THE IMPACT OF HEAT STRESS ON HEALTH AND BEHAVIOR OF DAIRY CATTLE

by

THIAGO NOGUEIRA MARINS

(Under the Direction of Sha Tao)

ABSTRACT

Deep understanding of the impact of heat stress on cows allows the implementation of effective strategies to enhance cow productivity and welfare during summer. In chapter 3-5, we evaluated the metabolism and inflammation of cows fed two zinc sources (Zn-hydroxychloride vs. Zn-Met complex) under two environmental conditions (cooling [CL] vs. non-cooling [NC]) prior to and following an intramammary lipopolysaccharides infusion (IM-LPS) during summer. Non-cooling reduced plasma metabolite concentrations, but increased insulin concentration. Dietary Zn source had no impact on systemic and mammary metabolism. Following IM-LPS, NC induced greater leukocyte migration into the mammary gland after IM-LPS, and NC maintained lower plasma glucose concentrations but had a more rapid and prolonged increase in serum insulin concentration than CL. Further, NC influenced the systemic and mammary mineral metabolism after IM-LPS, however, dietary Zn source had no impacts. In chapter 6, we evaluate hormonal, inflammatory and immunological responses of lactating dairy cows fed an immunomodulatory supplement (OmniGen® AF, OG) and under CL vs. NC prior to and following an intravenous LPS infusion (IV-LPS) during summer. Feeding OG enhanced cortisol release under basal conditions and after IV-LPS in CL but not NC, suggesting that heat stress inhibits the OG mediated cortisol release. Further, NC enhanced inflammatory responses of circulating lymphocytes stimulated with

mitogens ex vivo than CL. Yet, OG promoted the proliferation of immune cells isolated from cows under NC. In chapter 7, we examined the behavioral responses of cows under CL and NC. NC reduced lying, rumination, and eating time but increased walking time and activity level. Cows under NC sorted more for long particles from the TMR than CL. Chapter 8 evaluated associations between behaviors and body temperature of lactating dairy cows on 3 commercial dairy farms, and we demonstrated that the effectiveness of cooling facility strongly affects the associations between lying/standing behavior and body temperature, but was not associated with daily walking activity and ruminating time under chronic heat stress condition and consistent management. These studies highlight the importance of implementing effective cooling systems and nutritional programs to reduce the adverse effects of heat stress on cow performance.

INDEX WORDS: Heat stress, Physiology, Performance, Health, Behavior, Dairy Cattle

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DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2025

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August 2025

DEDICATION

This work is dedicated to my father, Paulo, and to the loving memory of my mother, Mara, who both did everything possible to give me the opportunity to pursue my education. Your dedication and support helped shape me into the person I am today. I also dedicate this work to my beloved wife, Larissa. Thank you for being my partner. Thank you for standing by me every step of the way and for never letting me give up on this journey. Thank you for your unconditional support and love. I love you.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Sha Tao, for believing in me at the very beginning of this long journey. I am forever grateful for your support and guidance. Thank you for always showing me respect and for giving me the opportunity to express my thoughts. Your openness and encouragement have meant a lot to me in all these years working together. Thank you for showing me the true value of hard work and dedication. Certainly, it helped me grow both personally and professionally.

I would also like to thank my committee members, Dr. Joao Costa, Todd Callaway, and Romdhane Rekaya for the time and role played in this important stage of my journey, contributing to my professional growth. Thank you for the lessons taught inside and outside the classroom.

I extend my sincere gratitude to Dr. John Bernard, Melissa Tawzer, Katie Wilson, and all the people from the Department of Animal and Dairy Science at Tifton Campus, where I began my journey at UGA and I always received support. Also, many thanks to all the people from the Department of Animal and Dairy Science at Athens Campus, including staff, professors, and graduate students for the incredible time that I spent in Athens. The welcoming and encouraging atmosphere played a vital role in this journey.

To Dr. Maria Lucia Gambarini, my advisor in Brazil, thank you for the opportunity and meaningful impact on my family's life, even if you may not realize it. I am where I am today because you gave me the chance to grow.

To Dr. Anderson Alves, a special thank you for your help and always being open to discussion whenever I needed it. As I have said before, I wish I had the chance to learn from you years earlier.

A special thank you to Jing Gao, who was, firstly, my intern student and, later, a graduate lab mate. Thank you for your support. Your courage and hard-working attitude are admirable. I have learned a lot from you.

Many thanks to my graduate labmates, Marisol, Yun-Chun, Carol, Yara, and Ansley for all the time we shared together. I am grateful to have had the chance to share my experiences and learn from all of you. Despite the stress and hard work, we always found time to support each other and enjoy great moments together.

To all intern students (Jainer Calix, Jordana Silveira, Caique Pessoa, Qian Yang, Diego Araujo, Juliana de Souza, Zhen Wang, Fabian Gutierrez-Oviedo, Victor Lacerda) thank you for the hard work in helping me in several projects throughout my journey in UGA. I am happy with the opportunity to learn from your different cultural and social backgrounds.

To Samir, Luquinhas, Lucão, Carol, and Pedro thank you for your support and friendship. I feel fortunate to have had the opportunity to share incredible moments with all of you. From brief conversations at our desks during the workday to spending an entire day enjoying a Brazilian barbecue and having heartfelt talks about life and the future, the joy and laughter we shared have made the journey much easier. I look forward to creating many more memories together.

A special thanks to my dear friends, Jessica, Fabricio, Jordão, and Hanna. Meeting you guys in Tifton was one of the best lucks I have had during this journey. We have shared not only many good moments but also some tough times, always standing together and supporting each other with laughter and hope. I truly feel we have created a family. I am grateful to have been part

of meaningful moments like each of your graduations, the births of Mateo and Noah, and so many others we have celebrated together.

To Dr. Branch and Mrs. Teresa for all the support during this time and for welcoming my wife and me with a true sense of family at dinners, birthdays, and shared moments. You have truly made a difference in our lives and have been an important part of this accomplishment.

I would like to express my deepest gratitude to all my amazing family and friends in Brazil and the U.S for their support throughout this journey.

Last but not least, to part of my family, my cats Gordinha, Matilda, Tangerina, Mufasa, Faisca, Cobinha, and Dilinha, thank you for your “furry” friendship and always bringing me joy and comfort when I am at home after tough days of hard work.

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CHAPTER 1

INTRODUCTION

The advancing of climate change poses heat stress as a significant concern on animal production and sustainability of livestock systems. Direct and indirect effects of heat stress compromise livestock health, lower feed efficiency, and increase livestock production costs worldwide (Lacetera, 2019). Heat stress causes a tremendous negative economic impact on U.S. livestock industries, and the dairy industry suffers a greater economic loss due to heat stress compared to any other livestock industries (St-Pierre et al., 2003). The reduced milk yield is one of the most recognized negative impacts caused by the exposure to heat stress, but it is also widely recognized that heat stress causes negative impacts on the immune system, health, behavior, and overall welfare of dairy cattle (Polsky and von Keyserlingk, 2017; Dahl et al., 2020). Animal welfare is a major concern to the development of dairy industry, and heat stress disrupts natural behaviors and emotional states of the cow, leading to changes in physiological responses and behaviors. Indeed, consumer awareness and concern regarding animal welfare in food production have challenged dairy producers and industry personnels to seek efficient methods to identify, manage, and mitigate the negative effects of heat stress on dairy cows (Weary and Von Keyserlingk, 2017; Cardoso et al., 2018; Hendricks et al., 2022).

The magnitude of heat stress experienced by dairy cows is influenced by production systems, the surrounding microenvironment, and management practices to which cows are exposed to. The adopted cooling strategies can be different depending on farm infrastructure, investment capacity, and management practices. However, it is essential to ensure the

implementation of cost-effective heat abatement strategies to relief body heat and mitigate the negative impacts of heat stress, which is crucial to maintain productivity and maximize economic returns in dairy operations (Gunn et al., 2019).

Comprehensive understanding of the effect of heat stress on performance, physiology, immune response, and behavior will facilitate the interpretation on how animals respond to their surroundings and corresponding reactions. This will also allow the implementation of nutritional (e.g., diet formulation, feed additives, etc.), environmental (e.g., heat abatement systems, barn design and hygiene, etc.), and management strategies to promote both productivity and welfare of dairy cows during summer.

Monitoring of heat stress level is critical for implementation of the cooling strategies and can be based on environmental parameters, such as air temperature and relative humidity, and THI which combined temperature and humidity. Compared with other regions in the U.S., the environmental conditions are extremely challenging in the Southeast, like Georgia. The subtropical climate in Southeast is characterized with longer, hotter and humid summers which pose challenges in heat abatement for dairy cattle (West, 2003; Bohmanova et al., 2007; Key and Sneeringer, 2014). In addition, animal-related measurements can be effectively used to identify and assess the severity of heat stress in individual dairy cows or groups, offering a more comprehensive approach compared to environmental parameters alone. In dairy cattle, physiological, behavioral, and production-related signs can be used to identify animals suffering heat stress. These parameters include, but are not limited to, increased respiratory rate (panting, open-mouth breathing, and excessive salivation and drooling), decreased milk production, reduced feed intake, increased water consumption, restlessness or agitation, and the increased body temperature (Polsky and von Keyserlingk, 2017; Chen et al., 2023). The assessment of animal

behavior also serves as a valuable approach to identify illness and animal responses to environmental stimuli, and it has been adopted on heat stress monitoring (Becker and Stone, 2020; Eckelkamp and Bewley, 2020).

Certainly, past research in the last decades have contributed to a better understanding of the impact of heat stress on dairy cattle, but additional studies are necessary to improve the management of cows under the adverse heat stress conditions, ensuring a more sustainable milk production and optimal cow health and welfare. This is particularly important on the face of climate change and the continuing rise in global temperature.

Therefore, this dissertation aims to combine applied and basic research to evaluate the impacts of environment, nutrition, and management on productive performance, systemic and mammary metabolism, immune function and health, hormonal and inflammatory responses, and behavioral patterns of lactating dairy cows exposed to heat stress. Chapter 2 provides a comprehensive literature review of heat stress in dairy cattle. Chapter 3 evaluates the systemic and mammary metabolism of lactating dairy cows fed two supplemental Zn sources under two environmental conditions during summer. Chapter 4 assesses the effect of dietary supplemental Zn source and evaporative cooling on intake, milk yield and composition, and the rate of leukocyte migration into the mammary gland following intramammary lipopolysaccharide (LPS) infusion in lactating dairy cows. Chapter 5 determines the effect of evaporative cooling and dietary supplemental Zn source on blood metabolites, insulin and mineral concentrations, and milk mineral concentrations of lactating dairy cows to an intramammary LPS infusion during summer. Chapter 6 evaluates the impact of an immunomodulatory supplement and heat stress on hormonal, inflammatory, and immunological responses of lactating dairy cows under basal condition and during induced acute systemic inflammation. Chapter 7 evaluates the effects of deprivation of

evaporative cooling on behavioral responses of lactating dairy cows prior to and following mammary inflammation induced by intramammary lipopolysaccharide infusion during summer. Chapter 8 assesses the association between daily body temperature and behavioral responses of multiparous lactating cows on commercial dairy farms with different cooling settings and milking routines. The general conclusions of this dissertation are presented in Chapter 9.

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CHAPTER 2

LITERATURE REVIEW: HEAT STRESS

Importance of studying heat stress in dairy cattle

Climate change has accelerated in the last decades (Hansen et al., 2025) and the ongoing rise in global temperatures has been a major concern for livestock systems worldwide. The mean global temperatures are projected to rise in the near future with direct and indirect implications for livestock industry (Cheng et al., 2022), including the supply chain for animal sourced food (Godde et al., 2021). Recently, NOAA (2025) announced that the global surface temperature in 2024 was 1.29°C above the 20th-century average, ranking as the highest global temperature in the period of 1850–2024. Additionally, in 2024, the average global temperature exceeded the pre-industrial (1850–1900) average by 1.46°C. The unprecedented global temperatures in 2024 (Copernicus, 2025) reinforce the challenge of heat stress on dairy cattle and emphasize the need of innovative, effective, and sustainable approaches to mitigate the negative effect of heat stress.

Heat stress causes a tremendous negative economic impact on U.S. livestock industries. It results in approximately $897 to $1500 million economic loss in the dairy industry, which is greater than any other livestock specie in the U.S. (St-Pierre et al., 2003). Estimation of the losses due to heat stress shows that negative economic impact can occur for the entire dairy sector in the U.S, with greatest losses occurring in the southern states (Key and Sneeringer, 2014). Due to climate change, the economic losses caused by heat stress may rise to $2.2 billion/year by the end of this century (Mauger et al., 2015). Gunn et al. (2019) reported that the milk loss due to heat stress was predicted to increase at a rate of 174 ± 7 kg/cow/decade in the 21st century, suggesting

that the adoption of cost-effective heat abatement strategies will be critical to maintain productivity of dairy cows and positive economic returns of dairy producers. Interestingly, heat stress during the dry period of dairy cattle also causes negative economic losses. A study suggested an annual loss of $810 million in the U.S. caused by the reduced milk production of cows who are exposed to heat stress during the dry period (Ferreira et al., 2016). Additionally, there is an annual $595 million loss for the US dairy sector arising from the lower survival and milk losses of heifers who are born to the heat stressed dry cows (Laporta et al., 2020).

In addition to economic return, animal welfare is also compromised by heat stress because of the impaired health and altered natural living and affective state, which cause changes in the physiological response and behavior patterns of animals (Polsky & von Keyserlingk, 2017; Silanikove, 2000). Indeed, consumer awareness and concern regarding animal welfare in food animal production has challenged dairy producers and industry personnel for seeking efficient methods to identify, manage, and mitigate the negative effects of heat stress on animal wellbeing (Weary and Von Keyserlingk, 2017; Cardoso et al., 2018; Hendricks et al., 2022). Certainly, research in the last decades has contributed to a better understanding of the impact of heat stress on dairy industry, but additional studies are necessary to further improve the management of cows under the adverse heat stress conditions and to ensure a more sustainable dairy production and optimal cow welfare. This is especially important with the continuous rise in global temperature.

Main concepts of heat stress

Stress refers to an external event or condition that alters the animal's homeostasis (Collier et al., 2017) while distress is a harmful state in which coping and adaptation processes fail to return an organism to physiological and/or psychological homeostasis (Carstens and Moberg, 2000). Heat

stress is defined as a sum of external forces acting on animals that lead to an imbalance in heat production and heat loss to consequently increase core body temperature (Dikmen and Hansen, 2009; Polsky and von Keyserlingk, 2017). In addition to the term of “heat stress”, the change in the thermal relationship between thermal regulation and the surrounding environment, causing hyperthermia, is also known as “thermal stress” and “heat load” (IUPS, 2001; Heinicke et al., 2018; Lees et al., 2019). Heat strains are measurable physiological, behavioral, or productivity-related responses or effects which differ from the corresponding base values within the thermoneutral zone and serve as indicators of animal's reaction to heat stress (Lee, 1965).

Body temperature (vaginal or rectal) or respiration rate (panting frequency) provides valuable information for the relationship between the animal and the environment (Polsky and von Keyserlingk, 2017; Liu et al., 2019). Dairy cattle are homeothermic animals that maintain body temperature by ensuring equilibrium between heat gain and heat loss. The circadian body temperature may be changed or disrupted by factors such as breed, lactation state, reproductive state, metabolic activity, the time of feed delivery, physical activity, disease, and stress events (Kendall and Webster, 2009; Montes et al., 2023). The set point of physiological body temperature of dairy cattle is 38.5 °C (Collier et al., 2019), varying in the range of 37.8 to 39.1 °C under healthy condition and within the thermoneutral zone (Kendall and Webster, 2009; Gupta et al., 2023). Further, respiration rate (RR) is an early measurement to demonstrate cattle’s initial response to heat stress (Gaughan et al., 2000). It is suggested that RR > 60 breath/min indicates cows undergoing heat stress (Kadokawa et al., 2012). However, the threshold of RR needs to consider different factors, such as life stage, breed, posture, location within pen, milk yield and gestation length, with the magnitude of RR varies depending on these factors (Tresoldi et al., 2025).

