, and growth rate in dairy calves, indicating the importance of health and nutritional status of the dam during late pregnancy. Tao and Dahl (2013) reviewed the literature and concluded maternal HS during late gestation compromises placental development and fetal growth of the offspring.

Wolfenson et al. (2000) reviewed the literature and concluded that most components of the reproduction system have been found to be susceptible to HS. In dairy cattle, the fetus grows at the fastest rate and accumulates 60% of its birth weight during the last 2 months of gestation (Bauman and Currie, 1980). In later pregnancy, intrauterine hyperthermia is associated with intrauterine growth retardation (Dreiling et al., 1991). Fetal and placental weights and total content of protein were reduced in heat stressed ewes (Early et al., 1991). Although, maternal HS during late gestation in

cows resulted in a shorter gestation length and lower birth weight (do Amaral et al., 2009; Chen et al., 2010), there was no effect on calf birth weight after a moderate decrease in energy intake in late gestation (Janovick and Drackley, 2010).

During gestation, the fetus is exposed to the environment of its dam uterus; hence it is subjected to her thermoregulation, whereas its own thermoregulation is inhibited during this period. In sheep and goats, during late gestation, normally fetal body temperature is higher than maternal core body temperature (Faurie et al., 2001). In contrast, Laburn et al. (2002) found that body temperature was raised less in the fetus than in the mother during HS, indicating that the fetus is granted thermal protection when the mother animal experiences thermal stress. Therefore, fetal body temperature still increases dramatically under maternal HS, just not at the same rate as the dam.

Similar to other stressors, such as environmental stress, psychological stress, and social stress, HS compromises fetal development and postnatal immunity (Merlot et al., 2008). A greater cortisol concentration in heat-stressed sows is associated with their piglets having higher serum cortisol at birth (Machado-Neto et al., 1987). Tao et al. (2012a) investigated the effect of dam HS during the final stage of gestation on the growth of calves. Calves that were born to cows exposed to cooling had greater birth weight than calves from HS cows. Moreover, the sheep fetus had lower circulating glucose and insulin after maternal HS (Thorn et al., 2009) but higher levels of epinephrine and norepinephrine concentrations (Leos et al., 2010). Thus, concluding that maternal HS has many effects on the offspring's development.

Immunocompetence of Dam

It is well known that the survivability and proliferation of bacteria are favored in hot environments (Money et al., 2010). HS has been shown to have detrimental effects on the immune system of cattle making them more susceptible to disease (Lacetera et al., 2005, 2006; do Amaral et al., 2009). However, Urdaz et al. (2006) investigated the relationship between HS during late gestation and the presence of disease postpartum and found that cows under HS showed similar incidences of diseases as the cooled treatment. In contrast, a more recent study concluded cows dried off in hotter months had a higher incidence of mastitis and respiratory problems than those dried off in cool months (Dahl et al., 2012). Hence late-gestation HS influences the health and immune function of dairy cattle.

The immune system includes the primary nonspecific innate immune response and the specific adaptive immune function. Innate immunity is triggered by recognition of the pathogen, for example, by a complex of TLR4 and cluster of differentiation (CD) 14 at the cell surface, which then signals the release of cytokines from macrophages (Gioannini and Weiss, 2007). Cytokines produced by activated macrophages in response to bacterial products include IL-1, IL-6 and TNF-α. IL-1 and IL-6 activate lymphocytes and increase antibody production. TNF-α activates vascular endothelium and increases vascular permeability, which leads to increased effector cells immigrating to the site of infection. All three cytokines play critical roles in inducing the acute-phase response, which benefit effective host defense by activating phagocytic cells (Möller and Villiger, 2006). CD18 is another cell surface molecule playing a role in recognition, which binds adhesion molecules forming a

tight interaction needed for diapedesis (Lee and Corry, 2004). Following the arrival to the site of infection, neutrophils, along with macrophages, act as potent killers of invading microorganisms through phagocytosis and oxidative burst (Mayer, 2006). Therefore, innate immunity can be evaluated through the ability of neutrophils to phagocytize and destroy pathogens. Neutrophil phagocytosis and oxidative burst were greater in cows with heat abatement relative to calving compared with heat stressed cows, suggesting that heat abatement during the dry period improved the innate immune status (do Amaral et al., 2011). HS was shown to also impair the acquired immune function as measured by a reduced lymphocyte proliferation (Lacetera et al., 2006). IgG secretion was improved with heat abatement in cows under HS (do Amaral et al., 2011). Cytokine gene expression has also been evaluated. Cows under HS had an increase in TNF-α gene expression during the transitional period (Tao et al., 2013). The increase in cytokines reflects an adaptive response before calving given the fact that inflammatory cytokines are related to the stress-induced acute-phase response in cattle (Lomborg et al., 2008). However, when non-cooled cows exposed to HS encounter a pathogen, there may be a decrease in immunological responses (do Amaral et al., 2010).

Moreover, the mechanism whereby HS affects the immune system may be mediated through changes in the prolactin signaling pathways. Prolactin signals modulate both the innate and adaptive immune function (Lopez-Meza et al., 2010). For example, the greater concentrations of prolactin in plasma of cows exposed to HS were associated with reduced lymphocyte proliferation compared with cooled cows caused by the binding of the prolactin to their receptors (do Amaral et al., 2010). In

addition to the HS effects on the dam's immune system, HS also has numerous effects on calf immunity.

Immunocompetence of Calf

Cortese (2009) reviewed the literature on neonatal immunology and suggested the combination of passive and active immunity together provide the protection to the neonate. The fetus becomes immune competent while in utero to a variety of diseases (Casaro et al., 1971; Pare et al., 1998; Ellsworth et al., 2006). Lymphocytes develop and differentiate during gestation. In general, the shorter the gestation period, the less developed the immune system is at birth (Halliwell and Gorman, 1989). Normally, the immune system is fully developed at birth in the neonate although it is still unprimed (Tizard, 1992) causing the calf to become susceptible to pathogens at birth. The fetus is protected primarily by the innate immune system, but its phagocytic activity is not fully developed until late in gestation (Barrington, 2001). Although the number of phagocytic cells is abundant in the neonate, there is a decrease in function in these cells, which can occur up to 4 months of age in the calf (Hawser et al., 1986). The calf will have all essential immune components at birth, but most of them are not functional until at least 2 to 4 weeks of age and some will continue to develop until puberty (Reber et al., 2006). In cattle, T cells do not reach peak levels until the animal is 8 months of age. This does not mean the calf cannot respond to antigens, but the response is weaker and slower. Total T cells account for 28 to 34% of total lymphocytes; 20% are helper T cells (CD4) and 10% are cytotoxic T cells (CD8) (Kampen et al., 2006). B cells in the fetus only make up 1% of total lymphocytes

compared to the 4% at one week after birth and to the 10% in mature calves (Kampen et al., 2006). This results in the lack of any endogenous antibody response until 2 to 4 weeks of age making the ingestion of colostrum extremely important in providing an immunologic defense to the calf during the first 2 to 4 weeks of life (Chase et al., 2008).

There is no trans-placental transfer of antibodies in cattle causing calves to be born nearly agammaglobulinemic. Therefore, ingestion of colostrum to obtain immunoglobulins for protection against infectious diseases (Barrington, 2001) becomes a major role in the newborn calf's defense mechanism. Colostrum is defined as the first secretions from the mammary gland present after birth. Adequate colostrum transfer has been recognized to have beneficial effects on the calf's immune response during early life (Furman-Fratczak et al., 2011). Constituents of colostrum include concentrated levels of antibodies and many immune cells. Although colostrum contains several types of immunoglobulins (IgG, IgA, and IgM), IgG constitutes approximately 85% of the immunoglobulins in colostrum (Butler, 1983). Neutrophils and macrophages have reduced phagocytic capacity in neonates, but this is increased after the ingestion of colostrum (Menge et al., 1998). It has been demonstrated that feeding colostrum containing maternal leukocytes to the neonatal calf accelerates the activation of lymphocytes in the calf (Reber et al., 2008) and enhances the development of antigen-presenting function (Reber et al., 2005). The cells are known to enhance defense mechanisms in the newborn through transfer of cell-mediated immunity, passive transfer of antibodies, local phagocytic activity in the digestive tract, and increased lymphocyte activity (Duhamel, 1993).

A calf's gastrointestinal tract is designed to temporarily allow the absorption of large molecules, including immunoglobulins, during the first 48 h of life after intake of colostrum (Besser et al., 1985). The absorptive capacity begins to decrease after 6 to 12 h of birth and ends by 48 h probably as a result of developmental processes occurring in the enterocytes (Sangild, 2003). Failure of passive transfer of immunity (FPTI) occurs when the calf fails to absorb an adequate quantity of immunoglobulin. FPTI has been linked with increased calf morbidity and mortality and a reduction in calf growth rate (Donovan et al., 1998). Rajaraman et al. (1997) demonstrated that there is a decrease in the immune response of neonatal calves up to d 3, reaching the lowest level. By d 5, their responses are back to the level of immune response seen at birth.

Moreover, cytokines present in the colostrum are very important for the development of the calf immune system. A study with pigs demonstrated that cytokines from the colostrum are absorbed and can be detected in the blood of the piglet (Nguyen et al., 2007). Among cytokines present in bovine colostrum, IL-1β, IL-6, and TNF-α are associated with pro-inflammatory responses and may aid in the recruitment of neonatal lymphocytes into the gut, thereby promoting normal immune development (Chase et al., 2008).

Neonatal corticosteroid levels must be high in order to increase colostrum absorption (Sangild, 2003). Therefore, situations such as cold stress, HS, dystocia, and premature birth that inhibit the release of cortisol by the neonate, also lead to inhibition of absorption of colostrum (Chase et al., 2008). Prenatal stressors also modify T- and B- cell function of the offspring (Merlot et al., 2008). Donovan et al.

(1986) observed lower plasma Ig concentrations in calves during summer months in Florida and higher mortality of those calves up to 6 months of age. In addition to compromising placental development and fetal growth of the offspring, maternal HS during late gestation has been shown to affect the immune function. Sows under HS during the last 2 wk of gestation had piglets with lower circulating IgG in colostrum compared to piglets from sows under a thermo neutral environment (Machado-Neto et al., 1987). Tao et al. (2012a) investigated the effect of dam HS during the final stage of gestation on the immune function of calves. Calves from HS cows had a lower serum IgG concentration compared to calves born to cooled cows during late gestation. Additionally, calves from uncooled cows had a lower proliferation rate indicating an impaired lymphocyte function after prenatal HS, but had no difference in anti-ovalbumin antibody production suggesting there was no influence on B-cell function (Tao et al., 2012a).

Alleviating Negative Effects of Heat Stress

Beede and Collier (1986) identified three management strategies to minimize the negative effects of HS including physical modification of the environment, genetic development of heat-tolerant breeds, and nutritional management practices. Shade is effective in protecting cows from solar radiation, but does not alter the air temperature or relative humidity around the cows to maximize sensible routes of heat loss (West, 2003). Shade usage was increased in cows exposed to increasing ambient air temperatures and solar radiation (Kendall et al., 2006). Studies comparing shade with no shade demonstrated improved milk yield and reproduction, as well as reduced

respiration rate (Kendall et al., 2007) and rectal temperature in shaded dairy cows (Collier et al., 2006). Schütz et al. (2011) concluded dairy cattle prefer to use shade in summer despite sprinklers being more efficient in decreasing heat load and insect avoidance behavior. During summer months, shaded cows had lower respiration rates and core body temperature than cows without shade (Blackshaw and Blackshaw, 1994). However, the benefit of shade on dairy cow performance depends on the breed (Collier et al., 1981) and coat color (Blackshaw and Blackshaw, 1994), and geographic location. Shade reduces heat load of cattle (Gaughan et al., 2010) and mortality in extreme weather conditions (Busby and Loy, 1996). In West Texas, feed intake and growth performance were significantly increased when shade was provided to feedlot cattle (Mitlohner et al., 2001).

Additionally, cooling systems using the principle of evaporation, combining water misting and forced ventilation through use of spray and fans, are used to alleviate heat load on dairy cows. The cooling system was shown to improve milk production and reproductive performance in cattle (Ryan et al., 1992). Dairy cattle allowed access to sprinklers had increased milk production (Turner et al., 1992), improved reproduction and improved conversion of feed to milk (Wolfenson, 2009). Cattle also utilize sprinkler systems to reduce body heat when they stand to eat. These systems have been shown to decrease milk yield loss, decrease mortality in postparturient cows, improve reproductive parameters, and improve appetite (Shultz and Morrison, 1987). Kendall et al. (2007) found that shade and sprinklers reduce respiration rate and body temperature in dairy cattle when THI was greater than 69.

In cattle, slick hair coats play an important role in heat tolerance (Olson et al., 2003). *Bos taurus-type* cattle are less adapted to tropical and subtropical climates than *B. indicus* or Zebu-type cattle. Compared to *Bos taurus* cattle, Sharma et al. (1983) reported that Jerseys were more resistant to HS effects on milk production than Holsteins. In a more recent study, an interaction between breed and temperature effected respiration rates and panting scores (Brown-Brandl et al., 2006). Angus and MARC III breeds had the highest respiration rate and panting score, followed by Gelbvieh, then Charolais. These results were likely due to the hide color differences that affected the adsorption of solar radiation. The effects of HS that may be experienced during high ambient temperatures appear to be restored when night temperatures fall (Akari et al., 1987), thus suggesting short-term tolerance of HS for cows under commercial production conditions.

West (2003) reviewed the nutritional strategies for managing dairy cows under HS and concluded nutritional strategies that support yield, metabolic, and physiologic disturbances induced by HS will help the cow maintain a normal metabolism leading to an enhanced performance. Additionally, as parturition approaches, diets with less roughage given 3 wk before calving may reduce the decline in DMI and lipid mobilization (Kanjanapruthipong et al., 2010). Moreover, supplementation of Niacin (vitamin B_3) is known to increase peripheral vasodilation to increase sweat gland activity in dairy cattle (Di Constanzo et al., 1997). Zimbelman et al. (2013) followed with a recent study establishing lactating cows with Niacin reduced vaginal temperature, but had different effects on milk production depending on the period of study which may be due to a more severe HS in period 1 or a more advanced stage of

lactation in period 2. Supplemental saturated fatty acids improved milk yield and milk fat content and reduced peak rectal temperatures in mid-lactation heat-stressed dairy cows (Wang et. al., 2010). Friedman et al. (2012) concluded adding progesterone post insemination improved fertility of cooled dairy cows during the summer months, which suggest low progesterone concentration may be associated with reduced fertility during HS.

Summary

Heat stress has detrimental effects on the immune system in both poultry and cattle. Thus, the research conducted herein strived to determine the effect of HS on the immune response in laying hens and dairy cattle. Through the first experiment, access to cooled perches were used to determine their efficiency in reducing the negative effects of both acute HS and high ambient temperatures on laying hens throughout the summer months. The second experiment studied the indirect HS from the dam and whether it affected the offspring's immune system. Collectively, the experiments allowed an overall comparison of how HS affects the immune system in avian and ruminant species.

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CHAPTER II

THE EFFECT OF COOLED PERCHES INSTALLED IN CAGES ON IMMUNE RESPONSE IN WHITE LEGHORN HENS DURING HEAT STRESS

Abstract

Heat stress (HS) is a common immune modulator across many species. The objective of this study was to determine if thermally cooled perches installed in conventional cages improve hen immunity during hot months of summer. White Leghorn chickens, 16 wk of age, were randomly assigned to 18 cages with 9 hens per cage. Cages were arranged as 3 banks with 3 tiers of cages per bank and 2 cages per tier. Each bank was assigned to 1 of the following treatments: 1) cooled perches (CP), 2) perches with ambient air (AP), and 3) cages without perches (NP). Hens were subjected to ambient temperatures throughout the experimental period from 16 to 32 wk of age with the exception of 4 h of an acute heating episode at 33.3°C at 27 wk of age. The study was conducted from June through September in Indiana (18-33°C). The heterophil to lymphocyte (H/L) ratio, plasma concentrations of total IgY and the cytokines of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF- α) were determined after the acute HS episode and at 32 wk of age. The mRNA expressions of these cytokines as well as toll-like receptor-4 (TLR-4) and inducible nitric oxide synthase (iNOS) were examined in the spleen of 32 wk-old hens. Cooled perches were not successful in improving any physiological indicator of stress perhaps because the HS was too mild or insufficient in length during 2013 summer. However, CP hens had a lower H/L ratio than AP or NP at 27 wk of age ($P < 0.01$) and it was still lower compared to NP hens ($P < 0.05$) at 32 wk of age, but not to AP hens. The lowered H/L ratio of CP hens suggests that they were able to cope with acute HS more effectively than hens without cooled perches.

Key words: cooled perch, heat stress, immunity, hen, White Leghorn

Introduction

During summer, heat stress (HS) is predictably one of the main environmental stressors adversely affecting laying hens. When adult chickens are subjected to ambient temperatures greater than 37.8ºC, more heat gain than heat loss occurs leading to an increase in core body temperature, and possibly death (Sahin et al., 2009; Felver-Gant, 2012). In laying hens, HS reduces BW gain (Scott and Balnave, 1988), feed intake (Franco-Jimenez et al., 2007), egg production (Whitehead et al., 1998), and egg quality (Mahmoud et al., 1996).

It is well established that high environmental temperatures affect the immune function in chickens causing immunosuppression (Subba Rao et al., 1970; Thaxton and Siegel, 1972; Niu et al., 2009). The degree of heat-induced immunosuppression depends on the breed and strain of the chickens (Regnier et al, 1980) as well as the length and intensity of the heat exposure (Henken et al., 1982; Kelley, 1983). In chickens, HS limits immunocompetence by suppressing antibody production (Mashaly et al., 2004) and altering the populations of immune cells, leading to an increase in the heterophil to lymphocyte (H/L) ratio which is used as an indicator of stress (Thaxton et al., 1968; Gross and Siegel, 1983; McFarlane and Curtis, 1989; Soleimani et al., 2011). With chickens having only rudimentary lymph nodes, they rely heavily on the bursa of Fabricius and the spleen as major immune organs. The bursa of Fabricius plays a key role in the development of the antibody-producing B lymphocytes (Mustonen et al., 2010) and the spleen is an important site for immune responses to antigens (Jeurissen, 1993). Hens with greater spleen weight have higher immunocompetence (Cheng et al., 2004). In addition, SW decreased in laying hens (Felver-Gant, 2012; Ghazi et al., 2012) and

broilers (Niu et al., 2009; Quinteiro-Filho et al., 2010) after exposure to high temperatures. An additional indicator of stress besides lymphoid organ regression is the production of pro-inflammatory cytokines (Kang et al. 2011); as immune mediators involved in cell signaling they are synthesized by a variety of cells including B- and T-cell lymphocytes. Cytokines play an important role in regulating host immunity by coordinating the humoral (B-cell) and cell mediated immune (T-cell) responses. Examples of cytokines include the interleukin (IL) family and tumor necrosis factor-alpha (TNF-α) produced by several immune cells including CD4 T-helper cells. The IL-1 cytokine stimulates B- and T-cell development and differentiation. The TNF-α is synthesized by monocytes and acts as a cytotoxin promoting apoptosis leading to tumor regression. In addition to cytokines, there are 10 toll-like receptors (TLR) identified in the chicken (Kannaki et al., 2010). One of them being TLR-4 found on the membranes of macrophages and dendritic cells, responsible for recognizing conserved sections of invading antigens triggering the activation of immune cells. In addition, a non-specific immune defense mechanism that hens may use during stressful conditions is nitric acid. Nitric oxide synthases consist of a family of enzymes responding for producing nitric oxide from L-arginine. Because of an unpaired electron, the nitric oxide produced by the inducible isoform of nitric oxide synthase (iNOS) acts as a free radical, attacking and destroying antigens such as viruses, bacteria, tumors, and parasites. Nitric oxide also influences inflammatory responses. There are many types of cells that synthesize iNOS in response to cytokines (Guzik et al, 2003; Li et al., 2009).

The majority of hens are currently housed in conventional cages in the United States; however, egg producers are updating their facilities with cages that can eventually

be enriched with a nest, perches, scratch pad, and/or nail trim area. Introducing perches stimulates a variety of motor patterns (Bizeray et al., 2002) causing an increase in bone strength in laying hens (Appleby and Hughes, 1990; Duncan et al., 1992; Hester, 2014). Allowing access to cooled perches during the summer may help alleviate HS for laying hens during times of increased environmental temperatures. Hens have a natural tendency to perch for resting and protection. More than 25% of the heat produced by chickens can be lost through their feet by modulating blood flow (Hillman and Scott, 1989). Increased conductive heat transfer from the feet to a thermally controlled perch helped to relieve HS in laying hens (Reilly and Harrison, 1988). Broiler breeder hens (Muiruri et al., 1991) and broiler chickens (Reilly et al., 1991) had improved bird performance with access to cooled perches during high temperatures (Muiruri and Harrison, 1991; Reilly et al., 1991). Broilers subjected to HS with access to cooled perches exhibited less panting and had less change of core body temperatures and increased BW (Zhao et al., 2012). Cooled perch availability increased BW gain and feed efficiency of broilers in high ambient temperatures regardless of stocking density (Zhao et al., 2013). In laying hens, perch temperature strongly influenced the bird's resting postures. Birds with access to cold perches had a higher percent of resting with their heads tucked backwards allowing for more coverage of un-feathered areas; indicating these birds were not subjected to HS (Pickel et al., 2011).

Currently, there is no study on cooled perches that evaluate the physiological responses of laying hens when exposed to high ambient temperatures. Therefore, the aim of the present study was to determine if cooled perches inhibit heat stress-induced immunological suppression in caged laying hens. Our hypothesis was that chickens with

cooled perches during hot weather will exhibit improved well-being indicators of decreased H/L ratios, down-regulation of splenic cytokines, TLR-4, and iNOS; with greater spleen weights and plasma IgY concentrations as compared to hens without access to cooled perches.

Materials and Methods

Birds and Management

In the current study, 172 Hy-Line W36 White Leghorn female chickens at 16 wk of age were transported to the Layer Research Unit at Purdue University's Poultry Research Farm. The 162 healthy hens from the 172 hens with similar BW were leg tagged, individually weighed, and randomly assigned to layer cages at 9 hens per cage. Feeder and floor space allotments from 16 wk of age to the end of the study at 32 wk of age were 8.4 cm and 439 cm^2 per hen, respectively. Two nipple drinkers were assigned to each laying cage. Dropping boards were located between tiers of cages. The manure was scraped from the boards as needed.

A pre-lay diet with calculated values of 3,009 kcal ME/ kg, 20.0% CP, 1.0% Ca, and 0.45% non-phytate P was fed from 16 to 17 wk of age. At 17 wk of age, chickens consumed a layer diet with 2,890 kcal ME/kg, 18.3% CP, 4.2% Ca, and 0.30% non-phytate P until the end of the study at 32 wk of age. Throughout the study, hens had free access to feed and water.

At 16 wk of age, light hours were gradually increased, achieving a photoperiod of 16L: 8D by 30 wk of age, where it remained until termination of the study. The protocol was approved by the Purdue University Animal Care and Use Committee.

Treatments

One of 3 banks of cages was assigned randomly to 1 of the following treatments: (1) thermally cooled perches (CP), (2) perches with ambient air (AP), and (3) conventional cages without perches (NP). Within a bank of cages, there were 3 tiers (decks) with 2 cages per tier for a total of 6 cages per bank. For the bank of cages assigned the cooled perch treatment, each tier had its own pump to distribute chilled water (10ºC) through the round galvanized steel pipe, 3.38 cm outside diameter (the perch), that ran parallel to the feeder. Holes were cut in the cage walls to allow for the passage of the perch pipe that was arranged in a loop. One loop was used for each tier. This looped arrangement provided 2 perches per cage giving each hen 16.9 cm of perch space. The cage dimensions and perch placement within the laying cages were reported previously (Hester et al., 2013; Figure 2.1). Perch height was 8.9 cm from the cage floor so the hens could perch without their heads hitting the cage ceiling and eggs could roll underneath the perches to the collection area. The front perch closest to the feeder received chilled water pumped directly from a common vertical manifold which was constructed of 13 cm polyvinyl chloride pipe that was 1.70 m tall (Figure 2.2). The back perch was the return loop that sent the water back to the common manifold to be re-chilled. A chiller was used to cool the water in the manifold; it had its own independent thermostat which kept the water at 10ºC. A separate 4th pump continuously circulated the deionized water between the water chiller and the manifold. A sensor for monitoring air temperature was installed to the controller of each tier to activate the circulation of chilled water through the perch loop when ambient temperature reached 25ºC or to stop circulation of water when the ambient temperature fell below 25ºC. The bank of cages

that was randomly assigned the air perches were also arranged in a loop system as described for the cooled perches, but no water was pumped through these perches. Air temperature and relative humidity within the room, the cage, the water temperature of supply and return lines of the cooled perches, and the air temperature of the air perches were recorded using a wireless monitoring system (Gates et al., 2014).

Evaporative cooling pads were not used at any time during the study to allow hens exposed naturally to hot summer days. The study was conducted in 2013 from June through September in Indiana. Chickens were exposed to ambient temperature throughout the study with the exception of an acute heating episode where the ambient temperature was elevated to a mean of 33.3ºC for 4 h at 27 wk of age by providing auxiliary heat. The heating episode was initiated 9 h following the beginning of their daily photoperiod after most hens had laid their eggs for that day.

Sampling

Two hens without eggs in their uterus, as determined through palpation, were randomly taken from each cage for sampling at 27 wk of age. The order of sampling was 1 hen per cage, beginning with CP, then AP, and finally NP treatment. The sampling process was repeated using the same order of treatments but using a different hen from each cage. The sample collection began at 2 h post initiation of the 4 h acute heating episode to ensure all samples were collected within the acute HS period. A 4 mL blood sample was collected from each bird via the brachial vein within 2 min of being handled. The blood was collected into K3 EDTA-coated test tubes. A leg ring was placed on the right shank before returning the hen to its cage.

At 32 wk, 2 hens per cage without a leg ring were randomly removed from their cage and sampled using the same sequence order as described for the blood collection at 27 wk of age. Hens were sedated by injecting 30 mg of sodium pentobarbital/kg of BW into the brachial vein. A 5 mL blood sample was collected into EDTA-coated test tubes from each hen by cardiac puncture. The hen was immediately killed by cervical dislocation and its spleen was collected and weighed, then stored at -80°C until further analysis.

All blood samples were stored on ice and transported to the laboratory to be centrifuged at 700 x g for 20 min at 4°C. The supernatant plasma was collected and stored at -80°C until analysis.

Quantitative Analysis of Blood Parameters

Immediately following blood collection, duplicate blood smears were made per hen by generating a thin layer of cells along the slide at both 27 wk and 32 wk of age. After drying overnight, the slides were stained with Wright's staining. Through light microscopy, heterophils and lymphocytes were differentiated based on a previous counting method (Walberg, 2001). Briefly, 100 white blood cells per slide, total 200 cells per hen, were counted, then the H/L ratio was calculated (Cheng et al., 2001b).

Enzyme-Linked Immuno-Sorbent Assay

Cytokines of IL-1β, IL-6, and TNF-α were measured using a commercially available ELISA kits (Life Sciences Advanced Technologies Inc.; St Petersburg, FL, USA; IL-1β, Catalog No. CSB-E11230Ch; IL-6, Catalog No. CSB-E08549Ch; TNF-α,

Catalog No. CSB-E11231Ch). The sample wells for IL-1β were pre-coated with goat-anti-rabbit antibody. 50 µL of the concentrated plasma sample was added to the corresponding well. Reactions were initiated by adding 50 µL HRP (horseradish peroxidase) and 50 µL of chicken IL-1β antibody to each well. After 60 min of incubation at 37ºC and followed 3 washes, 50 µL of substrate A and 50 µL of substrate B were added to each well. The reaction was stopped by adding 50 µL of stop solution (dilute sulfuric acid). Samples were analyzed in duplicate with absorbance readings of 450 nm. Curves were standardized through comparison with 0.5 ml of Chicken IL-1β standard at concentrations of 0, 1, 4, 16, 50, and 200. IL-1β present in sample plasma was reported at pg/ml.

A chicken antibody specific to IL-6 was pre-coated onto the sample wells. 100 µL of diluted plasma samples (1:10,000) were added to the corresponding well. After 2 h of incubation at 37ºC, the liquid was removed and 100 µL of Biotin-antibody (1x) was added to each well. After an additional 1 h incubation at 37ºC, the plate was washed 3 times and then 100 µL of HRP-avidin (1x) was added to each well. After 5 washes, 90 µL of TMB Substrate was added to each well. The reaction was stopped by adding 50 µL of stop solution to each well after 15 min of incubation at 37ºC. Samples were analyzed in duplicate with absorbance readings of 450 nm. Curves were standardized through comparison with 250 µL of chicken IL-6 standard at concentrations of 0, 15.6, 31.2, 62.5, 125, 250, 500, and 1000. IL-6 present in sample plasma was reported at mg/ml.

The sample wells for TNF-α were pre-coated with chicken-specific TNF-α. 50 µL of the concentrated plasma sample was added to the corresponding well. Followed by the addition of 50 µL HRP (horseradish peroxidase) to each well, there was a 40 min

incubation period at 37ºC. After 5 washes with included wash buffer, 50 µL of TMB Substrate was added to each well. The reaction was stopped by adding 50 µL of stop solution to each well after another 20 min incubation period at 37ºC. Samples were analyzed in duplicate with absorbance readings of 450 nm. Curves were standardized through comparison with 100 µL of chicken TNF-α standard at concentrations of 0, 0.27, 0.82, 2.47, 7.41, 22.2, 66.7, and 200. TNF-α present in sample plasma was reported at pg/ml.

Immunoglobulin Y was measured using a commercially available ELISA kit. (Bethyl Laboratories, Inc.; Montgomery, TX, USA; IgG Catalog No. E33-104). Plasma samples were initially diluted to 1:100,000 with included sample buffer (1X dilution buffer B). Sample wells were pre-coated with anti-chicken IgY antibody. Reactions were initiated by adding 100 µL of chicken IgY detection antibody to each well. Followed by the additions of 100 µL SA-HRP (streptavidin-conjugated horseradish peroxidase) and 100 µL TMB Substrate Solution (3,3',5,5'-tetramethylbenzidine) between washes. The reaction was stopped by adding 100 µL of stop solution (dilute sulfuric acid). Samples were analyzed in duplicate with absorbance readings of 450 nm. Curves were standardized through comparison with 500 ng/ml of chicken IgY standard through a serial dilution. IgY present in sample plasma was reported at mg/ml.

Gene Expression

Interleukin (IL)-1β, IL-6, TNF-α, iNOS, and TLR-4 mRNA expression in the spleen were detected by real-time PCR using the primers and probes (Table 2.1) developed elsewhere (Applied Biosystems) as reported previously (Felver-Gant et al.,

2012). Data of the interested genes were expressed as ΔCt in relative abundance to the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The method in determining the mRNA expression was similar to that described by Felver-Gant et al. (2012) using the same kits, but slightly different reagent quantities. In short, after RNA extraction, reverse transcription was conducted using 61.5 µL of master mix, made of 2.5µL of Multi-Scribe reverse transcriptase, 22 µL of 25 mM MgCl, 5 µl random hexamers, 2µL RNase inhibitor, 20 µl dNTPs, and 10 µL of TaqMan reverse transcription buffer provided in the TaqMan Reverse Transcription Reagent Pack (Applied Biosystems, Foster City, CA). The 61.5 µL of master mix was then added to the quantified RNA sample and RNase-free water (Ambion Inc.) for a total of 100 µL. Reverse transcription and amplification was done using the Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO) under the same cyclic conditions previously used by Felver-Gant et al. (2012). Stock primers and probes were diluted to 10 µM solutions. The conditions for PCR were a ratio of 1.625 µL of TaqMan probe, 2.25 µL of gene- specific TaqMan forward and reverse primers each, 12.5 µL of PCR Mastermix (Applied Biosystems), 3.875 µL RNase-free water (Ambion Inc.), and 2.5 µL of sample cDNA. Cycling conditions for real-time PCR were also consistent with Felver-Gant et al. (2012). Samples were measured in duplicates and standards in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

Statistical Analysis

Data from the randomized design were subjected to an ANOVA (Steel et al., 1997) using the MIXED model procedure of the SAS Institute (2008). The fixed effect

was perch type. The cage was the experimental unit (6 cages per treatment). Subsampling error terms included tiers within bank (3 tiers per bank per treatment) and hens within cages within tiers (2 hens per cage per tier per treatment). Pooling of error terms occurred when $P > 0.25$. The data were normally distributed and reported as least square means ± SEM. Significant treatment effects were subjected to the SLICE option (Winer, 1971). Significance was set at $P < 0.05$ for all statistical analysis.

Results

There was no significant difference in spleen weight among the hens regardless of treatments ($P > 0.05$, respectively; Table 2.2).

The H/L ratio was lower in CP hens compared to both AP and NP hens at 27 wk of age following acute HS ($P < 0.01$, respectively; Figure 2.3), and the differences were continuously seen at 32 wk of age following fluctuating ambient temperatures (18-33°C; 45%) but between CP hens and NP hens only ($P < 0.05$; Figure 2.3). The H/L ratio was not different between AP and NP hens following both acute HS at 27 or 32 wk of age ($P > 0.05$, respectively; Figure 2.3).

Total IgY concentrations were not different among hens regardless of treatments after the acute HS episode at 27 wk of age ($P > 0.05$; Table 2.3); however following accumulated chronic HS at 32 wk of age, total IgY concentrations were significantly lower in both CP hens and AP hens compared to NP hens ($P < 0.05$, respectively; Table 2.3).

There were no differences in the plasma concentrations of IL-1β, IL-6, and TNF-α among treatments at 27 wk of age ($P > 0.05$; Table 2.3). In addition, there were no

treatment effects on IL-1β, IL-6, TNF-α, iNOS, and TLR-4 mRNA expression in spleen tissue among hens at 32 wk of age following accumulated HS ($P > 0.05$, respectively; Table 2.2).

Discussion

With the exception of the H/L ratio changes, giving access to thermally cooled perches during the 2013summer months had little effect on physiological parameters as indicated by spleen weight; plasma concentrations of IgY at 27 wk of age; plasma cytokines (IL-1ß, IL-6, and TNF-α) at both 27 wk and 32 wk of age); and the gene expressions of cytokines, TLR-4, and iNOS in the spleen at 32 wk of age. The mild summer of 2013 in West Lafayette, IN and the brevity of the acute heating episode (4 h) were most likely the reasons for little change in physiological parameters of hens at both 27 and 32 wk of age. However, the acute heating episode at 27 wk of age did alter the behavior of the laying hens in a parallel study. Specifically, heat exposed hens in the current study with access to thermally cooled perches showed a delay in the onset of panting and wing spreading as compared to hens with access to air perches or hens with no perches. Furthermore, once panting and wing spreading was initiated in the hens with access to cooled perches, the proportion of hens panting was always lower during and immediately following the 4 h heating episode compared to the hens with access to the air perches as well as the hens without perches (Makagon et al., 2014). These behavioral changes during the heating episode where hens sought out the cooled perches causing less panting and wing spreading helped them cope with the stressful heating events as exemplified by reduced rectal temperature as compared to hens with no perch but not the

air perch (Liedtke et al., 2014). Moreover, with the behavioral changes, the current physiological results confirm previous findings that HS causes behavioral changes to occur earlier than physiological changes in chickens (Felver-Gant et al., 2012). It agrees, in general, that an animal response to a stressor (stimulation) is to initiate appropriate behavioral responses to avoid or minimize further damage or to adjust to the hot environment, then, as a disturbing factor persists, physiological and behavioral plasticity occurs (Young et al., 1989; Duckworth, 2007).