Within the thermal neutral zone, which covers a range of environmental temperatures, the animal doesn't have to work actively to maintain the core temperature and can maintain a balance between heat gained and heat loss without modification on metabolism, physiology, and productivity (Kadzere et al., 2002; Allen et al., 2013). The thermoneutral zone for dairy cattle lies in between 5 and 25 °C. When ambient temperature exceeds the upper temperature threshold, known as upper critical temperature, stress or discomfort begins to occur inducing heat strains (Kadzere et al., 2002). However, the thermoneutral zone may vary based on factors like age, species, breed, diet, prior acclimation to temperature, production demands, housing, insulation from tissues and coat, and the animal's behavior (Kadzere et al., 2002; West, 2003). In addition to air temperature, the effects of relative humidity, solar radiation, and wind speed should be considered due to their influence on surrounding microenvironment, thermoregulation and heat dissipation (Dikmen and Hansen, 2009; Renaudeau et al., 2012). Air temperature or ambient air temperature typically refers to the dry bulb temperature, which is the air temperature measured by a thermometer unaffected by air moisture. The relative humidity measures the ratio of the actual amount of atmospheric moisture in the air to that of the saturated air at a specific temperature (NRC, 1971; Bahadori et al., 2013).

Animals have developed coping mechanisms to reduce the impact of environmental stressors on their biological systems, and their responses to a given set of stressors may change over time. Acclimation is defined as the process through which animals adjust their phenotypic (physiological or behavioral) characteristics in response to changes to a particular environmental stressor over a relatively short period, while acclimatization is a coordinated response to numerous simultaneous stressors (e.g., temperature, humidity, wind speed, and solar radiation) (Bernabucci et al., 2010; Collier et al., 2019). Since it is difficult to have only one changing variable, typically

an animal is undergoing acclimatization to improve fitness to the environment (Collier et al., 2019). In cases where the stressful environment persists over a long period that compromises successive generations, animals will have evolutionary changes at genetic level that are referred to adaptations. Adaptation will present characteristics that give higher chances for the animal and offspring to survive against threatening environments (Collier et al., 2019).

Dairy cattle are highly sensitive to heat stress, and the accumulation of heat is due to direct solar radiation, high ambient temperatures, heat production by rumen fermentation, and metabolic processes to produce milk (Tucker et al., 2008; Liu et al., 2014). Physical activity may also increase the amount of heat produced by skeletal muscles and body tissues (West, 2003). Metabolic heat production of a dairy cow is strongly associated with milk production, which increases her susceptibility to heat stress (West, 2003). Similar to other homeothermic animals, cattle regulate internal body temperature by thermoregulatory mechanisms to match the amount of heat gained with the heat dissipation to the surrounding environment (Hansen, 2004). Thus, thermoregulation refers to the innate physiological adjustments that animals make to keep their body temperature within a comfortable range (Kadzere et al., 2002; Hansen, 2004). The routes of heat loss can be influenced by both the surrounding temperature and relative humidity. Sensible heat loss through conduction, convection, and radiation is influenced by the surrounding temperature while latent heat loss through evaporation achieved by sweating and panting is influenced by relative humidity (Hansen, 2004). In a condition where the ambient temperature is lower than body surface temperature sensible heat loss is the predominant. Convective heat loss transfers heat from the body to moving molecules such as air or liquid and is influenced by the temperature gradient between ambient temperature and body surface temperature, air movement, the surface area, and fluid velocity. The conductive heat loss occurs when there is a temperature difference between two

objects or regions that are in direct contact with each other, which is influenced by surface area, thermal conductivity, temperature gradient, thickness of material, and physical contact. Radiation occurs through the emission and absorption of electromagnetic waves, specifically in the form of infrared radiation, and is influenced by factors such as temperature difference, emissivity, absorptivity, atmospheric condition, and distance. In contrast, when the animal's body surface temperature exceeds the surrounding ambient temperature, evaporative cooling is the only efficient process to dissipate heat. Evaporation is a form of heat loss that transfers heat (energy) from a surface to the surrounding environment through the conversion of liquid to vapor, where the magnitude of heat transfer is influenced by the temperature and heat energy, surface area, air movement, humidity, and vapor pressure (Hansen, 2004; Shephard and Maloney, 2023).

Originally developed for humans as a discomfort index under hot conditions (Thom, 1959), the temperature humidity index (THI) was developed to assess and manage heat stress in various animal species, including dairy cows. It is an index calculated based on air temperature and relative humidity. There are many formulas of THI which have different weights in temperature and humidity (Bohmanova et al., 2007; Dikmen and Hansen, 2009). In dairy cattle, comparison of different THI formulations has been examined using animals' physiological state (e.g., milk yield, rectal temperature). The THI placing larger emphasis on humidity are appropriate for evaluating heat stress in areas with high relative humidity, while formulas with larger emphasis on temperature are better indicators for heat stress in the semiarid climate (Bohmanova et al., 2007; Dikmen and Hansen, 2009). The critical THI threshold varies depending on the animal parameters used (e.g., milk yield, milk composition, dry matter intake, rectal temperature, respiration rate, behavior) and the specific climate conditions (Bohmanova et al., 2007; Pinto et al., 2020; Lee et al., 2023). Pinto et al. (2020) determined the THI threshold using respiration rate, heart rate, and

rectal temperature of multiparous lactating Holstein-Friesian cows as indictors under a continental climatic zone in Germany and suggested that strategies to mitigate heat stress in high-yielding dairy cows should be implemented when THI exceeds 65. Further, Yan et al. (2021) used rectal temperature and respiration rate of Holstein lactating dairy cows under different heat stress conditions in China to determine heat stress thresholds and found that THI thresholds of 68.0 and 64.7 for increased rectal temperature and respiration rate, respectively. Gernand et al. (2019) demonstrated that reproductive traits may be affected at lower THI values and reported that the rates of insemination and pregnancy per artificial insemination began to decrease when average THI on the day of insemination exceeded 57 and 65, respectively.

When using milk production to determine THI, the critical THI threshold for heat stress has changed over time with the increased milk production of cows. The THI = 72 has been used for a long time as the heat stress threshold above which the cow's milk production begins to drop (Armstrong, 1994; Ravagnolo et al., 2000; Bohmanova et al., 2007). However, as the milk production of a dairy cow increases, the THI threshold of heat stress reduces. For example, Zimbelman et al. (2009) demonstrated that, in an environmentally controlled chamber, the milk production of high-producing multiparous Holstein cows starts to decline when daily average THI exceeds 68. Mbuthia et al. (2022) analyzed a large dataset of milk test-day records in Germany and reported that the heat stress threshold at which milk yield and protein yield started to decline was the air temperature = 16.0 °C or THI = 60. In a study with the objective to identify the THI thresholds at which heat stress starts negatively affecting milk production traits in Canadian Holstein cows, Campos et al. (2022) reported the estimation of daily maximum THI of 68, 57, and 60, and daily average THI of 64, 50, and 58, for milk, fat, and protein yield, respectively. These indicate that milk component yields are more sensitive to heat stress. In a recent study conducted

in South Korea, the milk traits (decrease in milk performance and increase in MUN and SCS) of Holstein cows was negatively affected when the THI exceeded 70 (Lee et al., 2023).

Additionally, dry cows are more heat tolerant relative to lactating cows due to their non-lactating status and low metabolic heat production (Hahn, 1999); but exposure to heat stress during the dry period negatively impact the cow's milk production in the following lactation and has a long lasting effect on the offspring's productive performance and health throughout her lifetime (Tao and Dahl, 2013; Laporta et al., 2020). Interestingly, Ouellet et al. (2021) demonstrated that dry cow respiration rate and rectal temperature increased abruptly at a THI of 77 when provided only shade and managed in a subtropical climate and suggested that dry cows should be closely monitored before THI reaches 77 to avoid further heat-stress related impairments in the subsequent lactation and to mitigate potential carry-over effects on the offspring.

The use of THI has been widely used to characterize thermal environment under a barn, but not suitable for pastures where shade is not available. Buffington et al. (1981) developed the Black Globe-Humidity Index (BGHI = tbg + 0.36tdp + 41.5, where tbg = black globe temperature °C and tdp = dew point temperature,°C) as a comfort index to count effects of radiant heat load and to better represent heat stress for animals under solar radiation, and determined that BGHI exceeds 75 cause heat stress for dairy cows based on the decreased milk production and increased rectal temperature. This index uses the black globe temperature which is obtained from a temperature sensor housed in a black cap that absorbs solar and radiant heat and defined as an indirect measurement of the radiant heat load of the environment that allows the thermal sensation of an animal to be inferred (Buffington et al., 1981). The black globe temperature integrates the effects of air temperature, solar radiation, and convective cooling due to wind (Li et al., 2009). The importance of the effect of solar radiation on dairy cattle in outdoor conditions has been

demonstrated. For example, Roman-Ponce et al. (1977) reported that blocking solar radiation by providing shade on pasture significantly improved the conception rate from 25.3% to 44.4%.

Heat stress on lactation performance

Heat stress is one of the key environmental factors limiting the production of dairy cattle, and the reduced milk yield is the most recognized negative impact caused by the exposure of heat stress (Tao et al., 2020). High-producing cows are more challenged by heat stress than lower-producing animals (Spiers et al., 2004). Increasing the milk yield from 35 to 45 kg/d may decrease the air temperature threshold for heat stress by 5 °C (Berman, 2005). The dairy industry has made significant efforts to increase milk production (Capper et al., 2009; von Keyserlingk et al., 2013), and this trend will continue due to the demand for high quality dairy products in the next decades (Britt et al., 2018). Average milk production per cow has increased due to the high pressure of selection for dairy cows toward higher performance, and improvements in nutrition and dairy cow management (Barkema et al., 2015). However, the continuous selection of high-producing dairy cows resulted in increased susceptibility to heat stress (Ravagnolo and Misztal, 2000; Collier et al., 2019) due to increased metabolic heat production from greater feed intake and milk production (Kadzere et al., 2002; Ravagnolo and Misztal, 2002; Aguilar et al., 2009).

Although greater milk production per cow is desired, the dairy production system needs to be viewed as a whole, where environmental factors, animal welfare, and management practices for a sustainable dairy business need to be considered in addition to productivity (von Keyserlingk et al., 2013). Milk production exhibits a negative linear correlation with THI (Tao et al., 2020). M'Hamdi et al. (2021) evaluated the productive performance and lactation curve of Holstein dairy cows in Tunisia and reported that cows exposed to THI between 68 and 72 had the highest peak

milk yields and the highest total milk yield during a 305-day lactation. Similarly, Rodriguez-Venegas et al. (2023) found a decrease in milk performance on both the farm and cow levels when THI exceeded 72 with the greatest reduction in milk production when THI ≥ 77. The THI threshold for the reduced milk yield by heat stress varies due to differences in breeds, production levels, housing facilities, and cooling facilities, etc. For instance, Herbut et al. (2018) reviewed the THI threshold ranges from 68 to 74 when the reduced milk yield is used as the trait.

Many mechanisms have been proposed to explain the decreased milk synthesis by heat stress. During heat stress conditions, the dry matter intake (DMI) of a cow decreases as a coping mechanism to reduce internal heat load, causing a decrease in the availability of nutrients used for milk synthesis (West, 2003; Rhoads et al., 2009; Collier et al., 2019). The decrease in DMI by heat stress contributes 35 to 50% of the total reduction in milk production in mid and late lactating cows (Rhoads et al., 2009; Wheelock et al., 2010; Tao et al., 2020). In contrast, Lamp et al. (2015) and Tao et al. (2018) suggest that, in early lactating cows, the decreased DMI entirely explained the reduction in milk yield by heat-stress. Additionally, the number and activity of mammary alveolar cells determines the milk producing ability of dairy cows (Akers, 2017). The adequate mammary blood flow and sufficient supply of substrates are prerequisites for milk synthesis of mammary epithelial cells (Davis and Collier, 1985; Prosser et al., 1996). Lactating dairy cows exposed to heat stress have decreased mammary blood flow mainly due to the reduced feed intake, resulting in reduced nutrient uptake by the mammary gland (Lough et al., 1990; Rodrigues et al., 2019). Interestingly, dairy cows subjected to heat stress during late gestation also experience decreased milk yield in the subsequent lactation (Tao and Dahl, 2013; Dahl et al., 2016). Cows exposed to heat stress prior to calving have lower mammary epithelial cell proliferations (Tao et al., 2011; Skibiel et al., 2018; Dado-Senn et al., 2019), impairing mammary growth and subsequent milk

production. Further, the offspring born from cows under heat stress in the late gestation have reduced milk production by 5 kg/day during the first 35 weeks in their first lactation (Dahl et al., 2016; Monteiro et al., 2016).

Heat stress not only decreases milk yield, but also negatively affects the milk quality by increasing somatic cell count and decreasing fat and protein contents of milk (Ravagnolo and Misztal, 2000; Bertocchi et al., 2014; Nasr and El-Tarabany, 2017). These alterations in milk components during heat stress are largely attributed to the decrease in DMI, the alteration of metabolic processes, and the reduced mammary gland blood flow (Gao et al., 2017, 2019; Guo et al., 2018). Bouraoui et al. (2002) reported that lactating Friesian-Holstein cows during the summer (average THI 78 ± 3.2) had decreased DMI by 9.6%, reduced milk production by 21%, and lowered fat and protein contents in milk by 30 and 23 %, respectively, compared with cows in the spring (average THI 68 ± 3.7). In a recent study, Campos et al. (2022) estimated that milk fat and protein yields of lactating dairy cows started to decline when daily maximum THI exceeds 57 and 60, or daily average THI exceeds 50 and 58, respectively. Ouellet et al. (2019) found the increased number of consecutive days of heat stress was associated with less ECM, protein (% and kg/d), and fat (% and kg/d) variables in Holstein cows under humid continental climate as in the province of Quebec (Canada). In agreement, in a recent study conducted in Spain, Besteiro et al. (2025) demonstrated that productive Holstein cows suffer the most from accumulated heat stress days, and a 2–3 consecutive days of heat stress is sufficient to reduce protein and fat content. The authors also established heat stress threshold of THI with 64 and 71 for protein percentage and yield, 63 and 69 for fat percentage and yield, and 68 for ECM.

These results suggest that milk component yields are more sensitive to heat stress and are affected at lower THI threshold compared to milk yield (Moore et al., 2024). The unfavorably

effect of heat stress on milk protein and fat yields has a deleterious economic impact, particularly in countries that heavily rely on cheese and butter production (Liu et al., 2017; Moore et al., 2023). However, in a recent meta-analysis including 34 trials, Chen et al. (2024) found that heat stress decreased DMI, energy correct milk (ECM), and milk protein concentration, but did not affect milk fat concentration or feed efficiency (ECM/DMI). Similarly, heat stress per se may affect milk protein content more significantly than fat content (Chang-Fung-Martel et al., 2021) due to the reprioritization of amino acids away from milk protein synthesis (Gao et al., 2017). In contrast, the potential reduction in milk fat content by heat stress may be associated with reduced fiber intake and consequent changes in energy and carbohydrate metabolism which may impair milk fat synthesis (Baumgard and Rhoads, 2012).