Because chickens having only rudimentary lymph nodes, they rely heavily on the bursa of Fabricius and the spleen as major immune organs. The bursa of Fabricius plays a key role in the development of the antibody-producing B lymphocytes (Mustonen et al., 2010) and the spleen is an important site for immune responses to antigens (Jeurissen, 1993). The spleen plays an important role in antigen-presentation and storage of many immune cells. Therefore, immunosuppressed or chronically stressed birds classically have smaller lymphoid organs (Pope, 1991). Additionally, hens have been found to have higher immunocompetence with greater spleen weight (Cheng et al., 2004). As a result, spleen weight is a potential biomarker of an improved immune response during a chronic HS. Felver-Gant et al. (2012) reported HS reduced spleen weight in laying hens, coinciding with the previous findings of HS studies in broilers (Niu et al., 2009; Quinteiro-Filho et al., 2010). In the current study, spleen weight was not significantly different among birds in response to HS. The lack of results may be attributed to the low intensity of heat exposed during the summer of 2013, as reported previously that the outcome of HS effects in chickens is dependent on the exposed heat intensity and duration (Kelley, 1983). Temperatures beyond 30ºC are generally adequate enough to

provoke HS in poultry (Ensminger et al., 1990). However, in the current study, the lack of results indicate that the 4 h of acute HS at mean 33.3ºC and fluctuating ambient temperature throughout the 16 wk period, mean 24ºC, was not sufficient enough to see an effect on the spleen weight of hens.

Regarding immune function, circulating catecholamines and corticosterone released by the adrenal glands that have undergone hypertrophy during stress bind to receptors of immune cells (Plaut, 1987), causing profound immunosuppression and lymphoid organ regression (Pope, 1991; Brown-Borg et al., 1993; Hessing, 1995; Walrand et al., 2001; Wurtman, 2002). The numbers of B- and T- lymphocytes involved in antibody- and cell-mediated immunity, respectively, are reduced, and their functions are impaired under conditions of chronic stress (Siegel, 1995). As a result of glucocorticoid induced lymphopenia, an increase in the H/L ratio occurs in blood and is used as an indicator of stress (Gross and Siegel, 1983; Maxwell, 1993), a response that occurred in the current study with the acute heating episode. The H/L ratio is a dependable biomarker of HS in poultry (Thaxton et al., 1968); and it was increased in chickens after exposure to HS (Prieto and Campo, 2010; Soleimani et al., 2011; Felver-Gant et al., 2012; Habibian et al., 2014). Given our understanding of the effect of HS on the H/L ratio, we expected that CP hens would have a lower H/L ratio, indicating a low and or delayed stress response. As expected, CP hens did have a lower H/L ratio than both NP and AP hens at 27 wk of age following acute HS, indicating the ability of cooled perches to improve hen immunity. The H/L ratio of hens at 32 wk of age suggest that perches per se may have alleviated long-term stress, especially for hens with access to thermally cooled perches because their H/L ratio was lower than hens without perches.

The H/L ratio response of hens with air perches was intermediate between hens with thermally cooled perches and hens without perches (Figure 2.3). Hens housed on a slat and littered floor with access to perches also had lower H/L ratios than hens without perches suggesting that perches helped reduce stress (Campo et al., 2005), but a similar effect was not reported for caged hens with and without perches (Barnett et al., 1997).

Chronic HS inhibits antibody production to specific antigens in chickens (Mashaly et al., 2004). Immunoglobulins that have not been synthesized in response to antigenic challenge also decreased in response to HS (Al-Ghamdi, 2008). The short-term HS of the current study and the mild summer of 2013 were not severe enough to induce changes in IgY concentration at 27 wk of age perhaps due to an unsustained increase in the concentrations of corticosterone and catecholamines. The increase in IgY in NP hens at 32 wk of age is perplexing, especially since the increased H/L ratio of these hens without perches (Figure 2.3) would imply fewer B- cell lymphocytes available to produce less Ig. However, this could be attributed to IgY having a primary role for protecting the body against infection by binding to pathogens and activating further mechanisms to clear it from the body. Therefore, CP hens may have required less IgY, indicating access to cooled perches during HS caused less cell damage compared to the NP hens. Furthermore, similar to the present results, several negative correlations between IgY levels and physical or physiological well-being have been found including BW, feeding efficiency, egg production and survivability in birds (Siegal and Gross, 1980; Martin et al., 1989; Cheng et al., 2001b).

In the current study, to further understand the effect of cooled perches used to alleviate HS through the hens' immune system, cytokine plasma protein and mRNA

expression were observed at 27 wk following acute HS and at 32 wk of age following accumulated chronic HS. The interleukin 1 family consists of 11 cytokines that help regulate inflammatory and immune responses. In particular, IL-1α and -1β are well known for their pro-inflammatory effects. They also influence the thermoregulatory center of the hypothalamus causing core body temperature to increase leading to fever. In addition, IL-6 can cross the blood brain barrier and targets the hypothalamus to synthesize prostaglandins E2 leading to an increase in body temperature (Weber and Iacono, 1997). The TNF-α is another cytokine involved in inflammation that is capable of inducing fever (Dinarello et al., 1986). Laying hens subjected to 12 d of HS (34ºC) experienced an increase in serum levels of IL-1 and TNF-α (Deng et al., 2012), but the cooled perches of the current study were ineffective in lowering the levels of these cytokines during a heating episode (33.3ºC for 4 h) even though rectal temperature was lower in CP hens compared to hens without perches (Liedtke et al., 2014).

Similar to plasma cytokines, the splenic expression of cytokines, TLR, and iNOS at 32 wk of age was unaffected in hens with access to cooled perches. Other studies with chickens have reported an up-regulation of some of these immune parameters perhaps because the stressors were more severe (Sahin et al., 2010; Deng et al., 2012**;** Xu et al., 2014). Specifically, the expression of pro-inflammatory cytokines of IL-1β, IL-6, and IL-18 in the spleen and blood lymphocytes increased in chickens subjected to a corticosterone-induced stress (Shini and Kaiser, 2009). The upregulation of TNF-α and iNOS in tissues of chickens could be indicators of stress as suggested by Kang et al. (2011) who reported that laying hens exposed to the stressors of overcrowding (high stocking density) in combination with feed restriction showed an increase in hepatic and

splenic lipopolysaccharide-induced TNF-α and hepatic but not splenic iNOS expression levels as compared to control hens.

Even though we had hypothesized that hens given access to thermally cooled perches would demonstrate lower SW, prevent lymphoid regression, decrease cytokine production, and decrease the splenic expression of TLR and iNOS, the 4 h heating episode was not long enough nor was the summer of 2013 hot enough to induce much stress. Studies of human and mouse iNOS and the associated NO production have reported that these molecules are often active in concert with cytokines, to protect against viral infection (Li et al., 2009), however, their role in avian species is less defined. Moreover, iNOS has been shown to increase in laying hens exposed to stress (Kang et al., 2011) and in chickens infected with influenza A virus subtype H5N1 (Burggraaff et al., 2011). However, NO production, as a measure of iNOS function, was observed only after 48 h following HS and recovered after 72 h post HS (Howard et al., 2010). Therefore**,** the lack of results in the current study could also be due to a delay in iNOS activity at 27 wk of age following a 4 h heat exposure or iNOS function returning to normal levels by 32 wk of age.

Moreover, the current findings correspond with the results reported by Felver-Gant et al. (2012), indicating the lack of change observed in our study can be better understood by the biomechanics of HS. Laying hens during HS have a reduction in antibody production (Mashaly et al., 2004) and a diminished macrophage activity (Barlett and Smith, 2003). It has also been observed that pathogen recognizing molecules like TLR-4 show a higher response following a pathogen challenge such as *Salmonella enteritidis* (Eicher and Cheng; 2003; Malek et al., 2004). Also, acute stress has been

reported to enhance immunity, whereas chronic stress has been shown to suppress immune function (Millán et al., 1996; Dhabhar and McEwen, 1997). However, the mechanisms by which these alterations occur have not been fully clarified. Therefore, with both a high response expected following the inflammatory pathway of these cytokines, as well as an inhibited response expected due to the effect HS has on the immune system, the current findings can be further explained. In addition, as previously reported, the stress response is dependent on the type of stress and the duration of stress in laying hens (Cheng et al., 2001a; Cheng, 2010). The lack of a physiological response due to the presence of cooled perches as result of too mild of a stressful event including the acute heating episode, is further corroborated by the fact that their egg production, shell quality, feed efficiency, foot health, and feather score (Cheng et al., 2014), bone mineralization and muscle deposition (Hester et al., 2014) were unaffected by the cooled perch treatment.

Conclusion

The reduced H/L ratio was the only immunological biomarker in hens that responded to the thermally cooled perches after exposure to an induced heating episode of 4 h. The results suggested that these hens were able to cope with acute HS more effectively than hens with air perches or without perches. Current studies are in progress to evaluate the effectiveness of thermally cooled perches under higher ambient temperatures.

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TABLE 2.1. Primers and probes used for real-time PCR

Gene	Primers (5' -3')	Application Efficiencies, %	Product Length, bp	Reference/ Accession no.
IL-1β[1]	(f[2]) TGCTGGTTTCCATCTCGTATGTAC (r[3]) CCCAGAGCGGCTATTCCA (p[4]) AGTACAACCCCTGCTGCCCCGC (VIC/MGB)	95	80	NC_006096.3
IL-6[4]	(f) CCCGCTTCTGACTGTGTTT (r) GCCGGTTTTGAAGTTAATCTTTT (p) TGTGTTTCGGAGTGCTTT (VIC/MGB)	86	139	NC_006089.3
TNF-α[5]	(f) CCCCTACCCTGTCCCACAA (r) ACTGCGGAGGGTTCATTCC (p) CTGGCCTCAGACCAG (VIC/MGB)	75	62	NC_006101.3
iNOS[6]	(f) GAGTGGTTTAAGGAGTTGGATCTGA (r) TCCAGACCTCCCACCTCAAG (p) CTCTGCCTGCTGTTGCCAACATGCT (VIC/MGB)	103	80	NC_006106.3
TLR4[7]	(f) TCTGAGACCCCCAAGTCCAA (r) CCTTAAGTTTTGCCAGAGGAGGTT (p) CCCACCACACCCACT (VIC/MGB)	98	197	NC_006104.3

[1]Interleukin 1β
[2]Forward primer
[3]Reverse primer
[4] Probe
[5]Interleukin 6
[6]Tumor necrosis factor α
[7]Inducible nitric oxide synthase
[8]Toll-like receptor 4

TABLE 2.2. Heat stress responses in mRNA expression and spleen weight[1]

Treatment[2]	IL-1β	IL-6	TNF-α	iNOS	TLR-2	SW, g
Wk 32						
Cool Perch	0.75 ± 0.11	0.79 ± 0.15	1.65 ± 0.57	1.33 ± 0.27	2.35 ± 0.56	1.24 ± 0.10
Air Perch	0.69 ± 0.11	0.60 ± 0.15	1.87 ± 0.57	1.30 ± 0.27	2.40 ± 0.56	1.28 ± 0.10
No Perch	0.68 ± 0.11	0.59 ± 0.15	2.10 ± 0.57	1.04 ± 0.27	2.04 ± 0.56	1.18 ± 0.10
P-value						
Treatment	0.8855	0.5942	0.4546	0.7100	0.8880	0.7584
CP*AP	0.6869	0.3932	0.5363	0.9516	0.9522	0.7656
CP*NP	0.6628	0.3732	0.2174	0.4626	0.7020	0.6643
AP*NP	0.9735	0.9695	0.5214	0.4994	0.6585	0.4671

[1]All means reported are least square means ± SE (n= 6 cages of 2 hens) in relative abundance to GAPDH created by mixed model analysis.

[2]CP = Cool Perch; AP = Air Perch; NP =No Perch. IL-1β = interleukin 1 beta; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha. iNOS = inducible nitric oxide synthase; TLR-4 = toll-like receptor 4; SW = spleen weight.

TABLE 2.3. Heat stress responses in hen IgY, IL-1β, IL-6, and TNF-α at 27 wk following a 2-4 h heat stress and 32 wk of age in plasma[1]

Treatment[2]	IgY, mg/mL	IL-1β, pg/mL	IL-6, mg/mL	TNF-α, pg/mL
Wk 27				
Cool Perch	6.92 ± 0.86	1.19 ± 0.33	0.93 ± 0.15	144.48 ± 14.59
Air Perch	6.94 ± 0.86	0.92 ± 0.33	1.05 ± 0.15	143.40 ± 14.59
No Perch	7.25 ± 0.86	1.37 ± 0.33	1.03 ± 0.15	140.60 ± 14.59
Wk 32				
Cool Perch	15.79 [b] ± 1.57	3.64 ± 0.73	1.31 ± 0.25	190.67 ± 4.53
Air Perch	14.67 [b] ± 1.57	3.70 ± 0.73	1.02 ± 0.25	194.20 ± 4.53
No Perch	19.35 [a] ± 1.57	3.85 ± 0.73	0.89 ± 0.25	188.75 ± 4.53
P-value				
Wk27 CP*AP	0.9755	0.5737	0.5798	0.9591
Wk27 CP*NP	0.6051	0.6961	0.6429	0.8535
Wk27 AP*NP	0.6264	0.3458	0.9273	0.8939
Wk32 CP*AP	0.3797	0.9546	0.4315	0.5893
Wk32 CP*NP	0.0122	0.8367	0.2565	0.7687
Wk32 AP*NP	0.0020	0.8813	0.7156	0.4081

[1]All means reported are least square means ± SE (n= 6 cages of 2 hens) created by mixed model analysis.

[2]CP = Cool Perch; AP = Air Perch; NP =No Perch. IgY = plasma immunoglobulin Y; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha.

[ab] Mean within a time differ ($P < 0.05$).

FIGURE 2.1.

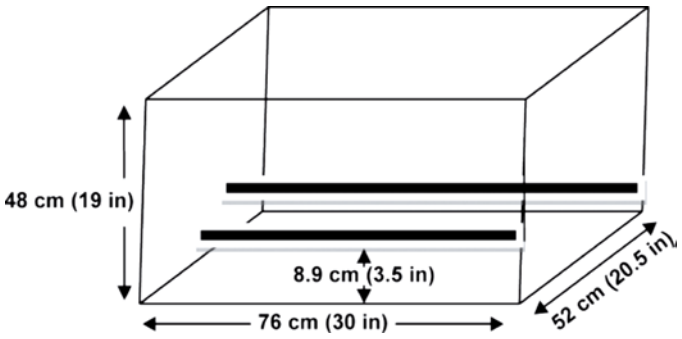

Figure 2.1. The cage dimensions and perch placement. Two perches were installed in a layer cage, arranged parallel to each other. Perch height was 8.9 cm from the cage floor. The distance between the 2 perches was 15 cm and the distance between the front perch and the feed trough was 18 cm. There was a distance of 15 cm between the rear perch and the back of the cage.

FIGURE 2.2.

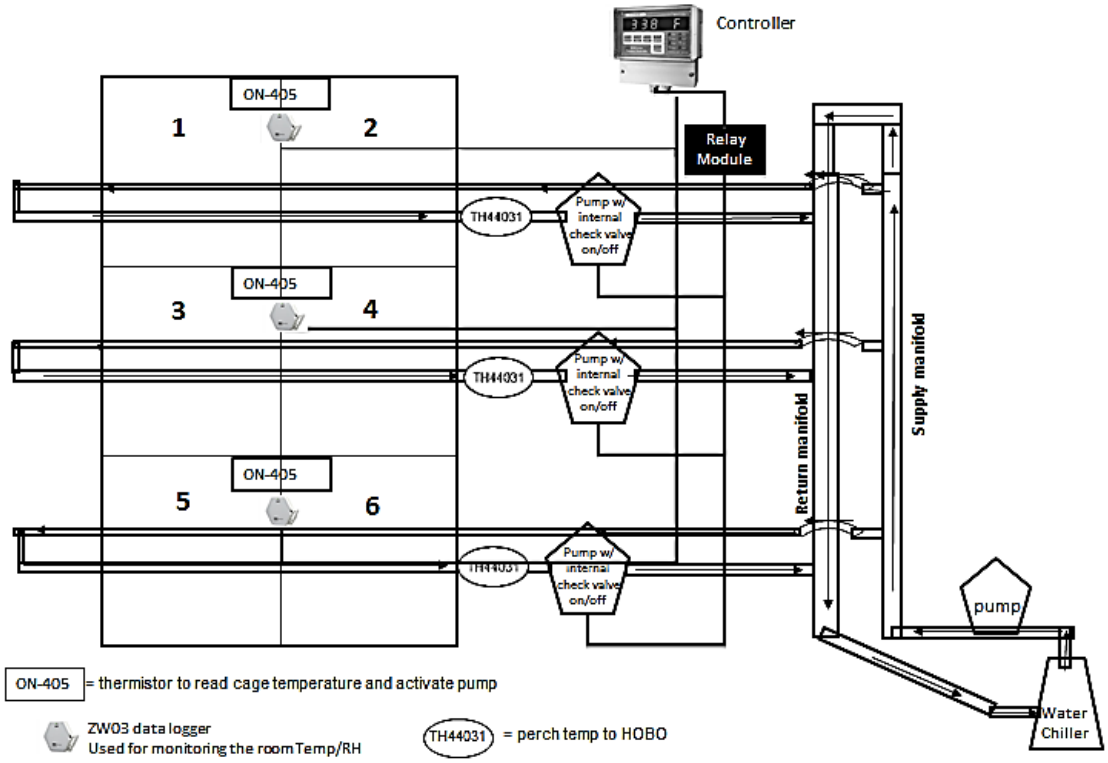

Figure 2.2. A schematic diagram of the cooled perch cage system. The front perch closest to the feeder received chilled water pumped directly from a common vertical manifold. The back perch was the return loop that sent the water back to the common manifold to be re-chilled. A chiller was used to cool the water in the manifold; it had its own independent thermostat which kept the water at 10ºC. A separate 4th pump continuously circulated the deionized water between the water chiller and the manifold. A sensor for monitoring air temperature was installed to the controller of each tier to activate the circulation of chilled water through the perch loop when ambient temperature reached 25ºC or to stop circulation of water when the ambient temperature fell below 25ºC.

FIGURE 2.3.