Heat stress on immune system, inflammation, and health/disease

The immune system plays a significant role in maintaining animal health and overcoming challenges during their production lifetime (Vlasova and Saif, 2021; Meade, 2023). The immune system is divided into innate (non-specific) and adaptive (antigen-specific) responses. The adaptive immune system is further divided into cell-mediated immunity, which involves T-lymphocytes, and humoral immunity mediated by the B-lymphocytes. The innate immune response is the first line of defense due to non-specificity and quick activation (Sordillo, 2016; Vlasova and Saif, 2021). Both innate and adaptive immune systems perform distinct tasks but work closely together through their specific roles to provide protection against external pathogens and regulate homeostasis, leading to optimal health and maximized productive performance (Sordillo, 2016; Cooke, 2019). In dairy cows, several factors (e.g., feeding management, social interaction, environmental contaminants, disease, etc.) may cause stress which consequently

generate negative effects on immune responses (Collier et al., 2017; Cooke, 2019). For instance, heat stress is a major environmental factor that negatively influences animal health across a cow's life cycle (Sejian et al., 2018; Dahl et al., 2020). The impact of heat stress on the immune system is complex and dynamic, and the underlying mechanisms are not fully understood, but deserve further investigation.

During heat stress, there is a complex interplay among endocrine secretions, heat shock proteins (HSP), and the immune system. The primary cellular protection mechanism against the high environmental temperature is the activation of the heat shock transcription factor (HSF) and increased expression of HSP, mainly HSP70 (Collier et al., 2008). High temperature causes misfolding of cellular proteins leading to the disruption of structures of cell organelles and impaired intercellular transport processes. Heat shock proteins facilitate the re-folding of proteins, protecting cells from damage caused by heat stress (Bernabucci et al., 2010; Collier et al., 2019). In addition to the increased expression of HSP at the intracellular level (Collier et al., 2019), it is reported that extracellular level of HSP is also elevated under heat stress. Min et al. (2015) reported an increased serum concentrations of HSF and HSP70 in lactating Holstein cows under heat stress conditions (average THI = 81.7) compared with cows in thermoneutral conditions (average THI = 53.4). Orellana Rivas et al. (2021) observed that deprivation of evaporative cooling during summer elevated the gene expression of HSP in mammary gland tissues from lactating cows. Similarly, Yue et al. (2020) found that Holstein cows exposed to heat stress (THI 72.5 to 86.9) had increased plasma concentration of HSP70 and upregulated gene expression of HSP70 in mammary gland tissue compared with cows in the thermal neutral condition.

Heat shock proteins are also linked to inflammatory response, modulating pro and anti-inflammatory pathways depending on the cellular environments where the HSP are located

(Moseley, 1998; Asea et al., 2002; Borges et al., 2012; Petrović et al., 2022). Although research exploring the immunomodulatory role of HSP is still little explored in dairy cattle, it is suggested that HSP are closely associated with both thermotolerance and immuno-modulation, contributing to the adaptation to the harsh heat stress environment (Basiricò et al., 2011; Hassan et al., 2019). For instance, Cartwright et al. (2022) reported that lactating Holstein dairy cattle identified as high immune responders showed greater circulating HSP70 concentration and greater peripheral blood mononuclear cells (PBMC) proliferation after in-vivo heat challenges, compared with cows identified as average and low responders, suggesting that high responders are not only more resilient against disease but may also be more resilient to heat stress.

Heat stress activates immune-inflammatory pathways, which is a complex biological process that involves interactions between the immune system and inflammatory responses. Under heat stress conditions, the neuro-endocrine response is affected due to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, increasing the production of glucocorticoids and its release into the circulation (Spencer and Deak, 2017; Sejian et al., 2018; Bagath et al., 2019). Because immune cells express glucocorticoid receptors, the immune response is initially stimulated by glucocorticoids during the acute exposure to stress (acute stress), but the immune response is impaired during the extended exposure to stress (chronic stress) (Dhabhar, 2009; Sordillo, 2016).

Acute phase response accompanying acute inflammation is a component of the innate immune response. In response to stress, tissue injury, or infection, leukocytosis, hormonal changes, releases of inflammatory mediators, increasing the production of acute phase proteins (Ceciliani et al., 2012; Trevisi and Minuti, 2018; Trevisi et al., 2025). There is a link between gastrointestinal barrier dysfunction and upregulated inflammatory responses in animals under stress conditions

(Lambert, 2009). Indeed, heat stress can compromise intestinal integrity and induce leaky gut in a variety of species (Pearce et al., 2013; Koch et al., 2019). For instance, Fontoura et al. (2022) reported that multiparous Holstein cows exposed to a heat-stress environment (THI: diurnal change 74 to 82) had increased total-tract gastrointestinal permeability. The combination of the altered rumen-intestine environment and a "leakier" gut under heat stress conditions may contribute to the upregulated inflammation. The loss of intestinal barrier integrity during heat stress allows the bypass of endotoxins (e.g., lipopolysaccharide) and histamine into the blood stream leading to the activation of the innate immune system and systemic inflammation with increased production of cytokines and acute phase proteins (Koch et al., 2019). It is also suggested that the immune system is activated during heat stress conditions which increases the consumption of glucose by the immune cells (Kvidera et al., 2017; Abdelnour et al., 2019).

During an inflammatory response, proinflammatory cytokines (e.g., TNFα, IL-1β, and IL-6) are produced by macrophages and mast cells to activate and stimulate the migration of polymorphonuclear leukocytes (neutrophils, eosinophils, basophils, and mast cells) and endothelial cells into the damaged or infected area, and to stimulate the release of positive acute-phase proteins (haptoglobin, serum amyloid A, fibrinogen, C-reactive protein, ceruloplasmin, ferritin, lactoferrin, lipopolysaccharide binding protein, etc.) (Bannerman, 2009; Trevisi and Minuti, 2018; Alhussien and Dang, 2019). The liver is the main site to produce acute phase proteins (APP) in response to pro-inflammatory cytokines (Horadagoda et al., 1999), but APP are also produced in the mammary gland (Hiss et al., 2004). Haptoglobin (Hp), serum amyloid A (SAA), and lipoprotein-binding protein (LBP) are the major APP in cattle (Saco and Bassols, 2023). Because the exacerbated systemic inflammation has negative effects on metabolism, immunity, performance, behavior, and wellbeing of the animal, assessment of inflammatory status has the

potential to improve the cow management (Huzzey et al., 2015; McCarthy et al., 2016; Kerwin et al., 2022). The acute phase proteins may be used as biomarkers for inflammation since their concentrations are positively associated with systemic inflammation (Bradford et al., 2015). Jo et al. (2021) reported that early lactating Holstein cows under short-term HS (4 days, THI = 86) in chambers had greater plasma cortisol and haptoglobin concentrations compared with cows under a cooler condition (THI = 71). Similarly, circulating LBP and SAA concentrations in lactating dairy cows are increased during hyperthermia induced by electric blankets (Al-Qaisi et al., 2020). However, in a recent study, Chen et al. (2023) did not observe differences in plasma and milk concentrations of haptoglobin and LBP of Holstein cows equipped with or without evaporative cooling during summer.

The release of cytokines is also part of the adaptive mechanisms that modulate the functions and development of T-helper 1 (Th1) and T-helper 2 (Th2) cellular responses, which are differentiation of CD4+ T helper cells based on cytokine signals from the antigen-presenting cells (Raphael et al., 2015; Bagath et al., 2019). Th1 cells promote cell-mediated immunity and release proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α), whereas Th2 cells activate humoral immunity and promote wound healing responses by releasing anti-inflammatory cytokines (e.g., IL-10 and IL-13). The ability of animals in regulating the balance of Th1:Th2 response is a key for optimal host responses (Cray, 2012; Sordillo, 2016; Bagath et al., 2019; Kuhla, 2020; Vlasova and Saif, 2021). The adaptive immune response under chronic heat stress is characterized by disruption of the balance of Th1:Th2 responses, causing a shift towards a Th2 response and suppressed cell-mediated immunity (Lacetera et al., 2005; Salak-Johnson and McGlone, 2007).

There are systemic inflammatory responses of a cow in response to heat stress. During acute stress and immune responses, there are simultaneous increases in proinflammatory cytokines

and cortisol. Glucocorticoids inhibit the cellular immune functions and the production of proinflammatory cytokines (Hirsch et al., 2012; Ciliberti et al., 2017; Ehrchen et al., 2019). The release of cortisol during stress is an attempt to control the inflammation (Sejian et al., 2018; Bagath et al., 2019). High concentration of cortisol in plasma, milk, and feces has been reported in dairy cows during acute exposure to heat stress; but during chronic heat stress, circulating concentration of plasma cortisol decreases to the level prior to heat stress exposure or even lower (Christison and Johnson, 1972; Roman-Ponce et al., 1981; Rees et al., 2016; Chen et al., 2018; Cartwright et al., 2023). The absence of a sufficient anti-inflammatory effect by cortisol could partly explain (Lemal et al., 2023) the increases in plasma proinflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) concentrations in cows exposed to prolonged heat stress (Min et al., 2016; Chen et al., 2018). In addition, the dysregulated systemic inflammation after prolonged heat stress exposure is also associated with oxidative stress and cellular and tissue damages, which release damage-associated molecular patterns (DAMPs), such as HSP, mitochondrial DNA, IL-1α, etc. (Sheldon et al., 2019; Lemal et al., 2023). The constant presence of these DAMPs or pathogen presence (pathogen-associated molecular patterns) activates the innate immune signaling pathways that stimulate persistent production of proinflammatory cytokines like TNF-α and IL-6 (Burhans et al., 2022; Lemal et al., 2023).

Heat stress was reported to suppress the functions of both innate and adaptive immune cells of dairy cattle. Lecchi et al. (2016) reported that the phagocytosis and ROS production of polymorphonuclear leukocytes are impaired after exposure to high temperatures in vitro. Similarly, do Amaral et al. (2011) reported that cows with heat stress abatement during the dry period had greater neutrophil phagocytosis and oxidative burst in early lactation compared with those without heat abatement. Heat stress also negatively affects the functions of lymphocyte of dairy cattle

(Bagath et al., 2019). Exposure to high temperatures in vitro reduced the proliferative responses of bovine peripheral blood mononuclear cells (PBMC) (Elvinger et al., 1991; Lacetera et al., 2005). do Amaral et al. (2010) reported that the cows without heat abatement during the dry period had lower PBMC proliferation and pro-inflammatory cytokine production in early lactation compared with cows with heat abatement when dry. Interestingly, Strong et al. (2015) reported that calves born from cows exposed to heat stress during late gestation had decreased circulating lymphocyte concentration and downregulation of expression of TNFα and toll-like receptor 2 in the first several weeks after birth.

Under heat stress conditions, the combination of compromised immune response with the potential negative energy balance due to the reduction in feed intake partially leads to increased disease incidence (West, 2003; Rhoads et al., 2009). Additionally, pathogens are more prevalent during the summer months due to the hot and humid conditions that promote their growth and proliferation (Lacetera, 2019). Mastitis and metritis are two major infectious diseases in mature dairy cattle, and there is evidence that HS increases the occurrences of both diseases (Dahl et al., 2020). In a study evaluating 22,212 Holstein cows from 15 large dairy herds, Gernand et al. (2019) reported a positive correlation between THI and the incidences of clinical mastitis, retained placenta and puerperal disorders from calving to day 10 post-partum. These data suggest that exposure to heat stress has detrimental effect on cow productivity and fertility, especially in early lactation. Similarly, Nasr and El-Tarabany (2017) reported that the greater THI during summer months were associated with higher SCC, with concomitant decreases in milk volume. During summer, the incidences of retained placenta and metritis were higher (24.05% vs. 12.24%) compared to the rest of the year (DuBois and Williams, 1980). Molinari et al. (2022) demonstrated increased incidences and greater persistence of metritis during the warm season than the cool

season. Because there is no significant association between vaginal pathogen contents and the season of calving, host factors are the major causes for the increased incidences of metritis during summer. Menta et al. (2022) reported that heat stress in dairy cows during the periparturient period was associated with the increased incidence of retained placenta, metritis, mastitis, and the removal from the herd. The increased lameness incidences are often observed in the late summer, which is partially explained by the increased standing time of heat-stressed cows (Cook et al., 2007). Additionally, heat stress affects feeding behavioral and lowers rumen pH and function, causing sub-acute ruminal acidosis (SARA) in lactating dairy cows, leading to losses in milk performance (Bilal et al., 2016; Chang et al., 2018). Burhans et al. (2022) described that heat stress can compromise health and causes mortality of dairy cows due to multifactorial causes involving dysfunction and imbalance of several interdependent systems such as electrolyte dyshomeostasis, unstable blood pH, gastrointestinal tract hyperpermeability, sepsis, severe immune activation-induced inflammation, disseminated intravascular hypercoagulation, systemic endothelial permeability, multiple organ failure, and circulatory failure.

Heat stress on behavioral patterns

Animal welfare has become an important topic of global livestock production due to the high pressure from society and the food production industry (Marchant-Forde, 2015; Buller et al., 2018). Animal behavior is a sensitive indicator for animal welfare (Gonyou, 1994) and considered as internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli (Levitis et al., 2009). Optimal welfare is only achieved when animals are healthy, express natural behavior, and are maintained in optimal environmental conditions (Polsky and von Keyserlingk, 2017; Beaver et al., 2020). Therefore, the assessment of

animal behavior is a valuable way to detect not only illness but also the immediate animal responses to environmental stimuli (Weary et al., 2009; Polsky and von Keyserlingk, 2017; Becker and Stone, 2020; Eckelkamp and Bewley, 2020). The changes in animal behavior provides information on how animals interpret their surroundings and their corresponding reactions to stressors, thus, allowing the implementation of environmental adjustment strategies and management practices that promote both animal production and welfare enhancements (Ratnakaran et al., 2017; Orihuela, 2021; Lee et al., 2022).

Visual observation or video recording are common methods for behavior assessment and are highly accurate; however, these methods require intensive labor and are time consuming, limiting their use over extended periods with large animal numbers. Additionally, these methods require trained personnel with skills to assess the behavior of animals correctly and otherwise are subject to human error (Elischer et al., 2013; Dong et al., 2018). Therefore, there are considerable interests in using automatic recording devices to monitor animal behavior continuously without disturbing their natural behavioral expression (DeVries et al., 2003; Borchers et al., 2016, 2021; Nielsen et al., 2018; Grinter et al., 2019; Dela Rue et al., 2020). Wearable devices (e.g., pedometers, neck collars, ear tags, and noseband halters) belong to Precision Dairy Technologies (PDT) that can be adopted to monitor the daily behavior of individual animals in both confinement and pasture-based production systems (Zambelis et al., 2019; Lee and Seo, 2021; Heins et al., 2023). The utilization of PDT not only improves the labor efficiency especially in large dairy herds but also facilitates rapid decision-making. These will eventually improve production efficiency and enhance the performance, health, and welfare of animals (Gargiulo et al., 2018; Bianchi et al., 2022).

Behavioral disturbance is an adaptive animal response to external threats, while the change in behavioral pattern serves as an indicator of stress resulting from the unfavorable or distressing environment (Ratnakaran et al., 2017; Herbut and Angrecka, 2018). In fact, heat stress is a major environmental factor that directly and indirectly modifies the behavior of cattle in both intensive and extensive production systems (Silanikove, 2000; Palacio et al., 2015). Dairy cattle's behavioral responses to heat stress vary according to the duration and intensity of thermal challenge and animal factors such as breed, age, parity, physiological state, and individual characteristics (Polsky and von Keyserlingk, 2017; Herbut et al., 2018). In addition to physiological responses, dairy cows use behavioral coping strategies to mitigate the increasing heat load and the negative impact of heat stress on health and performance (West, 1999; Polsky and von Keyserlingk, 2017). For instance, in hot environments, dairy cows show behavioral changes related to drinking activity (West, 2003). In an observational study, McDonald et al. (2020) found that, as THI increased, cows drank more water, made more visits to and spent more time at the drinker, and were engaged in more competitive events at the drinker. Further, dairy cattle are highly motivated to use shade in hot weather with increased competitive and aggressive behaviors for shade-seeking (Schütz et al., 2008, 2010).

Feeding behavior is regulated by the intricate interplay of appetite and satiety. Changes in feeding behavior represent an essential homeostatic response that motivates animals to seek and acquire vital nutrients. The feeding behavior of dairy cattle, including preferences and timing of consumption of available feed, exerts a substantial influence on cow health and overall productivity (Llonch et al., 2018; DeVries, 2019). Feeding behavior is affected by cow factors (e.g., weight, age, size, social behavior and temperament, health status), farm system (e.g.,

housing, feeding management), and environmental conditions (e.g., heat stress) (DeVries and von Keyserlingk, 2006; von Keyserlingk and Weary, 2010; Llonch et al., 2018; Leliveld et al., 2022).

The negative impact of heat stress on dry matter intake has been reported in both lactating and dry dairy cows (Karimi et al., 2015; Fabris et al., 2019; Corazzin et al., 2021; Chen et al., 2024) and it is suggested that the decreased dry matter intake is partly due to the direct effect of hyperthermia on the appetite center located in hypothalamus (Baile and Forbes, 1974; Becker et al., 2020). The reduced intake may work as a strategic response to minimize the metabolic heat production generated from feed digestion (Kadzere et al., 2002). However, the decrease in dry matter intake of heat-stressed cows is also associated with compromised rumination activity, reduced body weight gains, lower milk production, and impaired health (West, 2003; Wheelock et al., 2010; Soriani et al., 2013; Collier et al., 2019).

Dairy cows sort the feed differently under heat stress conditions. Beede and Collier (1986) and West (1999) suggested that dairy cows under heat stress sort in favor of concentrate and against forage. This is because the heat increment generated from fiber digestion is greater than concentrate. In contrast, Miller-Cushon et al. (2019) reported that heat-stressed lactating dairy cows sorted for long particles coming from forage. This response is likely because cows are motivated to maintain forage intake in response to the reduced rumen pH during heat stress (Burhans et al., 2022). Feed sorting impairs digestive efficiency by altering rumen environment (Miller-Cushon and DeVries, 2017). Thus, the different feed sorting behavior in heat stressed cows should be concerned (Miller-Cushon et al., 2019). However, studies that explore the sorting behavior of heat-stressed cows are few, and the impact of heat stress on feed sorting is still debatable. Thus, there is a need to further research the feed sorting in heat-stressed lactating dairy cows.

Eating and rumination activities of dairy cows provide useful information regarding the health and welfare of dairy cows (Schirmann et al., 2016; Beauchemin, 2018). Eating time is defined as the time spent prehending, chewing, and swallowing feed (Beauchemin, 2018). Under optimal conditions, a dairy cow spends 3 to 5 h/d eating (Grant and Albright, 2001); however, the daily eating time can be significantly influenced by feeding management, dry matter intake, physical and chemical composition of the diet, and environmental conditions (Grant and Ferraretto, 2018; Corazzin et al., 2021; Florit et al., 2023). There is also an inherent variability among animals (Beauchemin, 2018). Heat stress may pose direct effects on eating behavior. Dairy cows spend less time eating with few eating frequencies as the ambient THI increases (Bernabucci et al., 2010; Ramón-Moragues et al., 2021; Hut et al., 2022). Bernabucci et al. (2010) reported a notable decrease in daily eating frequency from 12 to 15 daily meals in thermal neutrality to 3 to 5 meals per day accompanied with larger meal size during heat stress. In addition, animals tend to reduce their daytime eating duration while extending their feeding activity into the night, taking advantage of the cooler environment during nighttime (Bernabucci et al., 2010; Ramón-Moragues et al., 2021). Hut et al. (2021) reported that, in a temperate climate, lactating cows on conventional milking system with pasture access decreased their eating time from 323 min/d to 248 min/d when daily average THI increases from 60 to 72. The authors also reported a similar decrease in eating time for cows milked on automatic milking system without pasture access, as mean daily THI increases. Additionally, there are seasonal effects on the eating time. Toledo et al. (2023) reported that early lactating cows housed in a barn equipped with evaporative cooling systems (soakers and fans) during the hot season (THI = 78.2 ± 0.4) spent less time eating (134 ± 13.1 vs. 199 ± 14.2 min/d) compared with cows during the cool season (THI = 54.4 ± 0.2).

Rumination is a physiological process that cows perform to physically breakdown feed to facilitate microbial colonization, the passage of small particles from rumen to the lower gastrointestinal tract, and saliva production (Beauchemin, 2018). Rumination closely correlates with productive performance, reproductive success, and disease incidence. Consequently, monitoring the dairy cow's rumination serves as a critical tool for timely health assessment and early disease detection and improves the management of a dairy farm (Gao et al., 2023). Rumination is triggered by the activation of tension receptors located on luminal surface of the reticulorumen. Although changes in rumination time are mostly explained by the feed management, DMI, physical and chemical composition of the diet, and inherent variability among animals (Beauchemin, 2018), climate factors are also associated with the daily pattern of rumination (Müschner-Siemens et al., 2020; Song et al., 2023).

There is a significant unfavorable association between hot environment and the cow's rumination time (Soriani et al., 2013; Abeni and Galli, 2017; Moretti et al., 2017), and the reduced daily rumination time has been proposed as an indicator of heat stress (Kadzere et al., 2002; Bernabucci et al., 2010). Soriani et al. (2013) found a negative correlation between THI and the rumination time of dairy cows on a free-stall barn with a conventional milking parlor. Specifically, when THI exceeds 76, each unit increase in THI is associated with a 2.2 minute decreases in rumination time. Interestingly, the same authors also reported that rumination during the nighttime accounted for 63.2% of the total daily rumination time, suggesting that cows may spend majority of rumination time during the cooler time of the day. Further, Moretti et al. (2017) confirmed that rumination time decreases as THI increases, and this decrease was more apparent in high-producing cows and early lactating cows. Corazzin et al. (2021) reported that late-lactating Italian Holstein Friesian under heat stress (THI > 72) had reduced daily rumination time (330 vs. 510.9

min/d) compared with cows under thermoneutral condition (THI < 72). Additionally, using sprinklers increased rumination time (493.9 vs. 397.9 min/d) when compared with cow without cooling (Corazzin et al., 2021). Interestingly, Toledo et al. (2023) reported that multiparous early lactating cows receiving evaporative cooling (soakers and fans) tended to spend less time ruminating during hot season (THI = 78.2; RT = 558 ± 25.8 min/d) in comparison with cool season (THI = 54.4; RT = 629 ± 28.2 min/d). Additionally, Talukder et al. (2023) reported that cows on a pasture-based production system with a voluntary automatic milking system had decreased rumination time when daily max THI exceeds 82. However, it is important to mention that the rumination time of cows in a confined system with pasture-access or a complete grazing system are negatively associated with the solar radiation (Cartes et al., 2025), with a larger proportion of rumination time occurring at nighttime (Iqbal et al., 2023).

Adequate lying time is considered an important aspect of optimal animal welfare (Fregonesi and Leaver, 2001). Tucker et al. (2021) reported that, under unstressful conditions, lower lying times are reported in cows housed in pasture-based systems, dry lots, or bedded packs (9 h/d) compared with cows in tiestalls or freestalls (10 to 12 h/d). More productive cows are found to lie down longer in a day than less productive herdmates (Lovarelli et al., 2020). The lying behavior of a cow provides insightful information on the cow's interactions with her surrounding environment. Intrinsic (e.g., reproductive status, days in milk, productive performance, parity, and disease) and extrinsic (e.g., environmental conditions and management practices) factors should be considered for a reliable interpretation of the cow's daily lying time (Tucker et al., 2021). Heat stress conditions pose direct negative effects on resting behavior. Exposure to increased THI reduces the lying time and consequently increases the standing time of dairy cows (Cook et al., 2007; Allen et al., 2015). Further, cows have longer time lying during the nighttime of a day when

THI usually decreases (Herbut and Angrecka, 2018). Cook et al. (2007) reported that multiparous the lying time of Holstein cows from a freestall-housed dairy herd decreased from 10.9 h/d during the coolest time (based on THI) to 7.9 h/d during the hottest time during a summer in Wisconsin. Toledo et al. (2023) reported that early lactating multiparous Holstein cows during hot season had decreased lying time (717 ± 21.1 vs. 814 ± 23.9 min/d) with increased standing time (720 ± 21.3 vs. 626 ± 24.0 min/d) and standing bouts (15 ± 0.7 vs. 12 ± 0.7 stands/d) when compared to cow during cool season.

It is suggested that dairy cows during heat stress increase their standing time to expose more body surface area to improve the efficiency of heat dissipation (Allen et al., 2015; Nordlund et al., 2019). Further, Cook et al. (2007) reported that cows spent more time standing in alleys near the evaporative cooling system from the coolest to hottest environment. This behavioral change suggests that cows attempt to increase the utilization of heat abatement systems in attempting to cool off, and also partially explains the increased standing time during heat stress.

The lying behavior and rumination of a cow are highly associated. Cows are able to ruminate while standing or lying (Schirmann et al., 2012). Acatincăi et al. (2010) reported that under non-heat-stressed condition, multiparous Romanian Black and White cows spent 63.4% of the total rumination time lying down and only 36.5% of the time in standing positions. However, during summer, cows spent 56% of the total rumination time in the standing position (Acatincăi et al., 2010). In a recent study, McWilliams et al. (2022) reported that greater ruminating time while lying tended to be positively associated with greater DMI and greater milk fat and protein contents in Holstein dairy cows in a free-stall pen and milked in an automated milking system, suggesting that encouraging lying while ruminating has positive benefits on dairy cow productivity.

Therefore, understanding how cow's lying behavior interacts with the environment and different managements is essential to improve cow comfort, and additional studies are needed to further understand how heat stress affects cow behavior to develop strategies for maintaining adequate lying time during the challenge of heat stress.

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CHAPTER 3

EFFECT OF SUPPLEMENTAL ZINC SOURCE WITH AND WITHOUT EVAPORATIVE COOLING ON SYSTEMIC AND MAMMARY METABOLISM OF LACTATING DAIRY COWS DURING SUMMER[1]

[1] Marins, T.N., A.P.A. Monteiro, X. Weng, J. Guo, R.M. Orellana Rivas, J. Gao, J.K. Bernard, D.J. Tomlinson, J.M. DeFrain, and S. Tao. 2020. *Journal of Dairy Science*, 103:10258–10263. Reprinted here with permission of the publisher.

ABSTRACT

The negative effects of heat stress partly result from disturbed systemic metabolic responses and possibly altered mammary gland metabolism of lactating dairy cows. Our previous research reported that supplemental dietary Zn sources may impact milk fat synthesis of lactating cows during summer. Thus, our objective was to evaluate the systemic and mammary metabolism of cows fed two supplemental Zn sources under two environmental conditions. Multiparous lactating Holstein cows (n = 72; days in milk: 99.7 ± 13.4 d; parity: 2.9 ± 0.3) were randomly assigned to 4 treatments in a 2 × 2 factorial arrangement. Treatments included two different environments: cooled (CL) using fans and misters or non-cooled (NC), and two supplemental Zn sources: 75 mg/kg of DM Zn hydroxychloride (IOZ) or 35 mg/kg of DM Zn hydroxychloride + 40 mg/kg of DM Zn-Met complex (ZMC). The 168 d experiment was divided into baseline and environmental challenge phases, 84 d each. During the baseline phase, all cows were cooled and fed respective dietary treatments, and during the environmental challenge phase cows continued receiving the same diets but NC cows were deprived of cooling. Temperature-humidity index averaged 77.6 ± 3.8 and 77.8 ± 3.8 for CL and NC pens during environmental challenge phase, respectively. Plasma was collected before the baseline phase and at 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75 and 81 d of the environmental challenge phase for metabolites and insulin analyses. Mammary biopsies were collected prior to baseline phase and at 7 and 56 d of the environmental challenge phase to measure mRNA abundance of proteins related to mammary metabolism. Compared with CL, NC reduced plasma glucose, non-esterified fatty acids, β-hydroxybutyrate, and triglyceride concentrations, but increased insulin concentration. Cows fed ZMC had greater plasma triglyceride concentration than IOZ. Treatments had no impact on mRNA abundance of protein related to mammary fatty acid and glucose metabolism except that NC cows had greater

mammary mRNA abundance of 6-phosphogluconate dehydrogenase and ATP-dependent 6-phosphofructokinase than CL cows. In conclusion, deprivation of evaporative cooling influenced the metabolism of lactating dairy cows, but dietary Zn source had no apparent impact.

Key words: heat stress, zinc, metabolism

INTRODUCTION

Heat stress influences the performance of dairy cows partly by reducing nutrient intake and disturbing metabolism. Studies conducted in environmental chambers highlighted the inhibitory effect of heat stress on adipose tissue mobilization and fatty acid utilization, and the increased glucose utilization by extra-mammary tissues in heat-stressed lactating dairy cows despite decreased DMI (Wheelock et al., 2010; Lamp et al., 2015). In a dry lot setting, Tarazon-Herrera et al. (1999) reported that during summer, lactating cows provided only shade had similar plasma non-esterified fatty acids (**NEFA**) concentrations compared with those provided shade and evaporative cooling. In a free stall barn, mid- to late-lactation cows without evaporative cooling consumed 19% less DMI compared with those with cooling (Weng et al., 2018), but how deprivation of evaporative cooling affects blood metabolites and insulin of lactating cows is unknown. In lactating goats, exposure to acute heat stress reduced mammary gland glucose uptake (Sano et al., 1985). Additionally, Weng et al. (2018) reported that deprivation of evaporative cooling during summer decreased milk lactose concentration suggesting altered glucose metabolism of mammary gland in lactating dairy cows. To the best of our knowledge, the cellular events related to metabolism in the mammary gland of cows with or without evaporative cooling have not been extensively studied.

In addition to environment, the source of supplemental trace minerals may affect mammary function. In our previous study, replacing 40 mg supplemental inorganic Zn/kg of DM with Zn-Met complex (total supplemental Zn: 75 mg/kg of DM) improved mammary epithelial integrity of lactating dairy cows during summer (Weng et al., 2018), providing evidence that the source of supplemental Zn alters the development of lactating mammary gland. Interestingly, in the same study, we observed that cows exposed to evaporative cooling and supplemented with Zn-Met complex had lower milk fat concentration compared with those fed an inorganic source of Zn. These data suggest that source of supplemental Zn in lactating cow diets could alter mammary fatty acid metabolism. Additionally, the impact of supplemental Zn sources on blood metabolites in heat-stressed lactating dairy cows is seldomly studied. Therefore, our hypothesis was that deprivation of evaporative cooling disturbs systemic glucose and fatty acid metabolism and alters mammary glucose metabolism of lactating dairy cows; and the source of supplemental Zn in diets alters mammary fatty acids metabolism of lactating cows exposed to different cooling environment. The objective of this trial was to examine the impact of supplemental Zn source and evaporative cooling on plasma concentrations of metabolites and insulin, and mRNA abundance of proteins related to fatty acid and glucose metabolism of mammary gland of lactating dairy cows during summer.