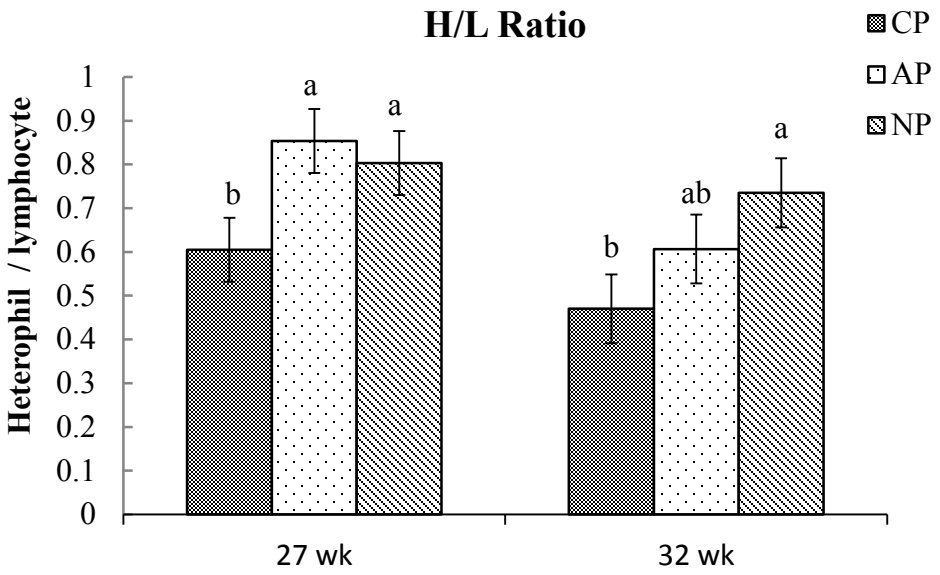

Figure 2.3. Heat stress responses in hen heterophil to lymphocyte ratio at 27 wk of age after 4 h of acute HS and at 32 wk of age. CP = Cool Perch; AP = Air Perch; NP =No Perch. Means reported as least square means ± SE (n= 6 cages of 2 hens) [a,b] Different letters indicate significant differences between treatments within each week of age ($P < 0.05$).

CHAPTER III

DAM HEAT STRESS EFFECTS ON CALF INNATE IMMUNITY

Abstract

Heat stress (HS), as one of the environmental stressors affecting the dairy industry, compromises the cow's milk production, immune function, and reproductive system. However, few studies have looked at how prenatal HS affects the offspring. The objective of this study was to evaluate the effect of HS during late gestation on calf immunity. Calves were born to cows exposed to evaporative cooling (CT) or heat stress (HS; cyclic 23-35°C) 3 wk before calving. Both bull and heifer calves (CT, n=10; HS, n=10) were housed in similar environmental temperatures after birth. Both CT and HS calves received 3.78 L of pooled colostrum within 12 h after birth and were fed the same diet throughout the study. In addition to tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 1 receptor antagonist (IL-RA), and toll-like receptor 2 (TLR2), and 4 (TLR4) mRNA expression, the expression of $CD14^+$, $CD18^+$, $Dec205^+$, and phagocytosis$^+$ ROS $^+$ were determined in whole blood samples at d 0, 3, 7, 14, 21, and 28. The neutrophil to lymphocyte (N/L) ratio, differential cell counts, and the hematocrit (HCT) were also determined. During late gestation, the HS cows had greater respiration rates (RR) ($P < 0.0001$), rectal temperatures (RT) ($P < 0.0001$), and tended to spend more time standing compared to the CT cows ($P = 0.09$). The HS calves had less expression of TNF-α and TLR-2 and greater levels of IL-1β, IL-RA, TLR-4, as well as greater phagocytosis$^+$ ROS^+ compared to CT calves ($P < 0.05$, respectively). The HS calves also had a greater percentage of $CD18^+$ compared to the CT calves ($P < 0.05$). Additionally, a greater percentage of NE and less lymphocytes were in the HS calves compared to the

CT calves ($P < 0.05$). The results indicate that biomarkers of calf immunity are affected by HS in the dam during late gestation.

Key Words: calf, heat stress, innate immunity

Introduction

Heat stress (HS) is a major environmental concern in the dairy industry. In dairy cattle, HS occurs when ambient temperatures are above about 25°C (Armstrong, 1994). Heat stress causes greater rectal temperatures (RT) and elevated respiration rates (RR) (Ominski et al., 2002; do Amaral et. al., 2011). Dairy cattle also spend less time lying when exposed to hot temperatures (Overton et al., 2002; Legrand et al., 2011). In addition to behavioral responses, HS in dairy cattle reduces feed intake (Adin et al., 2009**)**, milk production (Collier et al., 2006), and reproductive performance (Hansen, 2009). Heat exposure can also cause negative effects on the animals' immune response resulting in a decrease in neutrophil phagocytosis and oxidative burst (do Amaral et al., 2011), reduced lymphocyte proliferation (Lacetera et al., 2006), lower IgG concentrations (do Amaral et al., 2011), and increased (Tao et al., 2012) or decreased (do Amaral et al., 2010) tumor necrosis factor alpha (TNF-α). The immune impairment due to HS can cause an increase in the susceptibility to many diseases (Lacetera et al., 2006; do Amaral et al., 2009; Dahl et al., 2012).

Maternal HS during late gestation also inhibits the immune response of the offspring, by modifying T- and B- cell function (Merlot et al., 2008), decreasing IgG concentration in the calf (Donovan et al., 1986; Tao et al., 2012), and compromising the proliferation rate of mononuclear cells (Tao et al., 2012). Moreover, sows under HS during the last 2 wk of gestation had piglets with lower circulating IgG compared to piglets from sows under a thermoneutral environment (Machado-Neto et al., 1987). The immune system in the neonate is fully developed at birth but it is unprimed (Tizard, 1992). The fetus is protected primarily by the innate immune system, but its phagocytic

activity is not fully developed until late in gestation (Barrington, 2001). B cells in the fetus only make up 1% of total lymphocytes compared to the 4% at one week after birth and to the 10% in mature calves (Kampen et al., 2006). This results in the lack of any endogenous antibody response until 2 to 4 weeks of age making the ingestion of colostrum extremely important in providing an immunologic defense to the calf during the first 2 to 4 weeks of life (Chase et al., 2008). Adequate colostrum transfer has been recognized to have beneficial effects on the calf's immune response earlier in life (Furman-Fratczak et al., 2011), by the uptake of cytokines (Nguyen et al., 2007; Chase et al., 2008) and the absorption of immunoglobulins during the first 48 h (Sangild, 2003).

Currently, there is little data on the effects of maternal HS during late gestation on the immune function in dairy cattle. Therefore, the objective of the present study was to evaluate the effect of maternal HS during the late gestation period on the postnatal immune function of dairy calves.

Materials and Methods

Animals and Experimental Design

This study was conducted at the Purdue Animal Sciences Research and Education Dairy Unit from May to September 2014. The experimental protocol was approved by the Animal Care and Use Committee at Purdue University. Twenty multiparous Holstein cows were randomly assigned to 1 of 2 environmental treatments, control (CT) or heat stress (HS), approximately 3 wk before calving. All cows were managed in a metabolism barn with tie stalls. A single treatment was in the room at one time, using 3 replications per treatment. Cows had an acclimation period of 7 d and were on treatment for the

following 7 d. The geothermal metabolism barn for the CT cows was supplemented with evaporative cooling equipment. For the HS cows, the temperature increased to 25ºC ± 6 for 12 h each day and was reduced to 20ºC ± 4 for 12 h each night. Cows from both treatments were moved to box stalls under thermal neutral (22ºC ± 2) environmental conditions approximately one week before their expected calving date. Both bull and heifer calves were used in the current study (CT: bulls: 6, heifers: 4; HT: bulls: 4, heifers: 6). Both CT (n=10) and HS (n=10) calves received 3.78 L of pooled colostrum within 12 h after birth and were fed the same diet throughout the study. Calves from both treatments were removed from their dam immediately after birth and housed in individual hutches exposed to environmental temperatures.

Measurements and Sample Collection

The respiration rates (RR), rectal temperatures (RT), and posture of cows in both treatments were monitored hourly for 12 h during 7 d of treatment. The RR were measured in breaths per one minute using a stopwatch. The posture of the cow, whether they were lying or standing at the moment before RR were counted, was calculated in percent observation. The average of 2 consecutive RR per cow was recorded hourly. The RT were measured using a digital thermometer hourly and disinfected between individual cows with 70% isopropyl. The room temperature (T) and humidity (H) were recorded hourly by taking the average of two readings and was also digitally recorded every 15 min by a data logger (Onset HOBO, UX100-003; 10526645). Each hourly observation was conducted in the same recording sequence; T, H, RT, posture, RR.

Calf jugular blood samples were collected at d 1, 3, 7, 14, 21, and 28 into both 8.5 ml tubes containing 1.50 ml of ACD for Flow Cytometry and PCR and 8.5ml tubes containing 0.10 ml of EDTA (BD, Franklin Lakes, NJ) for hematology analysis. Immediately following blood collection, blood was stored on ice, transported to the laboratory and then placed in a water bath at 37°C for 30 min.

Gene Expression

Cytokines of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 1 receptor antagonist (IL-RA), and toll-like receptor 2 (TLR-2), and 4 (TLR-4) mRNA expression in whole blood were detected by real-time PCR using primers and probes diluted to 10 µM solutions (Table 3.1) and developed with Primer Express (Applied Biosystems). The RNA was extracted per instructions using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA; Cat. No 52304). Extracted RNA was then quantified using the GeneQuant pro RNA/DNA calculator (Amersham Bio- sciences Corp., Piscataway, NJ). All surfaces used were treated with RNase Zap (Ambion Inc.) before reverse transcription. Conditions for reverse transcription were 61.5 µL of master mix, made of reagents included in the TaqMan Reverse Transcription Reagent Pack (Applied Biosystems, Foster City, CA), including 2.5µL of Multi-Scribe reverse transcriptase, 22 µL of 25 mM MgCl, 5 µl random hexamers, 2µL RNase inhibitor, 20 µl dNTPs, and 10 µL of TaqMan reverse transcription buffer (TaqMan reverse transcription reagents, Applied Bio- systems). Equal amount of sample RNA was added with RNase-free water (Ambion Inc.) to make a total 35 µL, which was then added to 61.5 µL of master mix for a total of 100 µL. Reverse transcription was conducted in the Hybaid PCR

Express thermo cycler (Midwest Scientific, St. Louis, MO) and amplified using cycling conditions: 50°C for 2 min activation followed by 30 cycles of 95°C for 15 s, 60°C for 1 min, and a final stage of 60°C for 5 min, with a holding temperature of 4°C. The conditions for PCR were a ratio of 3.5 µL of TaqMan probe, 4.5 µL of gene- specific TaqMan forward and reverse primers each, 25 µL of PCR Mastermix (Applied Biosystems), 7.75 µL RNase-free water (Ambion Inc.), and 5 µL of sample cDNA. Cycling conditions for real-time PCR consisted of an initial step at 50°C for 2 min followed by 40 cycles of 10 min at 95°C, 20 s at 95°C, and 1 min at 60°C. To ensure accuracy and consistency, all samples were measured in duplicates and standards in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%. Data of the gene of interest is expressed as relative abundance to the stable (CV < 2%) reference gene 18S as ΔCt.

Immunological Cellular Markers and Cell Function

Cellular surface expression of CD14, CD18, and Dec205, and cell function phagocytosis$^+$ ROS$^+$ assays were measured using flow cytometry. For phagocytosis, 100 µL of reconstituted opsonizing reagent was added to 100 µL of E. coli bio particle. The mixture was incubated for 1 h at 37°C in a water bath, washed with 1 ml of 1X Hank's Balanced Salt Solution (HBSS**)** using a low speed centrifugation (1500g for 15 min at 4°C), and re-suspended in 100 µL of 1X HBSS. Meanwhile, 200 µL of Dimethyl sulfoxide (DMSO) was added to each Dichlorofluorescin diacetate (DCFDA) tube. Of this stock, 5.7 µL of DCFDA was added into 500 µL of whole blood for phagocytosis$^+$ ROS$^+$ and then incubated for 30 min at 37°C in a water bath. After incubation, 12 µL of

bio particle was added to the phagocytosis tube, and a total volume of 5 µL of labeled monoclonal mouse anti-human CD14 (Serotec; Raleigh, NC; Catalog No. MCA1568PE) and fluorescein isothiocyanate (FITC) labeled monoclonal mouse anti-human CD18 (BD, San Diego, CA; Catalog No. 555923) was added to the single CD14 and CD18 tube. Cluster of differentiation 14 is cross reactive with bovine. Additionally, 5 µL of fluorescein isothiocyanate (FITC) labeled monoclonal mouse anti-bovine Dec205 (Serotec; Raleigh, NC; catalog no. MCA1651) was added to the Dec205 tube. The control for CD14, CD18, and Dec205 contained only 500 µL of cells only and the phagocytosis$^+$ ROS$^+$ control had cells plus 5.7 µL of DCFDA. All tubes were incubated for another 30 min at 37°C in water bath. After incubation, 900 µL of sterile cold water was added to each tube of blood. After 45 s in contact, 100 µL of 10 x HBSS was added. The tubes were centrifuged for 3 min at 1800g and supernatant was then discarded. 1 ml of 1X HBSS was added, centrifuged again for 3 min at 1800g, and then supernatant was discarded. Lastly, cell pellets were re-suspended in 350 µL of 2% paraformaldehyde and these samples were kept at 20°C until analysis. Expression of CD14, CD18, Dec205, and phagocytosis$^+$ ROS$^+$ were then investigated using a flow cytometer (Accuri C6; Accuri, Ann Arbor, MI). For each sample, a total population of 10,000 cells was analyzed. The percentage of cells that were positive and the average relative fluorescence intensity of cells expressing CD14 and CD18 or showing phagocytosis$^+$ ROS$^+$ above the control cells were calculated.

Hematology Data

White blood cell (WBC) counts (K/μL), the N/L ratio, the hematocrit percentage (HCT) and the percentage and cell counts of neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), and basophils (BA) were measured using a hematology analyzer (Genesis™; Oxford Science, Inc, Oxford, CT). Plasma protein (PP) concentrations (g/dL) were measured using a refractometer (A 300 CL Clinical; Japan).

Statistical Analysis

Cow and calf data were checked for normal distribution and transformed when necessary and analyzed as a randomized complete block design using the MIXED procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC). Flow data were transformed to $\log_{10}$ prior to statistical analysis. No transformations were necessary for cow or calf PCR and hematology data. The calf model included treatment, day, and treatment × day interaction. The cow model included treatment. Statistical inferences of significance were based on $P < 0.05$ and trends on $P < 0.10$.

Results

In the present study, HS cows had greater minimum, mean, and maximum RR and RT than the CT cows ($P < 0.05$, respectively; Table 3.2). The HS cows also tended to spend more time standing compared to the CT cows ($P = 0.09$; Table 3.2).

The TNF-α and TLR-2 concentrations were less in the HS calves than the CT calves ($P < 0.05$, respectively; Table 3.3). The HS calves had greater IL-1β, IL-RA, and TLR-4 expression than the CT calves ($P < 0.05$; respectively; Table 3.3). As a day effect,

TNF-α decreased after d 0 while IL-1β, IL-RA, and TLR-4 concentrations decreased after d 7 ($P < 0.05$, respectively; Figure 3.1). Day had no effect on TLR-2 ($P > 0.05$; Table 3.3). There was no treatment by day effect on TNF-α, IL-1β, IL-RA, TLR-2, or TR-4 ($P < 0.05$, respectively; Table 3.3).

Considering all leukocytes without gating, the HS calves had greater percentages of $CD18^+$ ($P = 0.0438$; Figure 3.2a) and phagocytosis$^+$ ROS^+ ($P = 0.0382$; Figure 3.5) compared to the CT calves. There were no differences in the percentages of $CD14^+$ ($P = 0.25$; Figure 3.3a) and $Dec205^+$ ($P = 0.26$; Figure 3.4a), or the mean florescence of CD18 ($P = 0.14$; Figure 3.2b), CD14 ($P = 0.86$; Figure 3.3b), or Dec205 ($P = 0.18$; Figure 3.4b) among treatments in all leukocytes. Additionally, without gating, the HS calves tended to have a greater percentage of phagocytosis$^+$ ROS^+ on d 3 and 21 ($P = 0.08$; Figure 3.7a), and tended to have a greater mean fluorescence of CD18 on leukocytes on d 7 compared to the CT calves ($P = 0.08$, Figure 3.7b).

There were no differences among treatments in the percentage or mean fluorescence for CD18 (Figure 3.2), CD14 (Figure 3.3), Dec205 (Figure 3.4), or phagocytosis$^+$ ROS^+ (Figure 3.5) in lymphocytes ($P > 0.05$, respectively; Table 3.5). Day had no effect on the percent positive or mean fluorescence of CD18, CD14, Dec205, or phagocytosis$^+$ ROS^+ ($P > 0.05$, respectively; Table 3.5). However, the HS calves had a greater percentage of cells positive for phagocytosis$^+$ ROS^+ on d 0 and 21, but less percentage of cells positive for phagocytosis$^+$ ROS^+ on d 28 compared to the CT calves ($P = 0.02$; Figure 3.8a). The HS calves also tended to have a greater mean fluorescence of CD14 on lymphocytes on d 0 and 14 compared to the CT calves ($P = 0.06$; Figure 3.8b).

Heat stressed calves had a greater percentage and mean fluorescence of CD18 on monocytes compared to the CT calves ($P < 0.05$; Figure 3.2). There were no differences in the percentage of $CD14^+$ (Figure 3.3), $Dec205^+$ (Figure 3.4), or $Phagocytosis^+$ ROS^+ (Figure 3.5) in monocytes ($P > 0.05$; Table 3.5). However, Dec205 tended to have greater mean fluorescence on monocytes in HS calves ($P = 0.07$; Figure 3.4b). The $CD18^+$ expression on monocytes increased after d 7 ($P = 0.05$, Figure 3.6a). There was also an increase in the expression of $Dec205^+$ on monocytes after day 7 ($P < 0.05$; Figure 3.6c). The mean fluorescence of CD18 on monocytes was higher in HS calves on d 7 compared to the CT calves ($P < 0.05$, Figure 3.10). The expression of $CD14^+$ was greater on monocytes in HS calves on d 3 and lower on d 28 compared to the CT calves ($P = 0.03$, Figure 3.9a).

There were no treatment differences in the percentage of $CD14^+$ (Figure 3.3), $Dec205^+$ (Figure 3.4), or $phagocytosis^+$ ROS^+ (Figure 3.5) in polymorphonuclear (PMN) cells ($P > 0.05$; Table 3.5). However, the percentage of cells positive for CD18 was greater in the HS calves on PMN cells ($P = 0.05$; Figure 3.2a). There was no day effect on the expression of $CD14^+$, $Dec205^+$, or $Phagocytosis^+$ ROS^+ in PMN cells ($P > 0.05$; respectively; Table 3.5). The HS calves had less mean fluorescence of CD18 on PMN cells compared to the CT calves ($P < 0.05$, Figure 3.10).