MATERIALS AND METHODS

The animals and experimental design were described previously in detail (Weng et al., 2018). Before the initiation of the trial, experimental procedures and animal handling were approved by the Institutional Animal Care and Use Committee of the University of Georgia (AUP#: A2014 10-003-Y1-A0). The study was conducted during the summer at the Dairy

Research Center of the University of Georgia-Tifton campus. Seventy-two multiparous lactating Holstein cows were blocked by DIM (99.7 ± 13.4 d) and parity (2.9 ± 0.3) and then randomly assigned to one of 4 treatments with a 2 × 2 factorial arrangement. There were 2 environmental treatments: with evaporative cooling (**CL**), or without evaporative cooling (**NC**); and 2 dietary treatments: 75 mg supplemental Zn/kg of DM supplied from inorganic source (Zn hydroxychloride **[IOZ]**, Micronutrients, Indianapolis, IN), or 35 mg/kg of DM Zn hydroxychloride and 40 mg/kg of DM Zn-Met complex (**ZMC**, Zinpro Corp., Eden Prairie, MN). The experiment was divided into two phases with: an 84 d baseline phase, and a subsequent 84 d environmental challenge phase. During the baseline phase, all cows were cooled and fed their respective dietary treatments.

During the environmental challenge phase all cows remained on the same diet but evaporative cooling was deprived from NC cows. The onset of environmental challenge phase was considered as d 1 of the experiment. The stocking density in both NC and CL pens was similar and maintained below 100%, and all cows were managed in the same manner. The evaporative cooling system included misters attached to the front face of fans mounted over feed bunks and freestalls. The fans and misters were turned off for non-cooled cows. Cows were fed a TMR once daily (1300 h). The TMR was supplemented with either IOZ or ZMC, and each Zn supplement was mixed with TMR before feeding. Cows were milked three times each day (0800, 1600, and 2400 h) and yield recorded at each milking (Alpro, Delaval, Kansas City, MO). Ambient temperature and relative humidity in both CL and NC pens were recorded every 15 min throughout the experiment to calculate temperature-humidity index (**THI,** NRC, 1971), and vaginal temperature of a subset of cows (n = 16/treatment) was measured every 5 min for 4 continuous d each week during the environmental challenge phase using an iButton (Mouser Electronics, Mansfield, TX) coupled to a blank controlled internal drug releasing device (CIDR).

Blood samples were collected from coccygeal vessels into vacutainers containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ) before baseline phase (d -86) and at d 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75 and 81 (~1230 h) relative to the onset of environmental challenge phase from a subset of animals (n = 14, 14, 14, 13, for IOZ-CL, IOZ-NC, ZMC-CL, and ZMC-NC, respectively, parity = 3.0 ± 1.2, DIM = 175 ± 40 d at the onset of environmental challenge [mean ± SD]) to collect plasma. Plasma concentrations of glucose (Autokit Glucose; Cat#: 439-90901; Wako Chemicals USA, Inc.), NEFA (HR Series NEFA-HR(2), Cat#: 999-34691, 995-34791, 991-34891, and 993-35191; Wako Chemicals USA, Inc.), BHB (Autokit 3-HB, Cat#: 439-90901; Wako Chemicals USA, Inc.), triglyceride (Triglyceride M assay, Cat#: 994-02891, 990-02991, 992-02892, 998-02992; Wako Chemicals USA, Inc.), and insulin (Bovine insulin ELISA, Cat#: 10-1201-01; Mercodia AB, Uppsala, Sweden) were determined using commercially available kits, with inter- and intra-assays CV of 8.3 and 3.9%, 4.9 and 2.0%, 14.0 and 3.7%, 14.9 and 3.5%, and 9.1 and 2.9%, respectively.

Weng et al. (2017, 2018) previously reported procedures for mammary biopsy collection, total RNA extraction from mammary tissues, reverse transcription, and primer design. Briefly, mammary biopsies were collected at enrollment before the baseline phase as baseline samples, and d 7 and 56 relative to the onset of environmental challenge phase from a subset of animals (n=8/treatment). One cow from ZMC-NC group developed clinical ketosis during the baseline phase and one quarter of the other ZMC-NC cows were automatically dried off after first tissue collection. Therefore, these two cows were excluded from following mammary biopsy collection. The average parity and DIM at the onset of environmental challenge phase of the remaining 30 cows were 2.4 ± 0.8 and 170 ± 36 d (mean ± SD). Total RNA was extracted from mammary biopsies using TRI-reagent solution (Sigma-Aldrich, St. Louis, MO) and PureLink^{TM} RNA Mini

Kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA). To perform the qRT-PCR, cDNA from 50 ng of purified RNA was used for each reaction with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) on a StepOnePlus Real-Time PCR System (Applied Biosystems) under the following conditions: 95 °C for 10 min for initial denaturation and enzyme activation, 40 cycles of 95 °C for 15 seconds and 57 °C for 1 min for amplification. The mRNA abundance of proteins involved in preformed fatty acids transport (lipoprotein lipase), de novo fatty acids synthesis (fatty acid synthase, acetyl-CoA carboxylase 1), NADPH synthesis (cytoplasmic isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate 1-dehydrogenase), glucose transportation (facilitated glucose transporter member 1), lactose synthesis (α-lactalbumin) and glycolysis (ATP-dependent 6-phosphofructokinase and hexokinase-2) was examined. Sequences of target genes are reported in Table 3.1, and *GAPDH* was used as the housekeeping gene. The mRNA abundance was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where CT is the threshold cycle, with data of baseline samples as calibrator.

PROC MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) was used to analyze repeated measures data, including plasma concentrations of metabolites and insulin. The statistical models included block, environment, supplemental Zn source, time, and their respective interactions. Cow nested within treatment was included as a random variable and time was included as a repeated measure. Data collected prior to the baseline phase were included in the model as a covariate. The mRNA abundance data were analyzed by PROC MIXED procedure of SAS 9.4, and the statistical models included block, environment, supplemental Zn source, time, and their respective interactions, with cow nested within treatment as the random variable. Least

squares means ± standard error of the mean are reported. Significance and tendency were declared when $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

RESULTS

The intake and milk yield and composition data were reported in detail by Weng et al. (2018). During the environmental challenge phase, daily THI averaged 77.6 (SD = 3.8) and 77.8 (SD = 3.8) in CL and NC pens, respectively. Within a day, the THI in both pens remained similar and ranged from 73 at 0700 h to 82 at 1500 h (Figure 3.1). Cows without evaporative cooling had greater ($P < 0.01$) daily vaginal temperature (39.94 vs. 39.03 ℃, Figure 3.2), and lower ($P < 0.01$) DMI (21.6 vs. 26.7 kg/d), milk yield (24.5 vs. 34.1 kg/d), and BW (664 vs. 711 kg) compared with CL cows. These results suggest that deprivation of evaporative cooling increased heat load and impaired the performance of lactating dairy cows. However, supplemental Zn source had no impact on these parameters ($P > 0.50$). Compared with cows under evaporative cooling, NC cows had lower ($P < 0.01$) milk lactose yet similar ($P = 0.80$) protein concentrations. Relative to other treatments, CL cows supplemented with ZMC had the lowest milk fat concentration (environment by diet interaction: $P = 0.04$).

Relative to CL cows, plasma of NC cows had lower ($P < 0.01$) concentrations of glucose, NEFA, BHB and triglyceride but greater ($P < 0.01$) insulin concentration (Table 3.2). Supplemental Zn source had no ($P > 0.25$) impact on plasma metabolites or insulin except that cows supplemented with ZMC had greater ($P = 0.05$) plasma concentration of triglyceride than cows fed IOZ. Environment, dietary treatment or their interactions had no impact ($P > 0.10$) on mammary mRNA abundance except that NC cows had greater ($P = 0.03$) mRNA abundance of

ATP-dependent 6-phosphofructokinase and tended ($P = 0.08$) to have greater mRNA abundance of 6-phosphogluconate dehydrogenase compared with CL cows (Table 3.3).

DISCUSSION

Compared with CL cows, lower plasma glucose concentration observed for NC cows may have resulted from lower DMI. In early and mid-lactation, heat-stressed and pair-fed thermal neutral cows had similar reductions in plasma glucose concentration (Wheelock et al., 2010; Lamp et al., 2015), suggesting that lower blood glucose concentration of heat-stressed cows is mainly due to reduction in DMI. Interestingly, despite lower glucose concentrations, plasma insulin concentrations were greater in NC cows compared with CL cows. This may indicate enhanced insulin secretion, delayed insulin clearance or both. Previous studies (Wheelock et al., 2010; Baumgard et al., 2011; Tao et al., 2012) reported that exposure to heat stress had no impact on insulin response to glucose infusion in lactating and dry dairy cows, reflecting unaltered pancreatic insulin release to glucose stimulation. During the dry period, NC cows had similar insulin clearance after an intravenous insulin infusion compared with CL cows, suggesting that heat stress had no impact on insulin clearance before calving (Tao et al., 2012). The exact mechanisms for increased plasma insulin concentrations by deprivation of evaporative cooling observed in the current study are not clear and require further investigation. Despite reduced DMI, NC cows had lower plasma NEFA concentrations than CL cows, which may be expected because NC cows had greater plasma concentration of insulin. It has been reported that exposure to heat stress blunts adipose tissue mobilization even when cows are under negative energy balance in early or mid-lactation (Wheelock et al., 2010; Lamp et al., 2015).

In the present study, replacing a portion of inorganic source of Zn with Zn-Met complex did not affect plasma concentrations of glucose, BHB, NEFA and insulin, suggesting that supplemental Zn source had no substantial impact on systemic metabolism of mid to late lactation dairy cows under both CL and NC environments. Nayeri et al. (2014) reported that replacing 66.6 mg/kg of DM $ZnSO_4$ with Zn-AA complex in dry cow diets and 40 mg/kg of DM $ZnSO_4$ with Zn-AA complex in lactating cow diets had no impact on plasma glucose and BHB concentrations in early lactation, but increased NEFA concentrations compared with cows supplemented with $ZnSO_4$. However, the increased NEFA release might be due to lower DMI and greater feed efficiency of cows supplemented with Zn-AA in early lactation rather than direct effects of Zn-AA on adipose tissue mobilization (Nayeri et al., 2014). In heat-stressed growing pigs, dietary supplementation of Zn-AA had no impact on plasma glucose, NEFA and insulin concentrations compared with those fed diet containing $ZnSO_4$ (Sanz Fernandez et al., 2014; Pearce et al., 2015). Interestingly, cows fed ZMC had greater plasma concentrations of triglyceride than those fed IOZ regardless of the environment. Few studies have reported the impact of dietary Zn sources on blood triglycerides in ruminants. In multiparous Sahiwal cows (*bos indicus*), supplementation of 60 mg/kg of DM of Zn as $ZnSO_4$ from 2 months before until 4 months after parturition had no impact on plasma triglyceride concentration compared with unsupplemented controls (Chandra et al., 2018). The origin of increased plasma triglyceride concentration in cows fed ZMC in the current study is not clear but unlikely due to the increased hepatic secretion as supplemental Zn source had no impact on plasma NEFA concentration. It has been reported that supplementation of Zn-AA complex increased gut integrity compared with inorganic source of Zn under stressed conditions (Sanz Fernandez et al., 2014; Pearce et al., 2015) indicating improved function of intestinal cells. Thus, it is possible that cows supplemented with ZMC had greater fat absorption

in the small intestine compared with IOZ cows supporting increased plasma triglyceride concentrations.

Heat stress may influence nutrient uptake of the mammary gland by reducing mammary blood flow and arteriovenous concentration difference of nutrients. Because of the reduced DMI, heat-stressed cows have decreased mammary blood flow compared with those under thermal neutrality (Lough et al., 1990; Rodrigues et al., 2019a,b). In mid-lactation goats, acute heat stress (4 d) reduced arteriovenous concentration difference of glucose and the mammary plasma flow resulting in a net decrease in mammary glucose uptake compared with thermal neutrality (Sano et al., 1985). In mid-lactation dairy cows, Rodrigues et al. (2019a, b) reported that short term exposure to heat stress (5 d) had no impact, but prolonged exposure (14 d) decreased trans-mammary disappearance of glucose compared with thermal neutrality. In the current study, mRNA abundance of facilitated glucose transporter member 1, the main glucose transporter in the bovine mammary gland (Zhao and Keating, 2007), was not altered suggesting that deprivation of evaporative cooling may not have substantial impact on the ability of mammary cells to uptake glucose. Interestingly, mammary mRNA abundance of 6-phosphogluconate dehydrogenase and ATP-dependent 6-phosphofructokinase were upregulated by deprivation of evaporative cooling indicating that prolonged exposure to heat stress may induce a shift of glucose utilization from lactose synthesis to pentose phosphate pathway and glycolysis in the mammary gland. This may partially explain the lower milk lactose concentration of non-cooled cows compared with cooled cows (Weng et al., 2018), but biological significance of the potential shift of glucose utilization is not clear. Deprivation of evaporative cooling did not affect mRNA abundance of lipoprotein lipase, acetyl-CoA carboxylase 1 or fatty acid synthase, potentially suggesting that prolonged exposure to heat stress (≥ 7 d) had no apparent impact on expression of enzymes that mediate triglyceride

hydrolysis and de novo fatty acid synthesis, at least at the mRNA level. This is consistent with the similar milk fat percentage between CL and NC cows (Weng et al., 2018).

In the current study, CL cows supplemented with ZMC had the lowest milk fat concentration compared with other treatments. Because mRNA abundance of proteins related to fat synthesis and glucose metabolism in the mammary gland was not affected by supplemental Zn source or its interaction with environment, the altered milk fat concentration by different supplemental Zn source at different environments cannot be attributed to expression of genes involved in fat synthesis of the mammary cells. Future study may focus on potential impact of supplemental Zn source on protein expression of enzymes related to mammary gland metabolism.

CONCLUSION

In the current study, deprivation of evaporative cooling resulted in decreased concentrations of plasma glucose but increased insulin concentrations, suggesting disturbed endocrine regulation of metabolism by heat stress. Deprivation of evaporative cooling did not influence mRNA abundance of proteins related to mammary fatty acid metabolism but may altered glucose utilization by mammary cells. Data from the current study suggest that source of supplemental Zn has minimal impact on systemic and mammary metabolism of lactating dairy cows.

ACKNOWLEDGEMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection. This project was supported by Zinpro Corporation (Eden Prairie, MN) and the National Institute of Food and Agriculture (Washington, DC) Hatch project (GEO00749).

DECLARATION OF INTEREST

D.J.T. and J.M.D. are employed by Zinpro Corporation. The remaining authors are associated with the University of Georgia and have no other conflicts of interest.

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Table 3.1: Names, symbols, GenBank accession numbers, primer sequences and amplicon sizes of genes examined.

Protein name	Gene symbol	GenBank accession#	Primer[1]	Sequence (5' - 3')	Amplicon size, bp
Lipoprotein lipase	*LPL*	NM_001075120.1	F	GGGCAATGTGCATCTCTT	138
			R	CTTGGAGCTTCTGCATACTC	
Acetyl-CoA carboxylase 1	ACACA	NM_174224	F	GTTCCGAGCAAGGGATAAG	176
			R	CTTCTGTGCCCACTTCTAC	
Fatty acid synthase	*FASN*	NM_001012669.1	F	CTGAGTCGGAGAACCTGGAG	156
			R	CGAAGAAGGAAGCGTCAAAC	
Isocitrate dehydrogenase, cytoplasmic	*IDH1*	NM_181012	F	GCTTCATTTGGGCCTGTA	129
			R	CCTCTGCTTCTACTGTCTTG	
Glucose-6-phosphate 1-dehydrogenase	G6PD	NM_001244135	F	TACCAGGGTGATGACTTCC	146
			R	CCCACGATGTAGGTGTCT	
6-phosphogluconate dehydrogenase	*PGD*	NM_001143738.1	F	CACCACTGCTCTCTCTTTC	108
			R	GCCAAGAGTTCGTAGGTATG	
Facilitated glucose transporter member 1	*SLC2A1*	NM_174602.2	F	GTGCTCCTGGTTCTGTTCTTCA	84
			R	GCCAGAAGCAATCTCATCGAA	
Alpha-lactalbumin	*LALBA*	NM_174378.2	F	CCCTCACTCAAGCAACATC	128
			R	CTTTATGGGCCAACCAGTAG	
Hexokinase-2	*HK2*	XM_015473383	F	GGAGTTGGCAGGATGATTG	107
			R	GAAAGACGCATGTGGTAGAG	
ATP-dependent 6-phosphofructokinase	*PFKM*	NM_001075268.1	F	GCACCGGATCATAGAGATTG	125
			R	CACAGGAGAGGGAAGTGA	
glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_001034034.2	F	GATGCTGGTGCTGAGTATG	112
			R	CAGAAGGTGCAGAGATGATG	

[1]F = forward; R = reverse

Table 3.2: The overall least squares means and standard error of the mean of plasma concentrations of glucose, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), triglyceride, and insulin of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC) exposed to either evaporative cooling (CL) or not (NC). Samples were collected on d 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75 and 81 relative to the onset of environmental challenge phase.

Parameter	**IOZ**		**ZMC**			***P*-value**		
	CL	**NC**	**CL**	**NC**	**SEM**	**Zn**	**CL**	**Zn×CL**
n	14	14	14	13				
Glucose, mg/dL	62.3	57.0	60.2	57.7	0.9	0.39	<0.01	0.10
BHB, mg/dL	4.94	4.01	4.80	4.48	0.21	0.37	<0.01	0.18
NEFA, µEq/L	164.9	133.9	155.1	127.7	6.74	0.53	<0.01	0.92
Triglyceride, mg/dL	5.56	4.91	6.22	5.20	0.19	0.05	<0.01	0.50
Insulin, µg/L	0.34	0.40	0.33	0.43	0.02	0.42	<0.01	0.52

Table 3.3: The overall least squares means and standard error of the mean of mRNA abundance of proteins associated with metabolism in mammary tissues collected from cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC) exposed to either evaporative cooling (CL) or not (NC). Mammary tissues were collected on d 7 and 56 relative to the onset of environmental challenge phase.

Genes	IOZ		ZMC			*P*-value		
	CL	NC	CL	NC	SEM	Zn	CL	Zn×CL
n	8	8	8	8				
LPL[1]	0.52	0.98	0.86	0.83	0.31	0.74	0.48	0.44
ACACA	1.26	2.53	1.38	0.56	1.07	0.37	0.90	0.31
FASN	2.44	3.71	0.81	0.84	1.83	0.25	0.79	0.81
IDH1	0.98	1.39	0.99	0.82	0.46	0.56	0.82	0.55
G6PD	1.21	1.32	1.49	0.52	0.79	0.62	0.51	0.44
PGD	0.79	1.27	0.89	1.26	0.23	0.82	0.08	0.80
SLC2A1	1.03	1.07	0.83	0.84	0.35	0.55	0.94	0.97
LALBA	0.81	0.71	1.16	0.73	0.27	0.52	0.36	0.58
HK2	0.07	0.81	0.30	0.38	0.33	0.67	0.13	0.20
PFKM	0.72	1.62	0.94	1.47	0.30	0.83	0.03	0.52

[1]*LPL*: Lipoprotein lipase; *ACACA:* Acetyl-CoA carboxylase 1; *FASN:* Fatty acid synthase; *IDH1:* Isocitrate dehydrogenase, cytoplasmic; *G6PD:* Glucose-6-phosphate 1-dehydrogenase; *PGD:* 6-phosphogluconate dehydrogenase; *SLC2A1:* Facilitated glucose transporter member 1; *LALBA:* Alpha-lactalbumin; *HK2:* Hexokinase-2; *PFKM:* ATP-dependent 6-phosphofructokinase

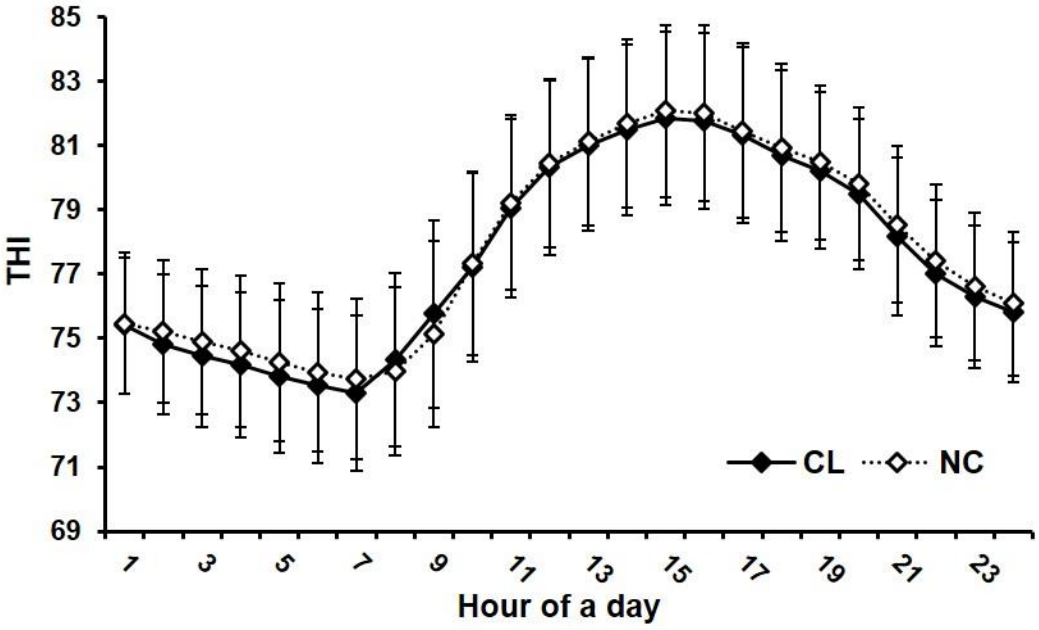

Figure 3.1: Temperature-humidity index (**THI**) of pens equipped with the evaporative cooling (**CL,** solid diamonds [♦]**)** or not (**NC,** open diamonds [◊]). Error bars represent standard deviation.

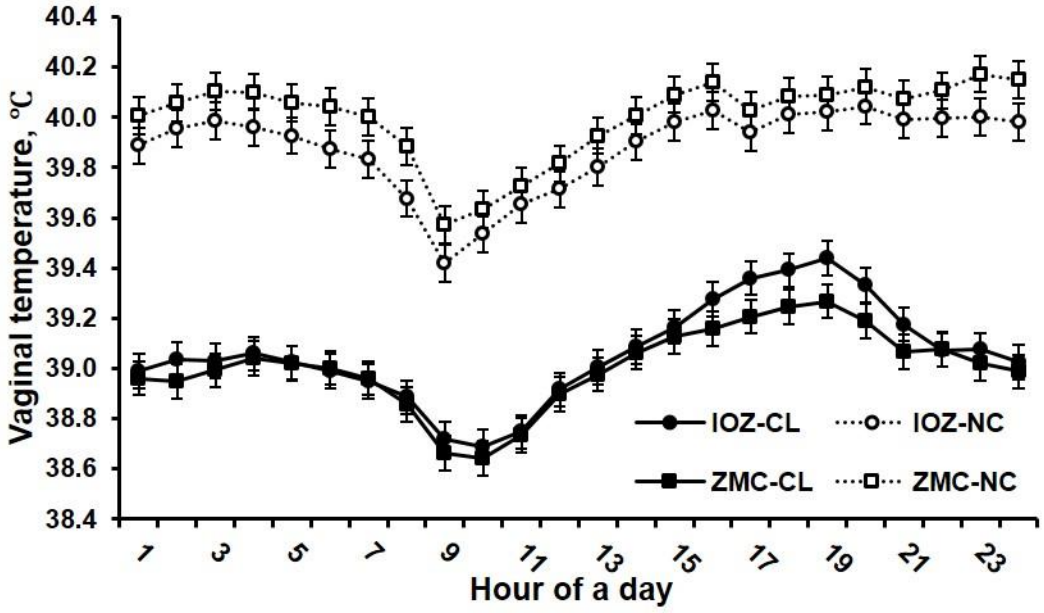

Figure 3.2: Vaginal temperature of cows fed diets supplemented with Zn hydroxychloride (IOZ) under evaporative cooling (CL) (solid circle [●], n = 16), cows fed Zn-MET (ZMC) under CL (solid square [■], n = 16), cows fed IOZ but without evaporative cooling (NC) (open circle [○], n = 16), and cows fed ZMC under NC (open square [□], n = 16). Error bars represent standard error of the means.

CHAPTER 4

RESPONSE OF LACTATING DAIRY COWS FED DIFFERENT SUPPLEMENTAL ZINC SOURCES WITH AND WITHOUT EVAPORATIVE COOLING TO INTRAMAMMARY LIPOPOLYSACCHARIDE INFUSION: INTAKE, MILK YIELD AND COMPOSITION, AND HEMATOLOGIC PROFILE[2]

[2] Marins TN, Monteiro APA, Weng X, Guo J, Orellana Rivas RM, Bernard JK, Tomlinson DJ, DeFrain JM, Tao S. 2019. *Journal of Animal Science*, 97(5):2053-2065. Reprinted here with permission of the publisher.

ABSTRACT

The objective of this study was to determine the effect of dietary supplemental Zn source and evaporative cooling on intake, milk yield and composition, and the rate of leukocyte migration into the mammary gland following intramammary lipopolysaccharide infusion. Multiparous Holstein cows (n = 72) were assigned to one of 4 treatments with a 2×2 factorial arrangement including two sources of supplemental Zn: 75 mg/kg Zn hydroxychloride or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex each with or without evaporative cooling. The cooling system was implemented by the use of fans and misters over the freestall and feeding areas. On d 34 of the experiment, cows (n = 16; days in milk = 263 ± 63 d) received an infusion of 10 μg of lipopolysaccharide, or a saline control, in the left or right rear quarters. Individual milk samples from both quarters were collected at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion and analyzed for composition and bovine serum albumin. Rectal temperature and respiration rate were assessed and blood samples were collected at the same time points (with an additional sample at 3 h) for analyses of lactose and cortisol. Complete blood counts were performed on samples collected within the first 24 h post infusion. Intramammary lipopolysaccharide infusion reduced ($P < 0.01$) milk yield, dry matter intake and feed efficiency regardless of dietary or cooling treatments. Non-cooled cows tended ($P = 0.09$) to have greater feed efficiency (=milk yield/dry matter intake) at 1 d after infusion than those subjected to cooling. Intramammary lipopolysaccharide infusion dramatically increased ($P < 0.01$) milk SCC but treatments had no apparent impact on milk SCC. Compared with cooled cows, non-cooled cows had greater ($P < 0.05$) plasma lactose concentrations, but lower ($P < 0.03$) blood concentrations of neutrophils and lymphocytes at 3 h post infusion. This suggests a greater leukocyte migration into the mammary gland of heat-stressed cows. In conclusion, non-cooled cows tended to maintain

greater feed efficiency and appeared to have greater leukocyte migration into the mammary gland immediately after intramammary lipopolysaccharide infusion compared with cooled cows. Dietary supplemental Zn source had no impact on measures assessed after intramammary lipopolysaccharide infusion.

Key words: cooling, dairy cow, intramammary lipopolysaccharide infusion, zinc

INTRODUCTION

Udder health is related to the productivity of animals and the economic return of dairy producers. A large portion of the economic loss due to impaired udder health (i.e. clinical mastitis) is attributed to decreased milk yield (Rollin et al., 2015). Therefore, it is important to understand how environment and nutritional factors influence udder health and alter cow responses to mammary infection. Milk somatic cell count (**SCC**) and incidences of clinical mastitis increase during summer (Hogan et al., 1989; Kadzere et al., 2002; Olde Riekerink et al., 2007), which can be attributed to both environmental and animal factors (Kadzere et al., 2002; Hogan and Smith, 2012). During summer, the hot and humid environment supports greater growth and survival of pathogens in the environment of the cow compared with cold and dry weather (Kadzere et al., 2002), increasing the chance of mammary infection. Further, immune cell function is compromised with heat stress. Peripheral blood mononuclear cells exposed to heat stress in vitro, or in vivo, have a lower mitogen-induced proliferative capacity (Kamwanja et al., 1994; Lacetera et al., 2006; do Amaral et al., 2010), suggesting impaired cellular immunity. Neutrophils cultured under elevated ambient temperature or isolated from heat-stressed cows have reduced phagocytosis and killing ability by oxidative burst (do Amaral et al., 2011; Lecchi et al., 2016). Heat-stressed lactating cows also have a reduced leukocyte migration into the mammary gland in response to an intramammary

chemotactic challenge of oyster glycogen (Elvinger et al., 1992). However, the impact of heat stress on lactating cows' responses, such as milk yield, intake and leukocyte migration into the mammary gland, to intramammary infection or to mammary inflammation is seldom studied.

Zinc, an essential micromineral, is involved in many critical functions in the body (NRC, 2001). For example, it is a cofactor in different metalloenzymes and metalloproteins involved in antioxidation, maintaining cellular redox balance and preventing oxidative stress systemically and in specific tissues, including the mammary gland (NRC, 2001; Alam and Kelleher, 2012). In dairy cattle diets, the specific source of supplemental Zn influences mammary health during lactation. Kellogg et al. (2004) reported that replacing inorganic source of supplemental Zn, such as Zn oxide or Zn sulfate, with Zn-Met complex in lactating cow diets reduces milk SCC. Nayeri et al. (2014) studied the ratio of Zn sulfate to Zn-AA complex of supplemental Zn in dry and lactating cow diets on performance of Holstein cows. These authors found that replacing 66.6 and 40 mg of Zn sulfate/kg of dry matter (**DM**) in the close-up and lactating cow diets, respectively, with Zn-AA complex reduced milk SCC in multiparous cows. But, it remains unknown if the dietary supplemental inorganic or amino-acid chelated Zn will affect the response of cows to mammary infection, or induced inflammation, particularly during heat stress.

The hypothesis of this study was that heat stress impairs leukocyte migration into the mammary gland and exaggerates the reduction of milk yield after intramammary lipopolysaccharide (**LPS**) infusion into lactating quarters of dairy cows, and that replacement of portion of the inorganic source of supplemental Zn with Zn-Met complex in the lactating cow diet will improve immune cell migration into the mammary gland and alleviate the reduction in milk yield after intramammary LPS infusion. The objective was to determine the effects of supplemental inorganic or amino-acid chelated Zn in the lactating cow diet and evaporative

cooling on milk yield and composition, dry matter intake (**DMI**), feed efficiency, hematologic profile, blood lactose and milk bovine serum albumin concentrations in the lactating dairy cow to an intramammary LPS infusion during the summer.

MATERIALS AND METHODS

Animals and Experimental Design

The animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia before initiation of the experiment. This study was conducted from April to September during a summer at the Dairy Research Center, located on the University of Georgia-Tifton campus.