The plasma protein concentration, the EO cell count, and the percentage of LY, MO were less in HS calves compared to CT calves ($P < 0.05$, respectively; Table 3.4). The percentage of NE was greater in the HS calves compared to the CT calves ($P < 0.05$; Table 3.4). There was no difference in WBC counts, the N/L ratio, the cell counts of NE, LY, MO, and BA or the percentage of EO and BA ($P > 0.05$, respectively; Table 3.4).

There was no difference among treatments in HCT ($P > 0.05$, Table 3.4). The PP concentration peaked at d 3 and decreased after d 7 ($P < 0.05$; Figure 3.11) and the WBC count peaked at d 0 and 7 ($P = 0.0004$; Figure 3.12). There was no day effect on differential cells counts, percentage of BA, or the HCT ($P > 0.05$, respectively; Table 3.4); however the N/L ratio was greatest on d 7 ($P = 0.03$; Figure 3.13). The percent of NE was greatest at d 0 and 7 ($P = 0.006$; Figure 3.14) and the percent of LY was greatest on d 3 compared to d 0 and 7 and increased to another peak at d 28 ($P = 0.01$; Figure 3.14). The percent of MO was greatest on d 3 and increased after d 7 ($P = 0.01$; Figure 3.14), but the percent of EO increased after d 3 and decreased after d 21 ($P = 0.07$; Figure 3.14). There was no treatment by day interaction for PP, N/L ratio, individual cell counts or the percentage of individual cells ($P > 0.05$, respectively; Table 3.4). The HS calves tended to have less total WBC at d 7 but a greater WBC count at d 28 compared to the CT calves ($P = 0.08$, Figure 3.12).

Discussion

Increasing RR and RT are two indicators of HS in dairy cattle (Ominski et al., 2002; do Amaral et. al., 2011). Lower RR and RT in CT cows suggest that less heat load is carried compared to HS cows (Tao et al., 2012). Dairy cattle also spend less time lying when exposed to high temperatures (Overton et al., 2002; Legrand et al., 2011). Like previous studies, our study showed cows exposed to HS had greater minimum, mean, and maximum RR and RT than CT cows. The HS cows also tended to spend more time standing than the CT cows. The results suggest HS cows are attempting to compensate for the added heat load by increasing their surface area to dissipate excessive heat. The

changes in RR, RT, and posture indicate there may be other changes occurring in the HS cows that could compromise the offspring environment and in turn lead to compromised calf immunity. Additionally as previously reported by Tao et al. (2012), the elevated RR and RT during the dry period of HS cows compared to CT cows provided evidence that the HS model in the present study was successful.

As part of the recognition of Gram-positive bacteria, TLR2 plays a role in mediating the innate immune response by releasing cytokines. In the present study, the expression of TLR2 and TNF-α were both less in the HS calves compared to the CT calves. The lower TLR2 expression in the HS calves could be associated with the lower monocyte percentage and eosinophil count found in the HS calves in the present study. Supporting the current results, Couret et al. (2009) found that maternal stress during late gestation decreased TNF-α production in piglets. In cows, TNF-α concentration was also decreased after exposure to HS (do Amaral et al., 2010). Additionally, calves born to dams during a heat event had less TNF-α expression than calves born in a thermal neutral environment (Deng, 2011). Eicher et al. (2004) also found that calves treated with growth hormone and dexamethasone had lower TNF-α expression in blood leukocytes at 2 weeks of age. In corroborating the previous findings, we hypothesize the lower TNF-α concentration in the current study could be caused by an increase in circulating stress hormones in the calves exposed to maternal HS. Additionally, calves pre-exposed to HS during fetal development may have a higher threshold to postpartum HS, as discovered in chickens during the incubation period (Loyau, et al., 2014).

A complex on the cell surface made up of TLR4 and CD14 recognize the lipopolysaccharide (LPS) of Gram-negative bacteria and activate macrophages to secrete

inflammatory cytokines (Gioannini and Weiss, 2007) and establishes the first line of defense against injury or disease (Uematsu and Akira, 2007). In the present study, the TLR4 mRNA level was higher in HS calves on d 0 and 7 and the expression of CD14 was also higher on monocytes in the HS calves, but on d 3. However, the expression of TLR-4 in human blood monocytes was decreased after strenuous exercise in the heat (34˚C) from an increase in circulating cortisol (Lancaster et al., 2005). Du et al. (2010) also reported that a reduction in TLR-4 causes major stress hormones to reduce the macrophage response in rats. Pearce et al. (2013) reported HS causes a decrease in intestinal integrity and an increase in endotoxin permeability. The increase in LPS gut permeability could explain the up-regulation in TLR-4 expression in calves exposed to prenatal HS in the current study. Another cell surface molecule, CD18, an adhesion molecule responsible for the recruitment of leukocytes to the site of infection had higher expression in the total leukocyte population, and more specifically on monocytes and PMN cells. Additionally, the up-regulation of CD18 expression along with the recognition of LPS of Gram-negative bacteria by the TLR4/CD14 complex has been shown to benefit the immune defense system leading to phagocytosis of bacteria (Nunes et al., 2010). Therefore we can speculate this mechanism is occurring in the current study.

Dec205 is a receptor expressed on dendritic cells and monocytes, which plays a role in antigen presentation to T cells as part of regulating the adaptive immune response (Jiang et al. 1995). Jiang et al. (1995) showed that the antigen-presentation function of lymphocytes is associated with high levels of DEC205 expression on antigen presenting cells. We were not expecting any treatment differences in Dec 205 expression among treatments, because of the association of Dec205 with mature dendritic cells (Butler,

2007). However, the HS calves tended to have a higher florescence intensity of Dec205 compared to CT calves. The higher florescence intensity in Dec205 was also seen in calves challenged to *Salmonella dublin* compared to control calves (Eicher et al., 2011). These findings indicate that maturation of dendritic cells was already being modulating in the HS calves compared to CT calves, suggesting prenatal HS may cause the neonate to have an altered adaptive response sooner than if they weren't pre-exposed to maternal HS.

In the present study, the mRNA expression of IL-1β and IL-RA were both up-regulated in the HS calves compared to the CT calves. Along with TNF-α, IL-1β is a pro-inflammatory cytokine mediated by recognition molecules responsible for a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Interleukin 1 expression is rapidly up-regulated following immune stimulation (Takashi and Kodama, 1994). In association with IL-1β, IL-RA acts as an inhibitor to IL-1 by competing with the IL-1 receptor. The levels of IL-1β and IL-RA were also both up-regulated in rats exposed to HS (Lin et al., 1995). Additionally, previous studies found that the pretreatment of IL-RA reduced damage caused by HS in rats (Lin et al., 1995) and rabbits (Lin et al., 1994). Thus based on previous HS studies in other species, the HS calves on d 0 and 7 may have increased IL-RA expression to compensate for the increase in IL-1β expressed to recover from the prenatal HS. Additionally, with the current results, we speculate that during HS recognition molecules may mediate specific cytokines; TLR2 and TNF-α were both greater in the HS calves while TLR4 and IL-1β were both less in the HS calves compared to the CT calves. Furthermore, IL-RA, IL-1β, and TLR-4 all decreased after day 7, while TNF-α decreased after day 0, which not only indicates they

are part of the first response of the innate system but also further evidence of their specific association. In another study, there was no seasonal effect on TLR-4, but there was an effect on the summer treatment, which had less TNF-α concentrations (Deng, 2011). This supports the speculation that certain recognition molecules may be associated with the release of specific cytokines during late gestational prenatal HS observed in the current study.

As the first line of defense against bacterial infection, the phagocytic and oxidative burst activities of neutrophils provide valuable information on the functional activity of these immune cells (Kampen et al., 2004). Oxidative burst is a process in which the pathogen is killed by toxic ROS after it has been phagocytized by a neutrophil or macrophage (Elbim and Lizard, 2009). During late gestation, heat-stressed cows had impaired neutrophil phagocytosis and oxidative burst relative to cooled cows on d 2 and d 20 (do Amaral et al., 2011). However, in the present study, the percentage of phagocytosis^{+} ROS^{+} was greater in the HS calves compared to the CT calves on d 3 and d 21. The mechanism behind the results are unclear but we can speculate that while it appears the calves in the current study didn't lose neutrophil function, they did require more oxidative burst from the residual effects of the prenatal HS.

It has been established that due to the increase in the number of neutrophils and the decrease in lymphocytes, the N/L is a suitable measure often used to assess the stress response in cattle (Friend et al., 1987; Stull and McDonough, 1994). An increased neutrophil and decreased lymphocyte and eosinophil percentage was also reported to occur after many types of environmental stressors including diseases of bacterial infection in cattle (Radostist et al., 1994). While there was no difference among

treatments in the N/L ratio, HS calves did have a higher percentage of neutrophils and a lower percentage of lymphocytes and eosinophils compared to the CT calves, indicating prenatal HS continued to carry over to the neonate. Additionally, Tao et al. (2012) observed decreases in the hematocrit, total plasma protein, and IgG concentrations in heifer calves born to cows exposed to late-gestational HS. Similarly, late-gestation HS in neonate calves (Stott, 1980) and piglets (Machado-Neto et al., 1987) caused a decrease in the concentration of IgG. In the current study, there was no difference in the hematocrit, but there was a lower PP concentration observed in the HS calves compare to the CT calves. The combined results indicate that passive immunity could have been compromised in calves exposed to prenatal HS during late gestation. Tao et al. (2012) found no differences among treatments in circulating cortisol levels and the stress hormones were not measured in the current study. Thus the primary mechanism behind the potential compromise in passive immunity from prenatal HS of the dam is still unclear.

Conclusion

The current data suggests immunological evidence of an altered immune system in neonates due to maternal HS during late gestation. Calves exposed to prenatal HS were associated with a greater percentage of neutrophils, a lower percentage of lymphocytes, and lower levels of plasma proteins. The expression of pro-inflammatory cytokines and recognition molecules were also affected in HS calves compared to the CT calves. From the current study, we conclude that prenatal HS compromises the calf's

immune system, and therefore greater measures are necessary for higher quality colostrum and more intense observation of those calves.

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Table 3.1. Primers and probes used for real-time PCR

Gene	Primers	Amplification Efficiencies, %	Product Length, bp	Reference/ Accession no.
TNF-α[1]	(f[2]) TGGGAAGCTTACCTTTTCCTTTC (r[3]) CTTCTTCATGACCCAGATACATCCT (p[4]) CCTCAAGTAACAAGCCG (VIC/MGB)	98	61	Bienhoff and Allen, 1995
IL-1β[4]	(f) TTCCTGTGGCCTTGGGTATC (r) TGGGCGTATCACCTTTTTTCA (p) CAAGAATCTATACCTGTCTTGT (VIC/MGB)	78	69	Ito and Kodama, 1996
IL-RA[5]	(f) CCTCCTTTCTCACCCCAGATC (r) AGAAAATGGAAGCCGCTTAGG (p) CAG GCGCTCACTTC (VIC/MGB)	96	64	Kirisawa et al., 1998
TLR2[6]	(f) CCACGGAAGGAGCCTCTGA (r) GCCATCGCAGACACCAGTT (p) CAGGCTTCTTCTCTGTCTT (VIC/MGB)	86	65	AF368419
TLR4[7]	(f) CCGGATCCTAGACTGCAGCTT (r) TCCTTGGCAAATTCTGTAGTTCTTG (p) CCGTATCATGGCCTCT (VIC/MGB)	104	71	AAG32061

[1]Tumor necrosis factor α
[2]Forward primer
[3]Reverse primer
[4] Probe
[5]Interleukin 1β
[6]Interleukin receptor antagonist
[7]Toll-like receptor 2
[8]Toll-like receptor 4

Table 3.2. Effects of heat stress on cow respiration rate, rectal temperature, and posture[1]

Treatment[2]	CT	HS	*P*-value
Min THI	< 64	75	-
Mean THI	69	83	-
Max THI	79	85	-
Min RR, breaths/minute	34.77 ± 1.02	46.90 ± 1.02	< 0.0001
Mean RR, breaths/minute	47.86 ± 1.29	64.36 ± 1.29	< 0.0001
Max RR, breaths/minute	62.56 ± 1.72	82.64 ± 1.72	< 0.0001
Min RT °C	37.73 ± 0.19	38.63 ± 0.19	0.0013
Mean RT °C	38.46 ± 0.03	38.96 ± 0.03	< 0.0001
Max RT, °C	39.02 ± 0.04	39.45 ± 0.04	< 0.0001
Standing, %[3]	53.35 ± 3.71	62.80 ± 3.71	0.089
Lying, %	46.65 ± 3.71	37.20 ± 3.71	0.089

[1]All means reported are least square means ± SE (n = 10 cows/treatment) created by mixed model analysis.

[2]CT = control cows; HS = heat stressed cows; Min THI = temperature and humidity index for the minimum room temperature; Mean THI = temperature and humidity index for the mean room temperature; Max THI = temperature and humidity index for the maximum room temperature; Min RR = mean of the minimum respiration rates; Mean RR = mean of the average respiration rates; Max RR = mean of the maximum respiration rates; Min RT = mean of the minimum rectal temperatures; Mean RT = mean of the average rectal temperatures; Max RT = mean of the maximum rectal temperatures.

[3]The percent of observations based on whether the cow was lying or standing before the respiration rates were counted (12 obsevations/day/cow)

Table 3.3. Effects of heat stress on calf mRNA expression in leukocytes[1]

Treatment[2]	TNF-α	IL-1β	IL-RA	TLR-2	TLR-4
		Treatment Effect			
CT	2.21 ± 0.26	0.16 ± 0.02	0.34 ± 0.05	0.41 ± 0.07	0.29 ± 0.03
HS	1.42 ± 0.26	0.21 ± 0.02	0.55 ± 0.05	0.20 ± 0.07	0.42 ± 0.03
		Day Effect			
d 0	4.16 ± 0.48	0.26 ± 0.04	0.61 ± 0.09	0.39 ± 0.12	0.52 ± 0.06
d 3	2.26 ± 0.44	0.26 ± 0.03	0.55 ± 0.08	0.34 ± 0.12	0.44 ± 0.05
d 7	1.72 ± 0.46	0.26 ± 0.04	0.61 ± 0.09	0.32 ± 0.12	0.50 ± 0.05
d 14	0.85 ± 0.44	0.11 ± 0.03	0.29 ± 0.08	0.23 ± 0.12	0.25 ± 0.05
d 21	1.06 ± 0.44	0.13 ± 0.03	0.32 ± 0.08	0.35 ± 0.11	0.24 ± 0.05
d 28	0.81 ± 0.45	0.10 ± 0.04	0.27 ± 0.09	0.21 ± 0.12	0.18 ± 0.05
P-value					
Treatment	0.0342	0.1044	0.0039	0.0361	0.0022
Day	< 0.0001	0.0002	0.0044	0.8875	< 0.0001
Treatment*Day	0.5501	0.1304	0.3714	0.4931	0.1327

[1]All means reported are least square means ± SE in relative abundance to 18S created by mixed model analysis (n = 10 calves/treatment).

[2]CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress; TNF-α = tumor necrosis factor alpha; IL-1β = interleukin 1 beta; IL-RA interleukin receptor antagonist; TLR-2 = toll-like receptor 2; TLR-4 = toll-like receptor 4.

Table 3.4. Heat stress effects on calf hematology measurements[1]

			P- value		
Treatment[2]	CT	HS	Treatment	Day	Treatment*Day
PP (g/dL)	6.34 ± 0.06	6.11 ± 0.06	0.0095	0.0004	0.3181
WBC (K/µL)	7.65 ± 0.47	8.17 ± 0.45	0.4271	0.0117	0.0813
N/L ratio	2.89 ± 0.38	3.36 ± 0.37	0.3762	0.0344	0.8656
NE (K/µL)	5.07 ± 0.51	5.33 ± 0.50	0.1639	0.5543	0.5522
LY (K/µL)	2.31 ± 0.17	2.22 ± 0.16	0.7040	0.2047	0.5140
MO (K/µL)	1.22 ± 0.33	0.49 ± 0.33	0.1200	0.3138	0.4566
EO (K/µL)	0.44 ± 0.11	0.12 ± 0.10	0.0288	0.6552	0.6003
BA (K/µL)	0.11 ± 0.05	0.01 ± 0.05	0.2122	0.3246	0.3431
NE (%)	54.57 ± 1.89	62.90 ± 1.86	0.0022	0.0057	0.9326
LY (%)	32.85 ± 1.89	27.92 ± 1.86	0.0650	0.0104	0.5938
MO (%)	8.01 ± 0.54	6.75 ± 0.53	0.0992	0.0128	0.7394
EO (%)	3.98 ± 0.78	2.27 ± 0.77	0.1209	0.0742	0.7424
BA (%)	0.60 ± 0.25	0.17 ± 0.24	0.2219	0.2891	0.3135
HCT (%)	24.34 ± 1.25	25.64 ± 1.20	0.4548	0.6068	0.8298

[1]All means reported are least square means ± SE created by mixed model analysis (n=10 calves/treatment).

[2]CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress; PP = plasma protein concentration; WBC = white blood cell count; N/L = neutrophil to lymphocyte ratio; NE = neutrophil count or percentage; LY = lymphocyte count or percentage; MO = monocyte count or percentage; EO = eosinophil count or percentage; BA = basophil count or percentage; HCT = percentage hematocrit.