Cows used for the intramammary infusion of LPS reported here were part of an experiment reported by Weng et al. (2018). Multiparous lactating Holstein cows (n = 72) were assigned to one of 4 treatments with a 2 × 2 factorial arrangement based on parity and days in milk. Treatments included two sources of supplemental Zn: 75 mg/kg Zn hydroxychloride (**IOZ** [inorganic Zn]**,** Micronutrients, Indianapolis, IN), or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (**ZMC,** Zinpro Corporation, Eden Prairie, MN), under two environments: cooling or no cooling. Cows were fed their respective dietary treatments for 12 wk before initiation of the environmental treatments. During the experiment, cows were housed in adjacent pens in the same barn according to their cooling treatments. The cooling system included misters attached to the front face of fans over feed lines and freestalls. Fans ran continuously when environmental temperature exceeded 18 °C and misters were activated continuously whenever ambient relative humidity was less than 85%. Cows were milked three times each day at 0800, 1600, and 2400 h, and were fed a total mixed ration (**TMR**) once daily at 1300 h. The TMR was supplemented with either IOZ or ZMC.

Each Zn supplement was mixed with TMR before feeding. The composition and nutrient content of experimental diets were reported by Weng et al. (2018). Milk yield of individual cows was recorded at each milking (Alpro, DeLaval, Kansas City, MO) and the daily feed intake was measured using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH). Barn air temperature and relative humidity were monitored by Hobo Pro Series Temp probes (Onset Computer Corporation, Pocasset, MA) every 15 min throughout the experiment and temperature-humidity index (**THI**) was calculated based on THI = (1.8×T+32) – ((0.55-0.0055×RH) × (1.8×T – 26)), where T = air temperature (°C), RH = relative humidity (%) (Dikmen and Hansen, 2009). The THI during the entire experiment averaged 77.7. Compared w–th cows receiving cooling, c–ws that were deprived of cooling had increased ($P < 0.01$) respiration rates (64.8 vs. 73.9 breath/min) and vaginal temperature (39.03 vs. 39.94 ℃), suggesting increased heat load. Supplemental Zn source did not affect vaginal temperature or respiration rates ($P > 0.25$). Non-cooled cows had lower ($P < 0.01$) milk yield (24.5 vs. 34.1 kg/d), DMI (21.6 vs. 26.7 kg/d), and feed efficiency (=milk yield/DMI; 1.21 vs. 1.34) compared to cooled cows. Supplemental Zn source did not influence these parameters ($P > 0.60$). Neither supplemental Zn source nor cooling impacted milk somatic cell score or concentrations of protein and fat ($P > 0.10$), but non-cooled cows had higher ($P < 0.01$) concentrations of milk urea nitrogen (**MUN**) relative to cooled cows.

Intramammary Infusion of LPS and Sample Collection

Intramammary infusion of LPS or saline was performed on d 34 of the experiment. Three days before infusion, individual milk samples from the left and right rear quarters of all cows were aseptically collected to measure SCC and cultured to detect common mastitis-causing pathogens. Twenty cows (n = 5/treatment) with culture negative milk samples and SCC below 200,000 cells/mL from both rear quarters were included in the infusion study. Because there are no studies

reported in the literature examining the impact of sources of supplemental Zn in the diet of lactating cows or heat stress on cow responses to intramammary LPS infusion, power analysis was performed based on data reported by Zarrin et al. (2014). Zarrin et al. (2014) studied the impact of induced hyperketonemia on mammary immune responses to intramammary LPS infusion of lactating dairy cows. The milk $\log_{10}$(SCC) data collected at 3 h after intramammary LPS infusion of hyperketonimic and control cows (5.10 vs. 5.65, SD = 0.24, respectively) were used to calculate the anticipated number of cows needed to provide sufficient statistical power. A sample size of 5 cows per treatment was calculated using a level of significance of 0.05 and 80% power to detect differences if present.

Before infusion, individual milk samples from the left and right rear quarters were collected to measure milk SCC at -12, -4, and 0 h relative to infusion of LPS or saline, and a California Mastitis Test (**CMT**) was performed on both left and right rear quarters of all cows to detect potential high SCC quarters. Only data collected from cows with negative CMT and SCC below 200,000 cells/mL for both rear quarters before infusion were included in statistical analyses. There were 4 cows that had SCC above 200,000 cells/mL in at least one of the rear quarters and their data were eliminated from final analyses, resulting in an n = 4, 4, 5, and 3 cows for cooled cows fed IOZ, non-cooled cows fed IOZ, cooled cows supplemented with ZMC, and non-cooled cows supplemented with ZMC, respectively. The average days in milk, parity and milk SCC at -12, -4, and 0 h before infusion (± SD) of the remaining 16 cows were 263.2 ± 63 d, 3.2 ± 1.4, 66.7 ± 55.5 $\times 10^3$ cells/mL, respectively.

During and after the intramammary infusion, all cows remained in their respective pens. Two hours after morning milking, 10 μg of *Escherichia coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO, Greco et al., 2015) dissolved in 5 ml pyrogen-free saline solution was infused into the

left rear quarter and 5 ml pyrogen-free saline solution was infused into the right rear quarter. Teats from infused quarters were massaged thoroughly to move the infused solution into the gland cistern. Daily milk yield and DMI were collected from 7 d before until 7 d after intramammary infusion. Rectal temperature and respiration rate were assessed, and individual quarter milk samples (50 mL) collected at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion. Rectal temperatures were measured using a GLA M700 digital thermometer (GLA Agricultural Electronics, San Luis Obispo, CA) and respiratory rates were assessed by counting flank movements for 1 min. Before milk sample collection, teats were dipped with an iodine-based solution (0.1%), cleaned using paper towels and alcohol pads, and the first 5 stream of milk was discarded. One aliquot of the milk sample was stored with bronopol-B-14 as a preservative for analyses of milk components (fat, protein, MUN, and SCC, Dairy One Cooperative, Ithaca, NY) using a Foss 4000 instrument (Foss North America, Eden Prairie, MN). A second aliquot was stored without a preservative at -20 °C for milk bovine serum albumin (**BSA**) analysis. Blood was collected following the same schedule as milk samples with an additional sample collected at 3 h after infusion. All blood samples were collected from coccygeal vessels into vacutainer tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately placed on ice and then centrifuged at 2,619 × g for 30 min at 4 °C to obtain plasma. Additional blood samples were collected at 0, 3, 6, 12, and 24 h relative to infusion into K3 EDTA vacutainer tubes (Becton Dickinson) to assess hematologic profile at the University of Georgia Tifton Veterinary Diagnostic Laboratory (Tifton, GA) using a hematology analyzer (ADVIA®2120i Hematology System, Siemens, Tarrytown, NY).

Sample Analyses

Plasma lactose (BioVision, Milpitas, CA), and cortisol (Cortisol EIA kit, Arbor Assay, Ann Arbor, MI) concentrations were measured using commercially available kits following the manufacturers' procedures. The intra- and inter-assay CV averaged 1.5 and 14.9% for plasma lactose, and 4.1 and 4.9% for plasma cortisol, respectively. Milk BSA concentrations were determined using a colorimetric method with bromocresol green dye (Sigma-Aldrich, St. Louis, MO) as described by Weng et al. (2018) and the intra- and inter-assay CV were 2.0 and 8.1%, respectively.

Statistical Analyses

Repeated measures data (DMI, milk yield and composition, feed efficiency [=milk yield/DMI], rectal temperature, respiration rate, plasma lactose and cortisol concentrations, hematologic data, and milk BSA concentrations) were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). The SAS models included cooling, Zn source, time relative to infusion, and their respective interactions, with cow nested within treatment as the random variable. Least squares means ± SEM are reported.

RESULTS

Milk Yield, DMI, Feed Efficiency, Rectal Temperature and Respiration Rate

Supplemental Zn source and its interaction with cooling or time had no ($P \geq 0.13$) effect on milk yield, DMI, feed efficiency, rectal temperature and respiration rate of cows following intramammary LPS infusion (Table 4.1). Regardless of the supplemental Zn source, cooled cows produced more ($P \leq 0.05$) milk from -7 to -1 d and at 2, 3, 4, 5, and 7 d relative to intramammary LPS infusion, but tended ($P \leq 0.08$) to have greater milk yield at 1 and 6 d after LPS infusion

compared with non-cooled cows (cooling × time interaction: $P < 0.01$, Figure 4.2A). Non-cooled cows consumed less ($P < 0.01$) DM and had greater ($P < 0.01$) rectal temperature and respiration rate relative to cooled cows (Table 4.1, Figure 4.1, Figure 4.2B). Interestingly, non-cooled cows tended ($P = 0.09$) to have higher feed efficiency at 1 d after intramammary LPS infusion compared with cooled cows (cooling × time interaction: $P = 0.01$, Figure 4.2C).

Milk Composition

Regardless of environmental and dietary treatments, intramammary LPS infusion increased concentrations of milk fat, protein, MUN and ln(SCC) in LPS infused quarters (time effect: $P < 0.01$, Table 4.2, Figure 4.3). In saline infused quarters, the percentages of milk fat, MUN and ln(SCC) increased but milk protein concentration decreased after saline infusion (time effect: $P < 0.01$, Table 4.2, Figure 4.4). There were no effects of supplemental Zn source or its interaction with cooling or time on milk composition in either LPS or saline infused quarters (Table 4.2). Lipopolysaccharide infused quarters of non-cooled cows had greater ($P = 0.05$) milk fat concentration at 24 h and tended ($P = 0.10$) to have greater milk fat concentration at 6 h after infusion compared with cooled cows (cooling × time: $P = 0.07$, Figure 4.3A). A cooling × time interaction ($P = 0.01$) was observed for milk ln(SCC) in LPS infused quarters (Figure 4.3C). Compared with cooled cows, the ln(SCC) of LPS infused quarters of non-cooled cows was greater ($P < 0.01$) at -12 h, lower ($P = 0.02$) at 96 h, and tended ($P = 0.10$) to be lower at 120 h relative to infusion (Figure 4.3C). Relative to cooled cows, milk collected from LPS infused quarters of non-cooled cows had lower ($P \leq 0.10$) protein concentrations before infusion (cooling × time interaction: $P < 0.01$, Figure 4.3B), and greater ($P \leq 0.06$) MUN concentrations at 12, 48, 96, 120, 144, and 168 h following infusion (cooling × time interaction: $P = 0.06$, Figure 4.3D). Similarly, MUN concentrations of saline infused quarters of non-cooled cows were greater ($P < 0.01$) at 168

h and tended ($P \leq 0.08$) to be greater at 72, 120 and 144 h following infusion (cooling × time: $P = 0.02$, Figure 4.4).

Hematologic Profile

Regardless of supplemental Zn source or cooling, intramammary LPS infusion resulted in temporary reductions of blood concentrations of plasma protein, white blood cells, eosinophils, monocytes, lymphocytes and neutrophils (time effect: $P \leq 0.01$) (Table 4.3). Supplemental Zn source, and its interactions with cooling or time had no ($P \geq 0.17$) impact on hematologic profiles (Table 4.3). Relative to cooled cows, non-cooled cows had lower ($P = 0.02$) blood monocyte concentrations (Table 4.3, Figure 4.5D). Cooling by time interactions ($P \leq 0.03$) were observed for blood concentrations of plasma protein, white blood cells, lymphocytes and neutrophils after infusion. Compared with cooled cows, non-cooled cows had lower ($P \leq 0.03$) blood concentrations of white blood cells, lymphocytes, neutrophils and plasma protein at 3 h after intramammary LPS infusion, and tended ($P = 0.07$) to have lower concentration of blood lymphocytes at 6 h after intramammary LPS infusion (Table 4.3, Figure 4.5A, B, C).

Plasma Lactose and Cortisol and Milk BSA

Intramammary LPS infusion increased plasma lactose concentrations (time effect: $P < 0.01$, Figure 4.6A). Consistently, milk BSA concentrations were increased (time effect: $P < 0.01$) in LPS infused quarters, but not in saline infused quarters (Figure 4.6B). There was no effect of supplemental Zn source, and its interaction with cooling or time ($P \geq 0.72$) on plasma lactose concentrations. Supplemental Zn source or cooling had no ($P \geq 0.28$) impact on milk BSA concentrations in LPS infused quarters following infusion. Compared with cooled cows, the plasma lactose concentrations of non-cooled cows were greater ($P < 0.05$) at 0 and 3 h and tended

($P = 0.09$) to be greater at -4 h relative to intramammary LPS infusion (cooling × time: $P < 0.01$, Figure 4.6A).

Relative to cooled cows, non-cooled cows had lower plasma cortisol concentrations (cooling effect: $P = 0.03$); however, there were no ($P = 0.24$) significant interactions between cooling by time for plasma cortisol concentrations (Figure 4.7A). Further, there was a tendency ($P = 0.10$) for a supplemental Zn source by time interaction, such that the plasma cortisol concentration of cows supplemented with ZMC was greater ($P = 0.05$) at 3 h and tended to be greater at 0 h relative to intramammary LPS infusion compared with cows fed IOZ (Figure 4.7B).

DISCUSSION

In the current study, the rectal temperature of cows peaked 6 h after intramammary LPS infusion, suggesting a successful induction of the febrile response (Lohuis et al., 1988a). The fact that there were no significant interactions between treatments and time for rectal temperature after infusion supports that dietary Zn source or cooling did not impact the febrile responses caused by intramammary LPS infusion. Regardless of dietary or cooling treatments, milk yield and DMI declined in response to intramammary LPS infusion, consistent with previous studies (Waldron et al., 2006; Silanikove et al., 2011). The decreased milk yield that occurs after mammary infection or inflammation is due to both systemic effects of inflammation (reduced DMI and altered metabolic responses) and local damage in infected quarters (Ballou, 2012). Intramammary infection causes apoptosis of epithelial cells, and is associated with necrotic damage to mammary epithelium, which partially explains the reduced milk yield after infection (Zhao and Lacasse, 2008; Long et al., 2001). In the present study, the reduced feed efficiency after intramammary LPS

infusion also supports the observation that the reduced DMI is not the sole reason for the decreased milk yield.

It is interesting that non-cooled cows tended to have greater feed efficiency 1 d after intramammary infusion of LPS compared with cooled cows. Because there was no interaction of cooling by time for DMI, both non-cooled and cooled cows had similar decreases in DMI after infusion. Thus, the greater feed efficiency of non-cooled cows compared with cooled cows 1 d after infusion may be due to the lower reduction in milk yield by a local mechanism within the LPS infused mammary gland. Due to heat stress, cooled cows maintained greater milk yield than non-cooled cows before and after intramammary LPS infusion except for 1 d after infusion when cooled cows only tended to have greater milk yield than non-cooled cows. This may further support that the mammary gland of non-cooled cows had a lower reduction in milk yield immediately after intramammary LPS infusion relative to cooled cows. Heat shock proteins (**HSP**) are evolutionarily conserved cellular stress proteins constitutively expressed at low levels in all somatic cells. Their expression can be induced by stress conditions including heat stress (Kregel, 2002). In the bovine, exposure to elevated temperature in vitro resulted in increased gene expression of HSP70 by mammary epithelial cells (Collier et al., 2006). The lactating mammary gland has greater HSP27, HSP70 and HSP90 gene expression when exposed to heat stress (Orellana et al., 2017). Because HSP have cytoprotective functions as molecular chaperones and inhibit apoptosis (Kregel, 2002, Schmitt et al., 2007), the enhanced mammary HSP expression induced by heat stress in lactating mammary gland may exert a protective function for cell survival and maintenance of milk synthesis after a secondary insult, such as intramammary infusion of LPS. Because milk yield of the individual quarter was not measured, this hypothesis was only partly tested in the current study but deserves further investigation.