Table 3.5. Effect of heat stress on the percentage of cells positive for surface expression or phagocytosis activity in calves [1]

			P- value		
Treatment[2]	CT	HS	Treatment	Day	Treatment*Day
No gate					
CD18, %	40.63 ± 3.95	51.96 ± 3.92	0.0438	0.8033	0.8984
CD14, %	24.23 ± 2.19	27.78 ± 2.17	0.2517	0.4703	0.4066
Dec205, %	3.70 ± 1.84	6.60 ± 1.83	0.2597	0.1839	0.4678
Phagocytosis$^+$ROS$^+$, %	2.24 ± 0.16	2.72 ± 0.16	0.0382	0.5221	0.0757
Lymphocytes					
CD18, %	0.08 ± 0.08	0.11 ± 0.08	0.7761	0.9151	0.9230
CD14, %	0.60 ± 0.07	0.66 ± 0.07	0.5042	0.1685	0.8824
Dec205, %	0.69 ± 0.01	0.71 ± 0.01	0.1874	0.4528	0.2605
Phagocytosis$^+$ROS$^+$, %	0.10 ± 0.05	0.16 ± 0.05	0.3554	0.6757	0.0243
Monocytes					
CD18, %	23.80 ± 2.97	32.12 ± 2.94	0.0493	0.0529	0.2241
CD14, %	2.22 ± 0.13	2.41 ± 0.12	0.2797	0.0492	0.0266
Dec205, %	1.78 ± 0.15	1.91 ± 0.14	0.5455	0.0009	0.1091
Phagocytosis$^+$ROS$^+$, %	8.27 ± 1.94	7.17 ± 1.94	0.6899	0.3962	0.1253
PMN					
CD18, %	40.69 ± 4.06	51.96 ± 4.02	0.0510	0.8033	0.8984
CD14, %	3.11 ± 0.11	3.19 ± 0.11	0.6077	0.8745	0.7669
Dec205, %	1.99 ± 0.20	1.93 ± 0.19	0.8126	0.7532	0.9474
Phagocytosis$^+$ROS$^+$, %	11.90 ± 3.08	15.23 ± 3.08	0.4469	0.7774	0.1714

[1]All means reported are least square means ± SE created by mixed model analysis (n=10 calves/treatment).

[2]CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress; CD18 = cluster of differentiation CD18; CD14 = cluster of differentiation CD14; Dec205 = cluster of differentiation Dec205; ROS = reactive oxygen species; PMN = polymorph nuclear cells.

Table 3.6. Effect of heat stress on the mean fluorescence of individual cells in calves[1]

			P- value		
Treatment[2]	CT	HS	Treatment	Day	Treatment*Day
No gate					
CD18	8.19 ± 0.16	8.51 ± 0.16	0.1447	0.0727	0.0842
CD14	7.62 ± 0.19	7.57 ± 0.20	0.8588	0.1701	0.7655
Dec205	6.10 ± 0.32	6.10 ± 0.40	0.1777	0.4157	0.4157
Lymphocytes					
CD18	4.16 ± 0.75	5.64 ± 0.52	0.1065	0.2329	0.4928
CD14	4.71 ± 0.62	5.79 ± 0.54	0.1965	0.9467	0.0614
Dec205	5.76 ± 0.61	4.96 ± 0.62	0.3561	0.5958	0.1068
Monocytes					
CD18	7.76 ± 0.17	8.41 ± 0.16	0.0058	0.0057	0.0308
CD14	8.25 ± 0.18	8.43 ± 0.17	0.4866	0.2090	0.8020
Dec205	6.60 ± 0.18	7.06 ± 0.17	0.0708	0.3586	0.1065
PMN					
CD18	8.19 ± 0.16	8.51 ± 0.16	0.1447	0.0727	0.0842
CD14	7.62 ± 0.19	7.57 ± 0.20	0.8588	0.1701	0.7655
Dec205	6.10 ± 0.32	6.80 ± 0.40	0.1777	0.4157	0.2404

[1]All means reported are least square means ± SE created by mixed model analysis (n=10 calves/treatment).

[2]CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress; CD18 = cluster of differentiation CD18; CD14 = cluster of differentiation CD14; Dec205 = cluster of differentiation Dec205; PMN = polymorph nuclear cells.

Figure 3.1.

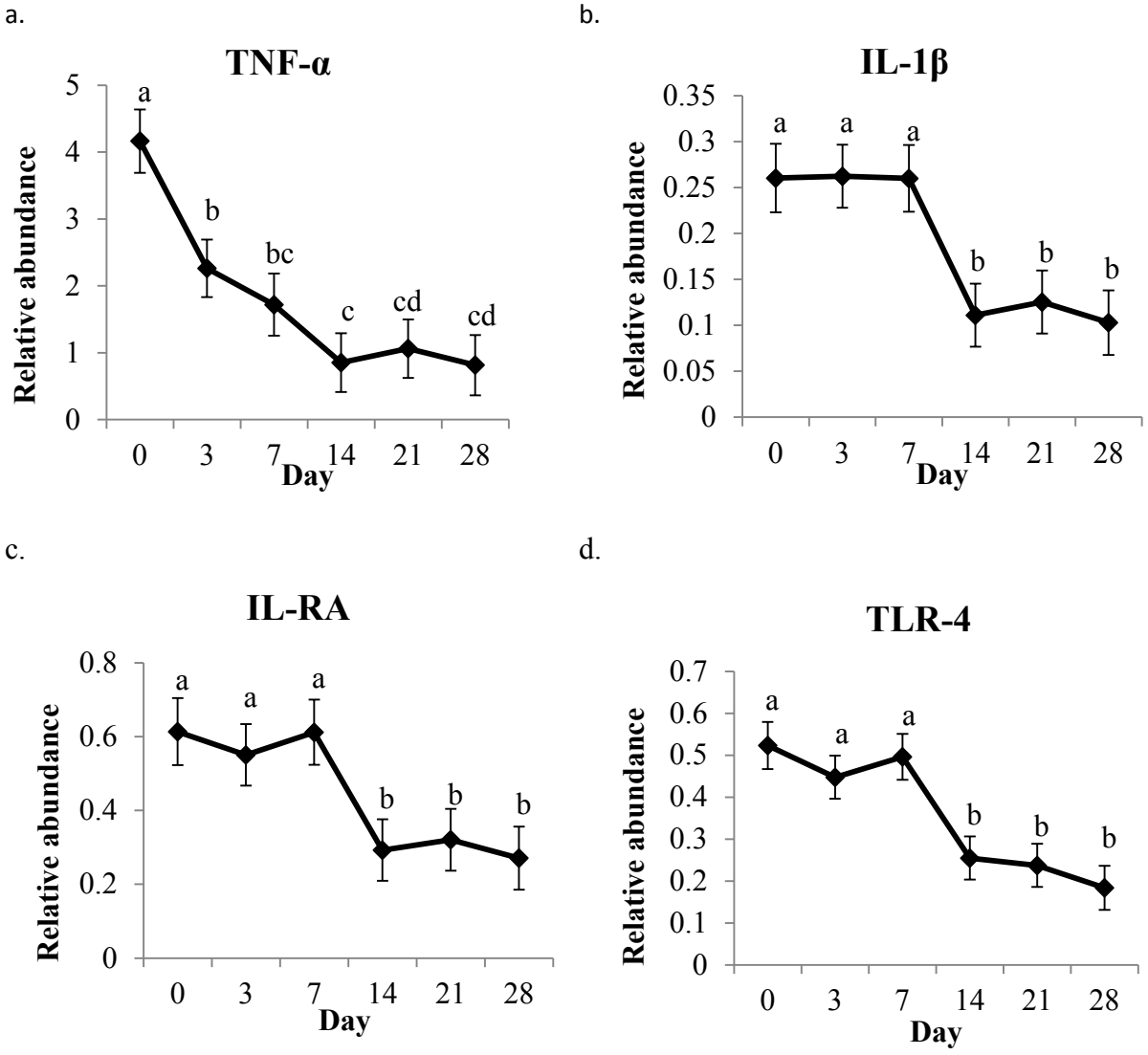


Figure 3.1. The day effect on tumor necrosis alpha (TNF-α) (Panel a), interleukin 1-beta (IL-1β) (Panel b), interleukin receptor antagonist (IL-RA) (Panel c), and toll-like receptor 4 (TLR-4) (Panel d) in leukocytes on days 0, 3, 7, 14, 21, and 28. Data is shown as least squares means ± SEM. Different letters indicate significant differences between days ($P < 0.05$). Data are relative abundance to a stable internal standard, 18S.

Figure 3.2.

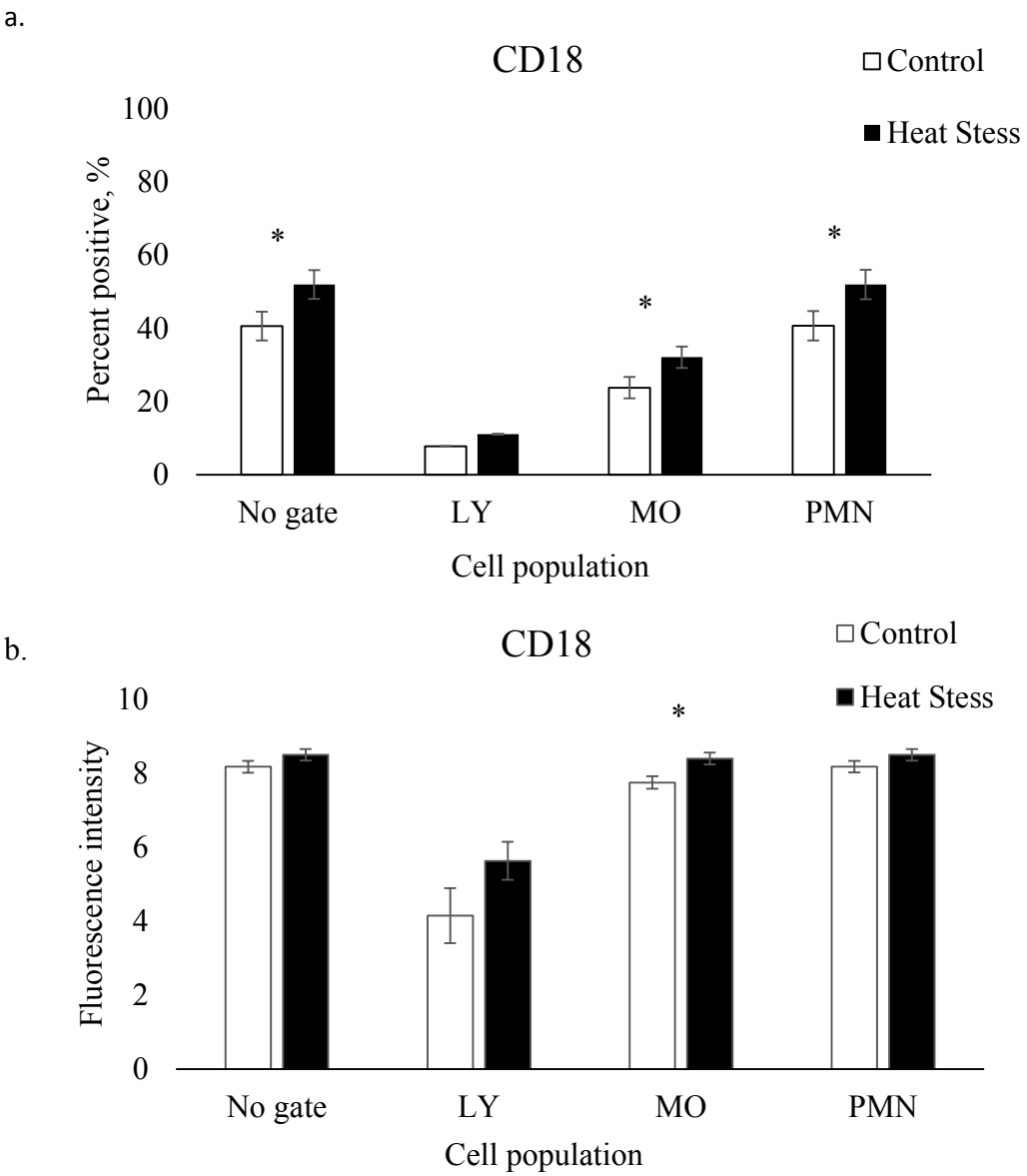
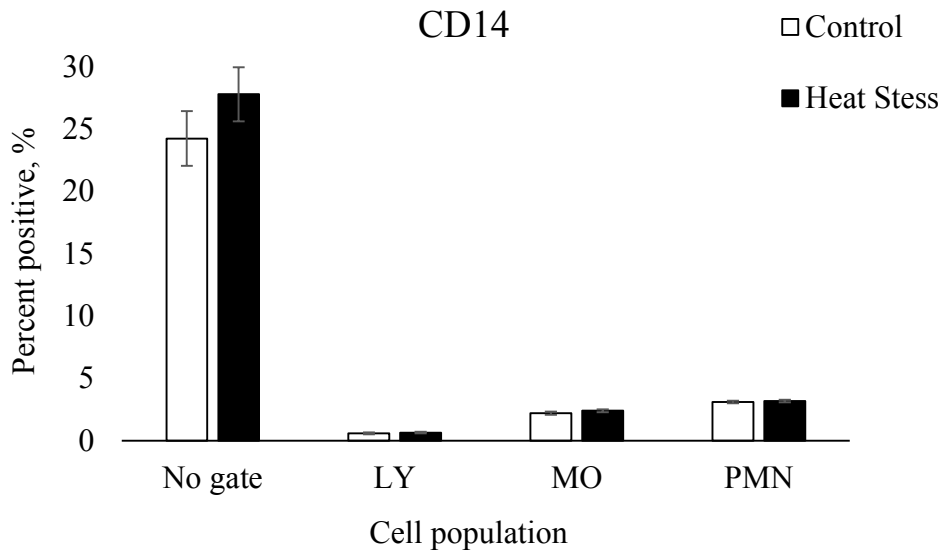


Figure 3.2. The percent positive of all leukocytes (No gate), lymphocytes (LY), monocytes (MO), and polymorph nuclear cells (PMN) that express cluster of differentiation (CD) 18 on their cell surface (Panel a) and the mean fluorescence expression of CD18 (Panel b). Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and fluorescence intensity data was log transformed before statistical analysis. * indicate significant differences between treatments within each cell population ($P < 0.05$).

Figure 3.3.

a.



b.

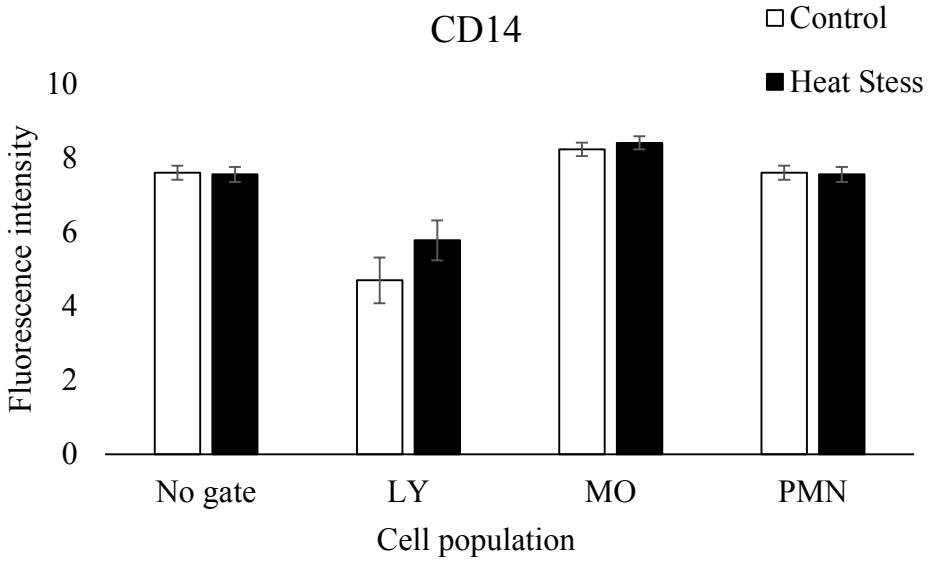
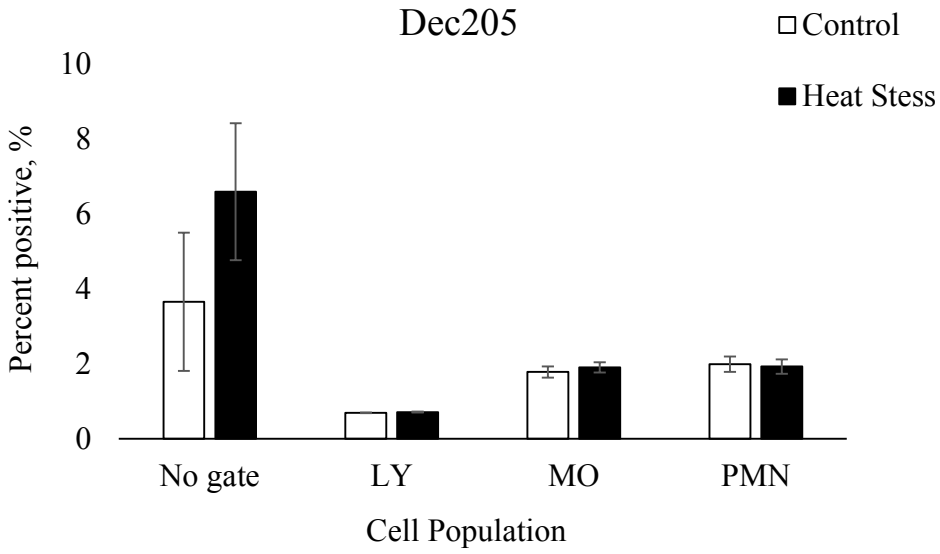


Figure 3.3. The percent positive of all leukocytes (No gate), lymphocytes (LY), monocytes (MO), and polymorph nuclear cells (PMN) that express cluster of differentiation (CD) 14 on their cell surface (Panel a) and the mean fluorescence of CD14 (Panel b). Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and fluorescence intensity data was log transformed before statistical analysis.

Figure 3.4.

a.



b.

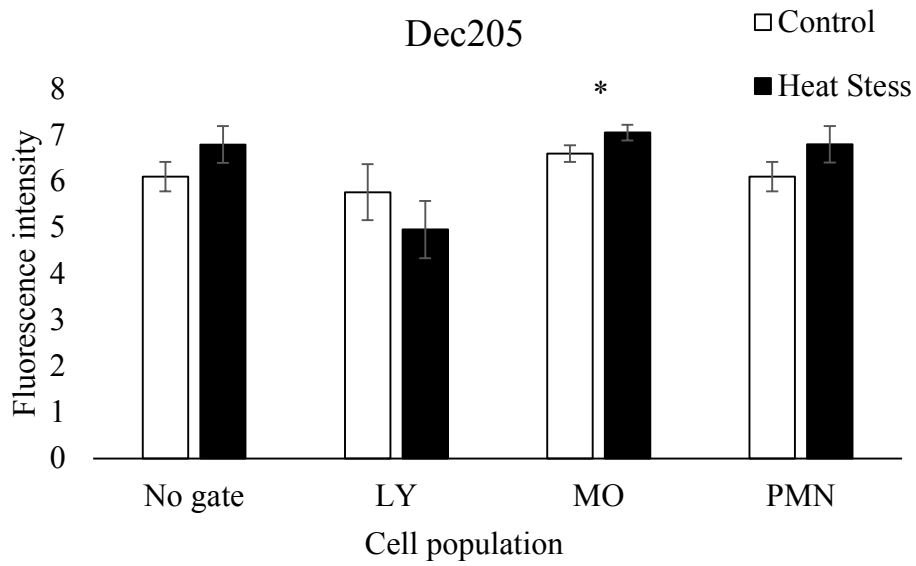


Figure 3.4. The percent positive of all leukocytes (No gate), lymphocytes (LY), monocytes (MO), and polymorph nuclear cells (PMN) that express Dec205 on their cell surface (Panel a) and the mean fluorescence of Dec205 (Panel b). Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and fluorescence intensity data was log transformed before statistical analysis. * indicate significant differences between treatments within each cell population ($0.05 < P < 0.01$).

Figure 3.5.

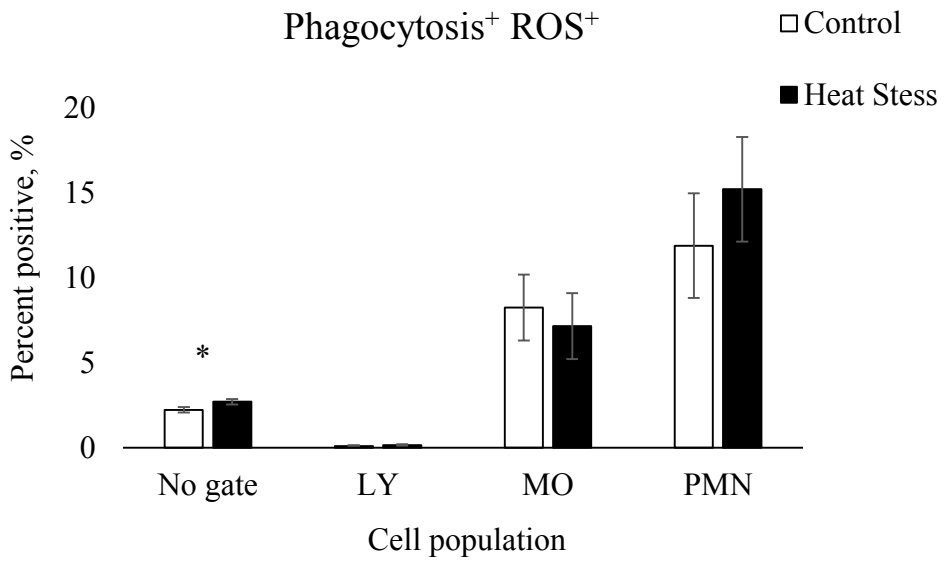
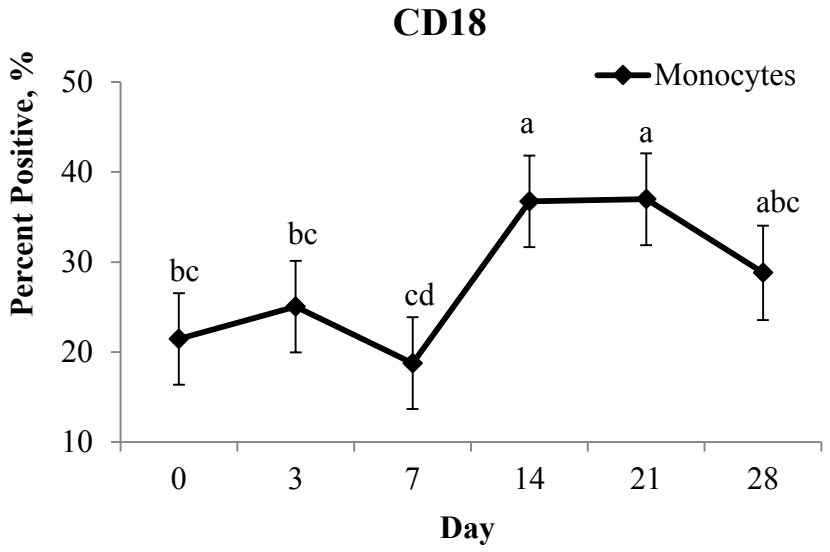


Figure 3.5. The percent positive of all leukocytes (No gate), lymphocytes (LY), monocytes (MO), and polymorph nuclear cells (PMN) for phagocytosis$^+$ ROS$^+$. Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data was log transformed before statistical analysis. * indicate significant differences between treatments within each cell population ($P < 0.05$).

Figure 3.6.

a.



b.

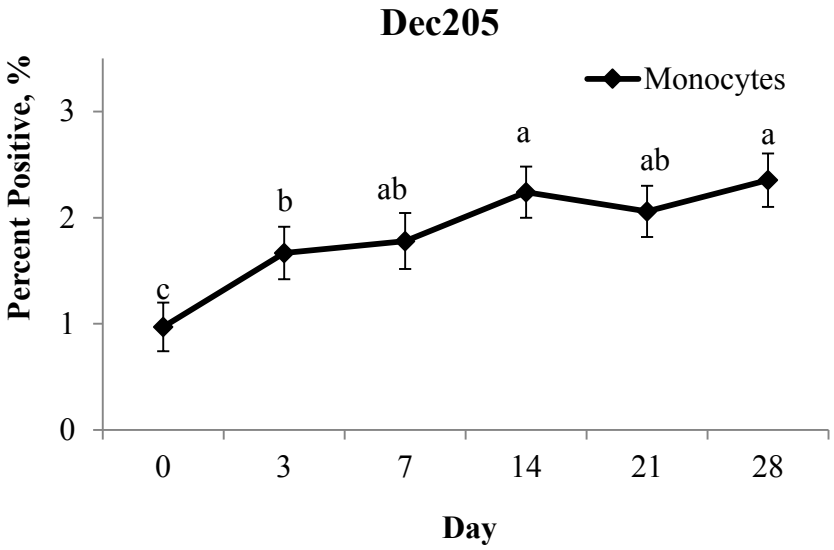


Figure 3.6. The day effect on the expression of cluster of differentiation (CD) 18 (Panel a) and Dec 205 (Panel b) on the monocyte population (MO). Data is shown as mean values ± SE. Percent positive data was log transformed before statistical analysis. Different letters indicate significant differences between treatments ($P \leq 0.01$).

Figure 3.7.

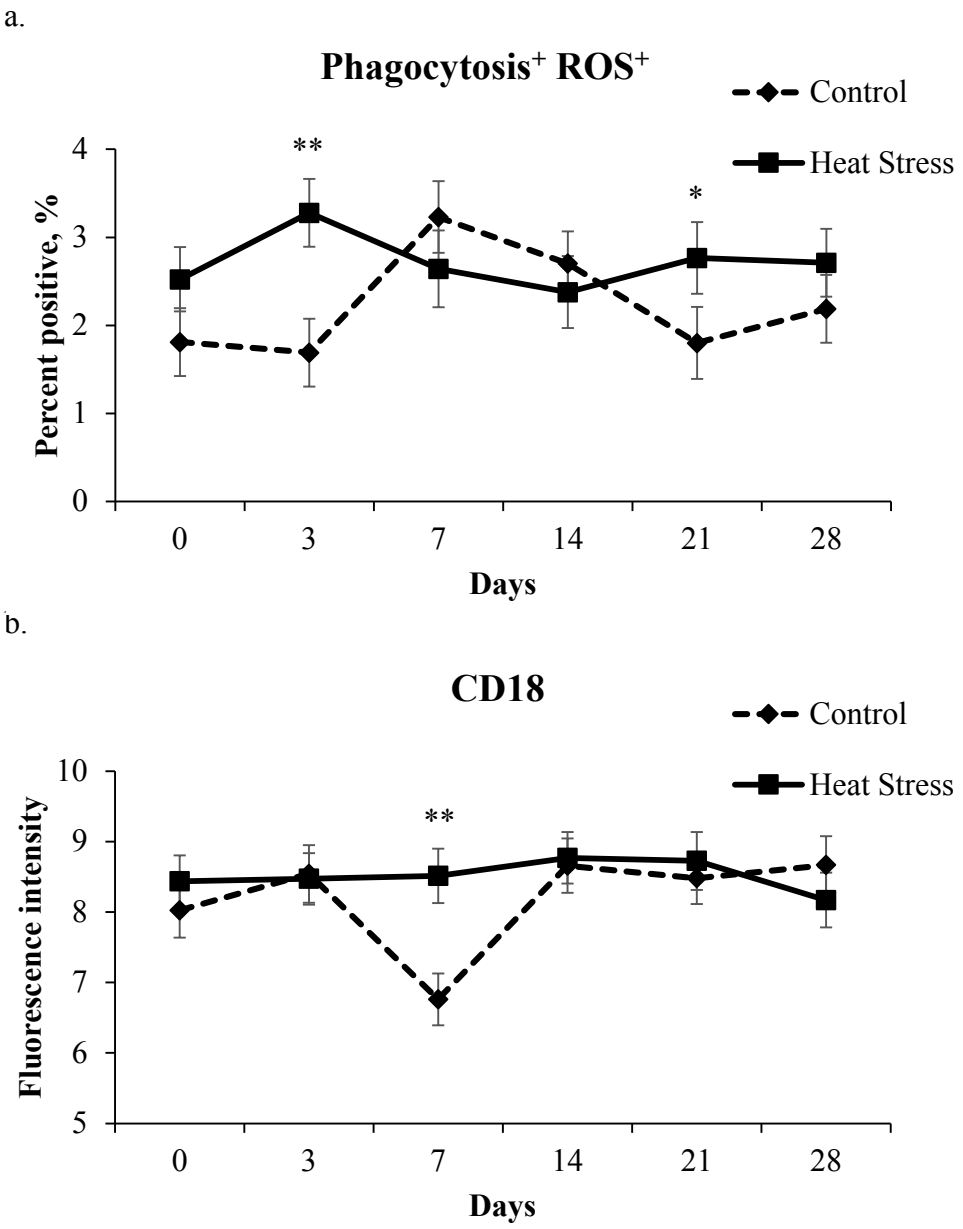
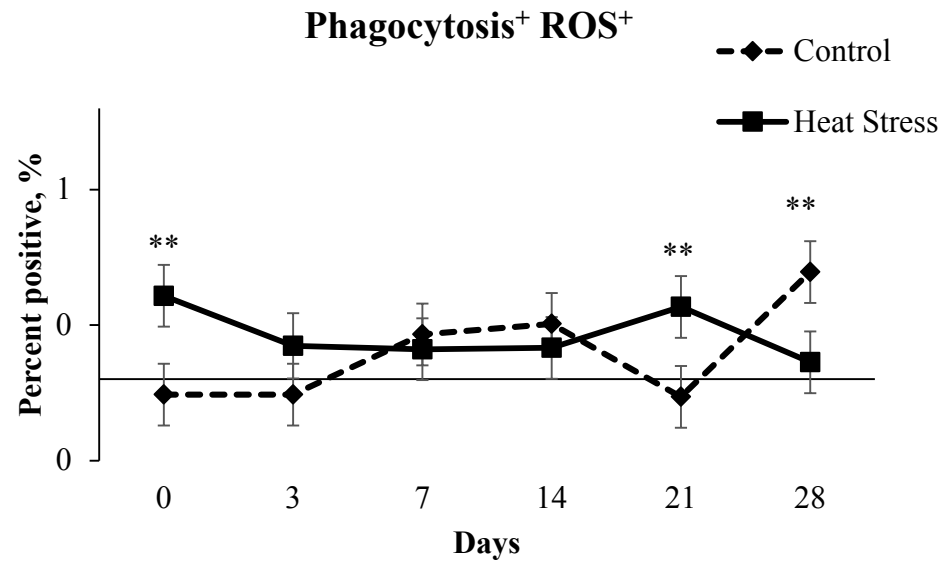


Figure 3.7. The treatment by day effect on the percentage of leukocytes expressing phagocytosis$^+$ ROS$^+$ (Panel a) and on the mean fluorescence of cluster of differentiation (CD) 18 (Panel b). Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and the fluorescence intensity was log transformed before statistical analysis. * ($0.05 < P < 0.01$) and ** ($P < 0.05$) indicate significant differences between treatments on that day.

Figure 3.8.

a.



b.

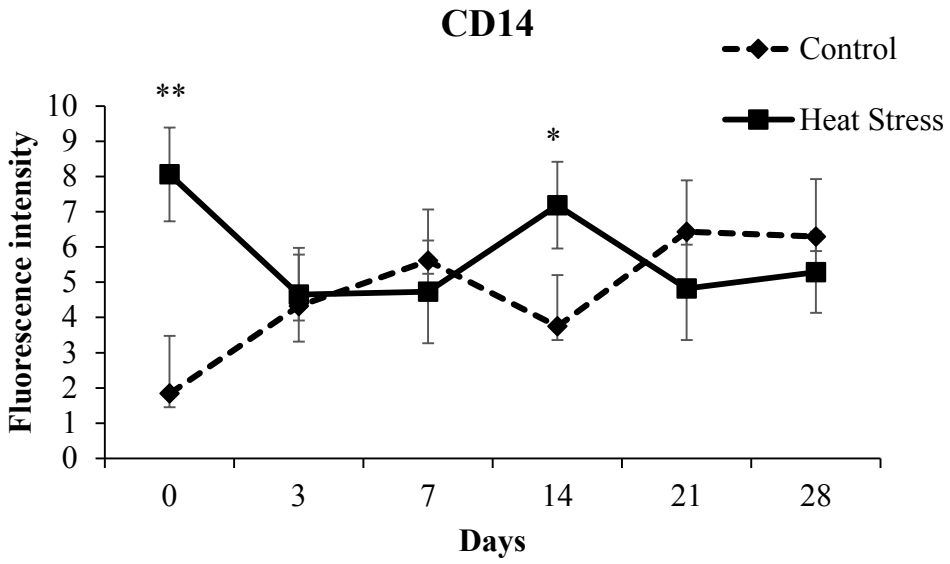


Figure 3.8. The treatment by day effect on the percentage of lymphocytes expressing phagocytosis$^+$ ROS$^+$ (Panel a) and on the mean fluorescence of cluster of differentiation (CD) 14 (Panel b). Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and the fluorescence intensity was log transformed before statistical analysis. * ($0.05 < P < 0.01$) and ** ($P < 0.05$) indicate significant differences between treatments on that day.

Figure 3.9.

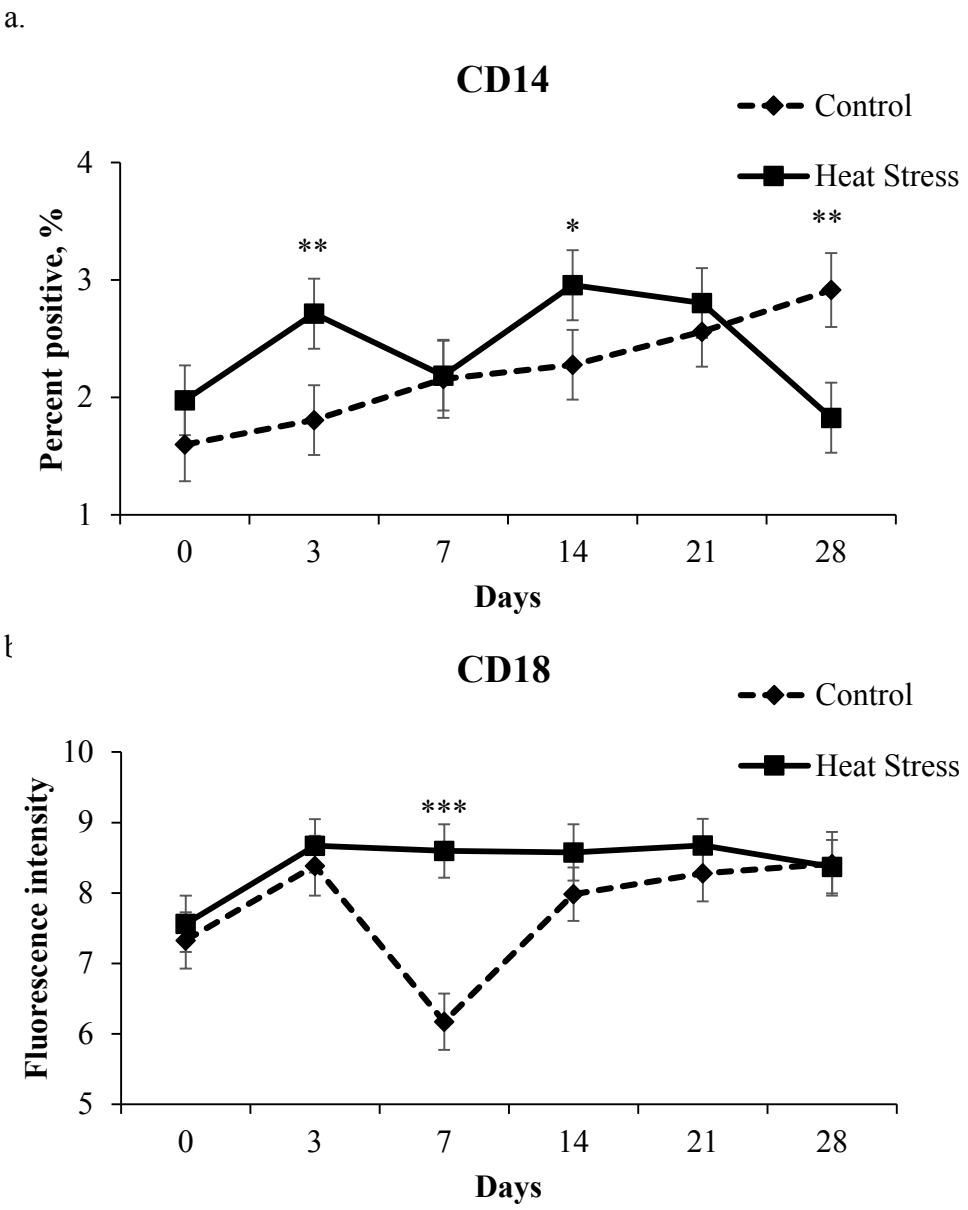


Figure 3.9. The treatment by day effect on the expression of cluster of differentiation (CD) 14 (Panel a) on the surface of monocytes and on the mean fluorescence of cluster of differentiation (CD) 18 (Panel b) on monocytes. Data is shown as mean values ± SE (n=10 calves/treatment). Percent positive data and the fluorescence intensity was log transformed before statistical analysis. * ($0.05 < P < 0.01$), ** ($P < 0.05$), *** ($P < 0.001$) indicate significant differences between treatments on that day.

Figure 3.10.

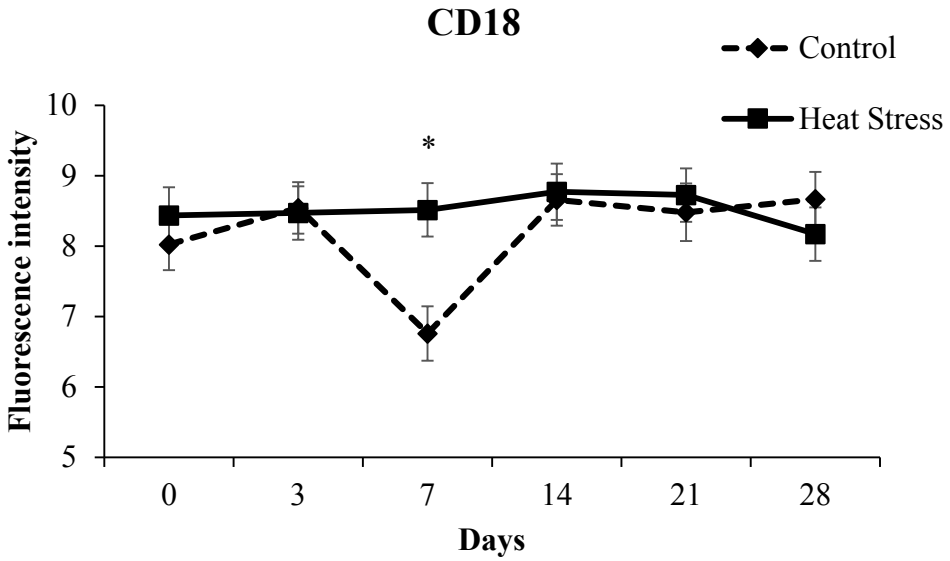


Figure 3.10. The treatment by day effect on the mean fluorescence of cluster of differentiation (CD) 18 in polymorph nuclear cells (PMN). Data is shown as mean values ± SE (n=10 calves/treatment). Fluorescence intensity was log transformed before statistical analysis. * indicates significant difference between treatments on that day ($P < 0.05$).

Figure 3.11.

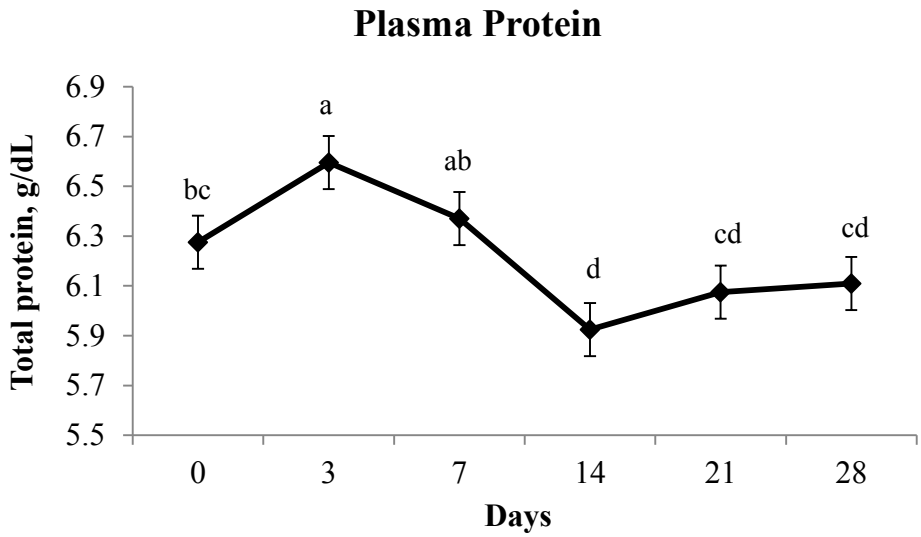


Figure 3.11. The day effect on plasma protein concentration. Data is shown as mean values ± SE. Different letters indicate significant differences between days ($P < 0.05$).

Figure 3.12.

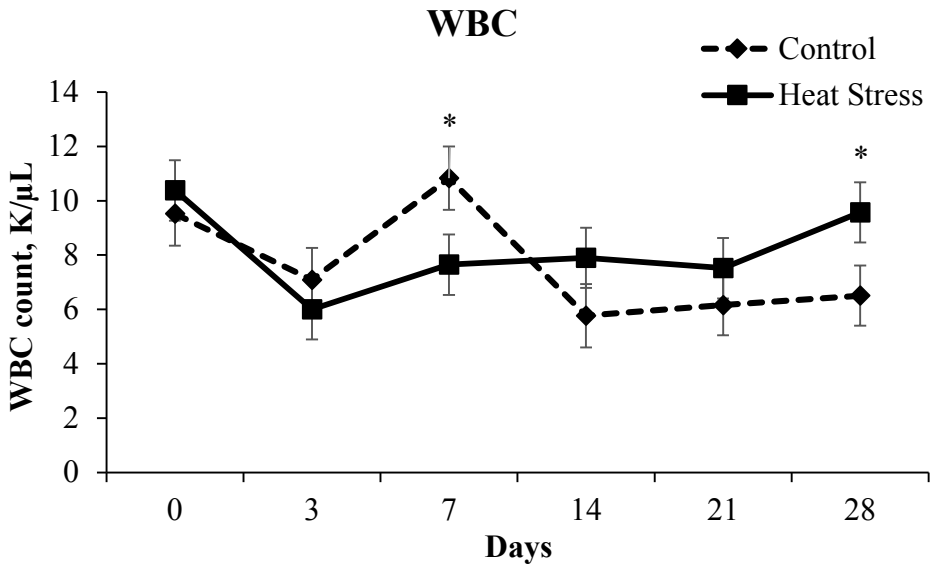


Figure 3.12. The treatment by day effect on white blood cell counts (WBC). Data is shown as mean values ± SE (n=10 calves/treatment). * indicates differences between treatments on that day ($0.05 < P < 0.01$).

Figure 3.13.

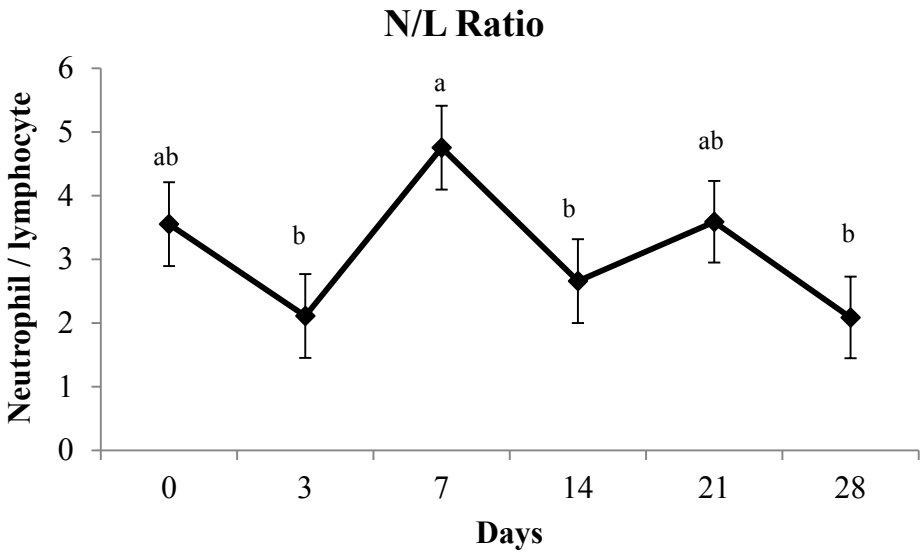


Figure 3.13. The day effect on the neutrophil to lymphocyte (N/L) ratio. Data is shown as mean values ± SE. Different letters indicate significant differences between days ($P < 0.05$).

Figure 3.14.

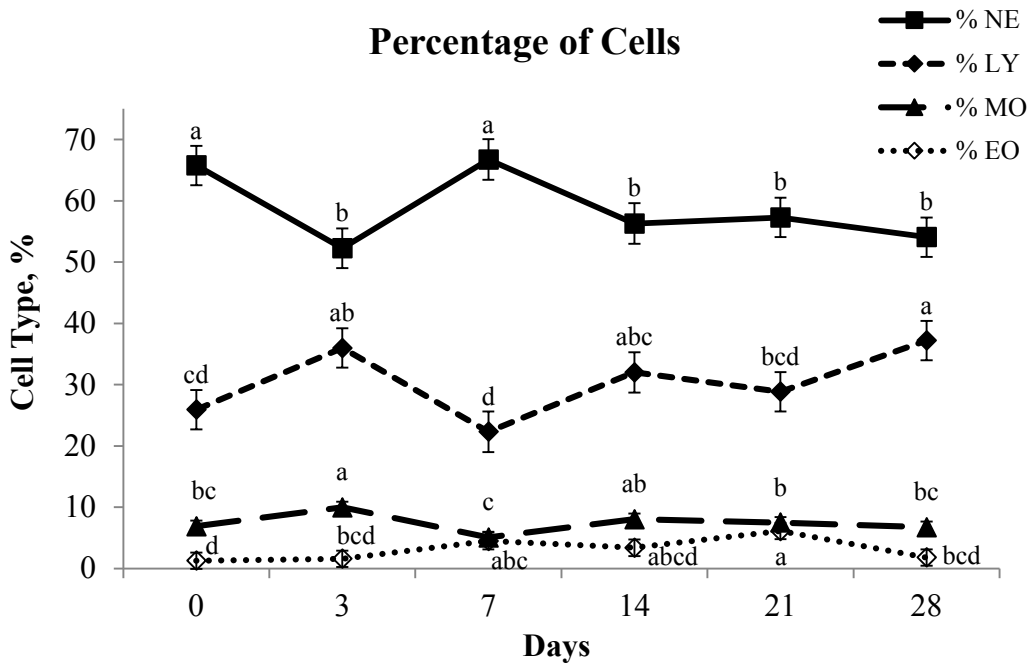


Figure 3.14. The effect of days on the percentage of neutrophils (NE), lymphocytes (LY), monocytes (MO), and eosinophils (EO). Data is shown as mean values ± SE (n=10 calves/treatment). Different letters indicate significant differences between days for each separate cell type ($P < 0.1$).

CHAPTER IV

SUMMARY AND FUTURE RESEARCH

With the increase in global climate change and the population growth driving the high demand for additional food production, HS is a major concern in the livestock industry across all species. The effects of HS are detrimental to the welfare of the animal, which leads to the reduction of their overall performance. Animals experience HS when exposed to high environmental temperatures outside their thermal neutral zone. The level of the effects is dependent upon the length and intensity of HS to which the animal is exposed to. Both physical and physiological changes occur in laying hens and dairy cattle as a result of HS, but there are some similarities and differences between the two species. Cattle have sweat glands, but chickens do not, therefore cattle have a better ability to dissipate heat during hotter temperatures. However, they both increase their respiration rates resulting in panting during high temperatures to adjust for the extra heat load.

Consequently, the changes that occur in the animal to compensate for the added heat load ultimately results in impaired animal welfare, a reduction in production, and also leads to higher incidences of morbidity and mortality. In laying hens, HS has been shown to decrease feed intake, increase water consumption, increase panting, reduce egg production, and impair egg qualities. In dairy cattle, HS has also caused a decrease in feed intake and an impairment in reproduction, as well as a decrease in milk production. As a result of these adverse effects of HS, there is need for extensive research on the effects of HS on the immune system, as well as potential methods to alleviate the stress in both dairy cattle and laying hens. Such research will in turn decrease morbidity and mortality during HS episodes and increase the production globally in hot environments outside livestock thermal neutral zones.

There is evidence that thermally regulated cooled perches can be used to alleviate HS in chickens. Hens have a natural tendency to perch for resting and protection and more than 25% of heat produced can be lost through their feet by modulating blood flow. Thus the increase in conductive heat transfer from the feet to the thermally controlled perch has previously been shown to help relieve HS in laying hens. It has also been demonstrated that broiler chickens prefer to roost on cooled perches when exposed to high environmental temperatures, resulting in a decrease in panting, a reduction in core body temperature, and an increase in BW gain. The aforementioned studies have determined the effectiveness of cooled perches to improve chicken performance during HS in broilers. However, there is insufficient evidence as to how cooled perches would affect laying hens during HS. The strategy of using cooled perches, as a method to alleviate HS in broilers, has provided evidence on the potential for cooled perches to improve laying hen immunity during HS.

In the first experiment, we observed the potential of cooled perches as a method to alleviate the immunological changes that occur from exposure to HS on laying hens of a popular commercial breed; Hy-Line W36. Measurements were taken at both acute and ambient environmental conditions; at 27 and 32 weeks of age respectively. Specific measurements suggested that cooled perches can be used to alleviate the immunological effects that occur from HS, such as a reduction in the H/L ratio, but to fully understand the extent of cooled perches on the immune system, an increase in stress on the birds is required. Additionally, the current data provides researchers with a basis of how thermally regulated perches have the potential to provide a strategy that can be implemented to improve hen immunity. There is a great opportunity for future research to

further expand upon our results by determining the effects of cooled perches on laying hens exposed to a longer duration of a more stressful environment, and an induced chronic HS over a longer production period.

As the first study implementing thermally regulated cooled perches in laying hens, there are a few considerations when moving forward before cooled perches can be applicable to the poultry industry. One area that needs to be greatly considered is the initial cost and management requirement for the use of thermally regulated perches. The current study did not provide sufficient evidence to outweigh the cost demand for the extensive cooled perch caging system. However, it did provide sufficient evidence to continue the research on the benefits of cooled perches in order to be implemented in future production cages to alleviate the negative effects of HS in laying hens. Secondly, no studies have looked at the benefits of cooled perches during HS throughout the life of the hen. The results of the current study were completed on young birds; however, HS has also been shown to have detrimental effects on older birds. HS has apparent short term effects on lying hens, such as a decrease in productivity, but the damage of HS on the hen beyond several weeks has been less studied. The long-term effects of HS are more of a concern in laying hens, due to their longer lifespan than broilers, allowing them to be a better model for the long term benefits of cooled perches used to improve hen immunity during HS. Thirdly, in experiment 1, very specific immunological parameters provided evidence that hens with access to cooled perches during HS improves hen immunity. However, little is known about the specific mechanism of how HS affects the immune system in birds. Therefore, to further expand upon how cooled perches alleviate HS, additional immunological factors that have been altered by HS should be determined,

which has been shown to increase in birds allowing protection from HS. These results would be expected to further support the claim that cooled perches improve hen immunity during HS. Lastly, it has been shown that birds are more susceptible to disease during HS and several studies have shown different methods to combat the effects, such as with nutritional supplementation and genetic selection. Thus, as more evidence is gathered on the benefits of thermally cooled perches on the immune system in birds, the next stage would be to see if access to cooled perches improved the immune function enough to reduce morbidity during HS.

Allowing access to regulated zone cooling through cooled perches has benefits on improving the immune system in laying hens during HS, especially during the summer months. The present study determined cooled perches have the potential to inhibit heat stress-induced immunological changes in laying hens during exposure to high temperatures. However, future research is required before thermally regulated perches can be implemented in the poultry industry.

The effects of HS on dairy cattle have been extensively studied, but less is known on the prenatal effects of HS on the neonate. Previous studies found that cows exposed to increased environmental temperatures have increased rectal temperatures and respirations rates. The cows also have a decrease in production and an impaired reproduction and immune system during HS. Our study was based on the hypothesis that with the physical and physiological changes occurring in the mother during HS in late gestation, there would be an effect on the offspring resulting in an impaired immune system. A few studies in pigs, mice, and sheep looked at the effects of HS on the offspring, but only one other study in dairy cattle has investigated the effects of prenatal HS during late gestation

on the growth and immune system of the neonate. Maternal HS during late gestation was found to inhibit the immune response of the offspring and lower birth weight. In that study there were limited immune factors analyzed, so in the current study we expanded the immunological parameters measured to determine how maternal HS during late gestation effects the offspring's immune function.

Like the other study, we anticipated that maternal heat load during late gestation would suppress the innate immune system in neonatal calves. In the second experiment, we observed immunological changes in the calves born to prenatal HS. Measurements were taken at 0, 3, 7, 14, 21, and 28 days of age while the calves were exposed to similar environmental temperatures after birth. We determined specific immunological parameters that suggest maternal HS does alter the calf innate immune system. Additionally, a previous study reported that 2 wk old calves are more vulnerable to bacterial infections since their maternal antibodies are declining but their own immune system is still not fully developed yet. Therefore, with the changes in immune function, shown in the current study, the calves may become more susceptible to disease after prenatal HS to microorganisms that may normally be nonpathogenic.

There is immense opportunity for future research to further expand upon our results in determining the effects of prenatal HS on neonate immunity by measuring additional maternal stress-related physiological changes, such as stress hormone levels to further understand the relationship between the maternal HS effects and the neonates' innate immune function. In addition, the implications of HS research are remarkable, and have the potential to be linked to the health and welfare of multiple species, including humans. Therefore, future studies regarding the prenatal HS effects on calf innate

immunity may have an application for better understanding and implementing strategies to prevent the maternal HS effects on offspring immunity in humans.

In conclusion, HS greatly impacts many different species and poses a wide threat on the health and wellbeing of animals due to the global climate changes and increased demands on the livestock industry. Thermally cooled perches, as a method to improve hen immunity during HS, has allowed additional knowledge for creating a long-term strategy to alleviate HS in laying hens. The changes found in neonatal immunity after exposure to late gestational prenatal HS has potentially opened other avenues of research to better understand the effects of prenatal HS on the offspring of both livestock and humans. While one study is alleviating the effects of direct HS and the other is investigating the effects of indirect HS, both experiments expand the much needed area of HS research in livestock production.