As expected, milk SCC increased dramatically in LPS infused quarters indicating a successful induction of mammary inflammation. In saline infused quarters, milk SCC was slightly elevated as well. Milk BSA concentrations in saline infused quarters were unchanged after infusion, indicating that the mammary epithelial integrity was not significantly disrupted and no immune cell infiltration occurred. Thus, the infusion of saline per se had no influence on milk SCC. When only saline is infused into the mammary gland of a healthy cow (without LPS infusion in other quarters), milk SCC of the infused quarter is not altered (Lavon et al., 2008). However, intramammary LPS infusion in one quarter reduces milk yield of non-LPS infused quarters of that cow. This arises from the systemic effects of LPS inducing inflammation that impacts all sites (Silanikove et al., 2011). Therefore, the increased milk SCC in saline infused quarters may result from a concentrating effect due to the reduced milk yield.

Consistent with previous research (Aditya et al., 2017), milk fat concentrations increased in both LPS and saline infused quarters. This is likely due to a dilution effect resulting from decreased milk yield. Compared with cooled cows, milk fat concentrations in LPS infused quarters of non-cooled cows were greater at 6 and 24 h post infusion. Relative to cooled cows, non-cooled cows had similar plasma β-hydroxybutyrate acid and triglyceride concentrations after intramammary LPS infusion, but lower concentrations of non-esterified fatty acids after 12 h post infusion (Marins and Tao, unpublished data, University of Georgia, Tifton). These results may suggest that cooling does not have a significant influence on milk fat synthesis after intramammary LPS infusion. Therefore, the greater milk fat concentrations at 6 and 24 h post LPS infusion may be explained by the lower reduction in milk yield in LPS infused quarters of non-cooled cows compared with cooled cows. Similar to Aditya et al. (2017), intramammary LPS infusion increased the milk protein percentage in LPS infused quarters, which may partially result from a dilution

effect due to reduced milk yield. Coliform mastitis or mammary inflammation induced by intramammary LPS infusion results in a disrupted mammary epithelial junctions accompanied with an influx of serum proteins, such as albumin, immunoglobulin, etc., into the mammary gland (Stelwagen and Singh, 2014). Moreover, intramammary LPS infusion increases the synthesis of antibacterial proteins and peptides and acute phase proteins in the mammary gland (Lohuis et al., 1988b; Isobe et al., 2009; Gott et al., 2015). This may also contribute to the increased milk protein percentage in the inflamed gland. In contrast, milk protein concentration was decreased after saline infusion regardless of the dietary and cooling treatments, suggesting that the systemic responses induced by intramammary LPS infusion reduced milk protein synthesis in saline infused quarters.

In the current study, after intramammary infusion, milk samples collected from both LPS and saline infused quarters in non-cooled cows displayed sustained elevation of MUN relative to cooled cows. This may result from a systemic inflammatory effect that alters protein metabolism. Intramammary infusion of LPS is associated with a slight reduction in rumen contraction rate, which would alter rumen function and protein metabolism (Lohuis et al., 1988a; Perkins et al., 2002). As discussed by Silanikove et al. (2011), the increase in MUN concentration following intramammary LPS infusion may be a result of enhanced systemic amino acid turnover due to the stress response, but, to the best of our knowledge, research to support this hypothesis is still not available. The sustained increase in MUN concentration in non-cooled cows compared with cooled cows observed in the current study may suggest that heat stress induces a prolonged systemic impact on protein metabolism in response to intramammary LPS infusion.

Compared with cooled cows, non-cooled cows had greater reductions in blood concentrations of white blood cells, neutrophils and lymphocytes immediately after intramammary infusion of LPS. This suggests greater leukocyte migration into heat-stressed mammary gland, and

trafficking to related lymph node in response to acute mammary inflammation. The greater plasma lactose concentrations at 3 h post infusion of non-cooled cows further supports greater immune cell infiltration into the mammary gland compared with cooled cows. Immune cell infiltration is associated with disrupted mammary epithelial junction resulting in greater lactose leakage from the gland into the blood. As reviewed by Evans et al. (2015), hyperthermic treatment enhances lymphocyte trafficking into lymphoid organs by increasing the expression of L-selectin on leukocytes and α4β7 integrin on high endothelial venules in lymph nodes, allowing the animal to mount more effective adaptive immunity. Hyperthermic treatment also augments neutrophil migration into inflamed, or infected, tissues through increased extra-vascular movement resulting from an enhanced inflammatory response (Evans et al., 2015). In contrast, previous studies reported that heat stress impairs neutrophil migration in response to chemo-attractants in vitro and in vivo in dairy cattle (Elvinger et al., 1991, 1992). Therefore, results of the current study may suggest that the mammary gland of a non-cooled cow exerts a stronger inflammatory response and subsequently releases more chemo-attractants to attract immune cells in response to LPS infusion in comparison with cooled cows. To test this hypothesis, future work needs to be conducted to examine the impact of heat stress on local inflammatory responses within the mammary gland after infection or induced inflammation. The biological significance of greater immune cell migration into the heat-stressed mammary gland immediately after intramammary LPS infusion is not understood. In dairy cattle, several lines of evidence suggest that heat stress compromises both adaptive and innate immunity. It is possible that the mammary gland of a heat-stressed cow requires more immune cells to compensate for impaired function caused by heat stress in order to cope with mammary infection.

Because there was no interaction between cooling and time, cooling did not influence cortisol release after intramammary infusion of LPS. Compared with cooled cows, non-cooled cows had lower average blood cortisol concentrations, consistent with Christison and Johnson (1972), who reported that prolonged exposure to heat stress reduces blood cortisol concentration. The level of supplemental Zn intake has been demonstrated to regulate hypothalamo-pituitary-adrenocortical activity and alters the glucocorticoid responses to stress or an adrenocorticotropic hormone challenge (Quarterman and Humphries, 1979; Takeda et al., 2016). However, as discussed by Quarterman and Humphries (1979), the hypothalamo-pituitary-adrenocortical sensitivity, and related glucocorticoid responses, to stress due to supplemental Zn are inconsistent across different species. To the best of our knowledge, impacts of levels and source of supplemental Zn on the stress response of lactating dairy cows have never been reported. In the current study, cows fed IOZ had a blunted cortisol response immediately after intramammary LPS infusion compared with those supplemented with ZMC; however, the different cortisol response did not appear to influence other inflammatory or production measures after intramammary infusion of LPS.

The source of supplemental Zn in the diet had no apparent influence on the parameters examined except for the cortisol responses. Zinc is an essential nutrient involved in mammary gland development and health. Mice consuming diets supplemented with marginal Zn (15 mg/kg, as fed basis) for 24 wks following puberty was associated with Zn accumulation, oxidative stress, macrophage infiltration and excessive collagen deposition in the mammary gland, leading to impaired mammary development compared with mice fed adequate Zn (30 mg/kg, as fed basis) (Bostanci et al., 2015). These defects in mammary gland function, due to marginal Zn intake, eventually result in compromised mammary secretion and lower milk production during lactation

of the mice (Dempsey et al., 2012). The mammary glands of mice that consumed marginal Zn (15 mg/kg, as fed basis) during the entire pregnancy and lactation cycle had incomplete involution following weaning and experienced stronger oxidative stress compared with mice that consumed adequate Zn (30 mg/kg, as fed basis) (Bostanci et al., 2016). In dairy cattle, the source of dietary Zn strongly influences mammary health. Previous studies (Kellogg et al., 2004; Nayeri et al., 2014) reported that feeding Zn-Met or Zn-AA complexes to dairy cows reduced milk SCC compared with Zn oxide or Zn sulfate, suggesting an advantage of AA chelated Zn over inorganic source on mammary health. However, previous research also found no difference in milk SCC between cows fed Zn-Met and Zn hydroxychloride under either cooled or non-cooled conditions (Weng et al., 2018). Data from our current study supports that the source of supplemental Zn (Zn-Met vs. Zn hydroxychloride) in lactating diets have no apparent influence on intake, milk yield and composition and leukocyte migration to mammary gland after intramammary infusion of LPS.

CONCLUSION

In the current study, non-cooled cows maintained higher body temperature, but tended to have greater feed efficiency and greater leukocyte migration into the mammary gland immediately after intramammary infusion of LPS compared with cooled cows. These results suggest that heat stress does not exaggerate the reduction in milk yield, but may influence a greater recruitment of immune cells into the mammary gland during mastitis. Because heat stress impairs innate immunity in cows (do Amaral et al., 2011; Lecchi et al., 2016), it is unresolved if heat stress impacts the clearance of bacteria from the mammary gland during clinical mastitis. This question deserves further investigation. The source of supplemental Zn in dairy cow diets influences mammary health, and many studies have reported that replacing a portion of inorganic source of

dietary supplemental Zn with AA chelated Zn reduces milk SCC. However, the results from the current study suggest that the source of supplemental Zn (Zn-Met vs. Zn hydroxychloride) has no impact on intake, milk yield and composition, and leukocyte infiltration into the mammary gland after intramammary LPS infusion. The results in the current study provide valuable preliminary data to further define the impact of heat stress and nutritional factors on cow responses during intramammary infection.

ACKNOWLEDGEMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection, and Drs. David J. Hurley and Geoffrey E. Dahl for their comments and review of the manuscript.

DECLARATION OF INTEREST

None declared.

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Table 4.1: Milk yield, dry matter intake (DMI), feed efficiency (milk yield/DMI), rectal temperature, and respiration rate of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either cooling (CL) or not (NC), in response to intramammary lipopolysaccharide (LPS) infusion. The daily milk yield and DMI were measured from -7 to 7 d relative to intramammary infusion, and rectal temperature and respiration rate were measured at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion.

	IOZ		ZMC		SEM	P value						
Item	CL	NC	CL	NC		Z[1]	C	Z×C	T	Z×T	C×T	Z×C×T
Milk yield, kg/d	33.6	25.6	33.1	23.5	2.8	0.66	0.01	0.78	<0.01	0.91	<0.01	0.85
DMI, kg/d	26.4	19.0	25.8	19.9	1.1	0.92	<0.01	0.51	<0.01	0.87	0.12	0.19
Feed efficiency	1.28	1.36	1.28	1.21	0.12	0.54	0.93	0.54	<0.01	0.98	0.01	0.23
Rectal temperature, °C	38.9	39.9	39.0	39.8	0.13	0.87	<0.01	0.48	<0.01	0.15	<0.01	0.26
Respiration rate, breath/min	55.1	68.0	57.3	77.6	3.6	0.13	<0.01	0.33	<0.01	0.94	0.19	0.25

[1]Z: Zn source effect; C: cooling effect; T: time effect.

Table 4.2: Milk composition of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either cooling (CL) or not (NC), in response to intramammary lipopolysaccharide (LPS) or saline infusion. Milk samples were collected from LPS and saline infused quarters at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion.

	IOZ		ZMC			P value						
Item	CL	NC	CL	NC	SEM	Z[1]	C	Z×C	T	Z×T	C×T	Z×C×T
LPS infused quarter												
Fat, %	3.40	2.80	2.68	3.28	0.34	0.74	0.98	0.10	<0.01	0.12	0.07	0.42
Protein, %	3.09	2.90	3.10	3.07	0.12	0.46	0.36	0.48	<0.01	0.65	<0.01	0.79
MUN, mg/dL	8.21	10.94	8.68	10.41	1.07	0.98	0.06	0.65	<0.01	0.99	0.06	0.22
Ln(SCC, 10^3 cells/mL)	7.13	6.74	6.76	7.05	0.19	0.89	0.80	0.09	<0.01	0.13	0.01	0.34
Saline infused quarter												
Fat, %	3.63[a]	2.73[b]	2.90[ab]	3.37[ab]	0.35	0.96	0.56	0.08	<0.01	0.33	0.50	0.13
Protein, %	2.86	2.58	2.84	2.75	0.09	0.45	0.07	0.35	<0.01	0.62	0.29	0.88
MUN, mg/dL	8.21	9.49	7.97	9.48	1.02	0.90	0.19	0.91	<0.01	0.85	0.02	0.80
Ln(SCC, 10^3 cells/mL)	4.18	4.74	4.99	4.87	0.27	0.11	0.44	0.23	<0.01	0.14	0.08	0.37

[1]Z: Zn source effect; C: cooling effect; T: time effect.
Superscripts: a vs b: $P < 0.10$.

Table 4.3: Hematologic profile of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either evaporative cooling (CL) or not (NC), in response to intramammary lipopolysaccharide infusion. Blood samples were collected at 0 h (before infusion) and at 3, 6, 12, and 24 h after infusion.

	IOZ		ZMC		SEM	P value						
Item	CL	NC	CL	NC		Z[1]	C	Z×C	T	Z×T	C×T	Z×C×T
White blood cells, $\times 10^3$ cells/μL	9.79	5.86	9.46	6.35	2.05	0.17	0.60	0.59	<0.01	0.38	0.03	0.28
Lymphocyte, $\times 10^3$ cells/μL	5.35	2.48	4.26	2.73	1.21	0.85	0.10	0.64	<0.01	0.71	<0.01	0.37
Neutrophil, $\times 10^3$ cells/μL	2.69	2.33	2.98	2.98	0.32	0.17	0.60	0.59	<0.01	0.38	0.03	0.28
Monocyte, $\times 10^3$ cells/μL	0.37	0.22	0.30	0.21	0.04	0.36	0.02	0.57	<0.01	0.83	0.22	0.91
Eosinophil, $\times 10^3$ cells/μL	1.60	1.52	1.64	1.44	0.09	0.78	0.13	0.52	<0.01	0.31	0.17	0.48
Basophil, $\times 10^3$ cells/μL	0.09	0.06	0.08	0.08	0.03	0.92	0.65	0.56	0.43	0.58	0.41	0.63
Hematocrit, %	28.4	27.9	27.4	27.4	0.52	0.20	0.63	0.60	<0.01	0.30	0.67	0.53
Plasma protein, g/dL	8.09	7.82	7.76	7.69	0.19	0.26	0.39	0.63	0.01	0.32	0.01	0.29

[1]Z: Zn source effect; C: cooling effect; T: time effect.

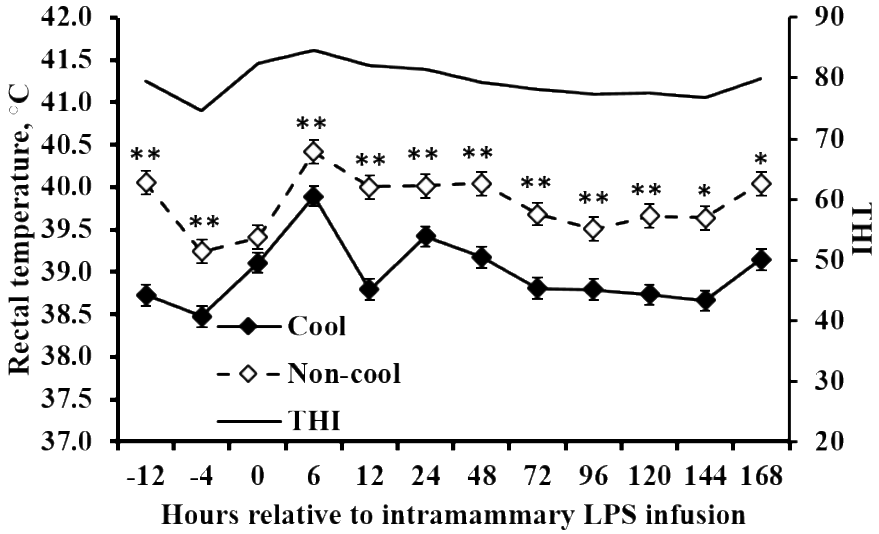

Figure 4.1: THI (solid line, no symbols), and rectal temperature of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) in response to intramammary lipopolysaccharide (LPS) infusion. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $\dagger P \leq 0.10$.

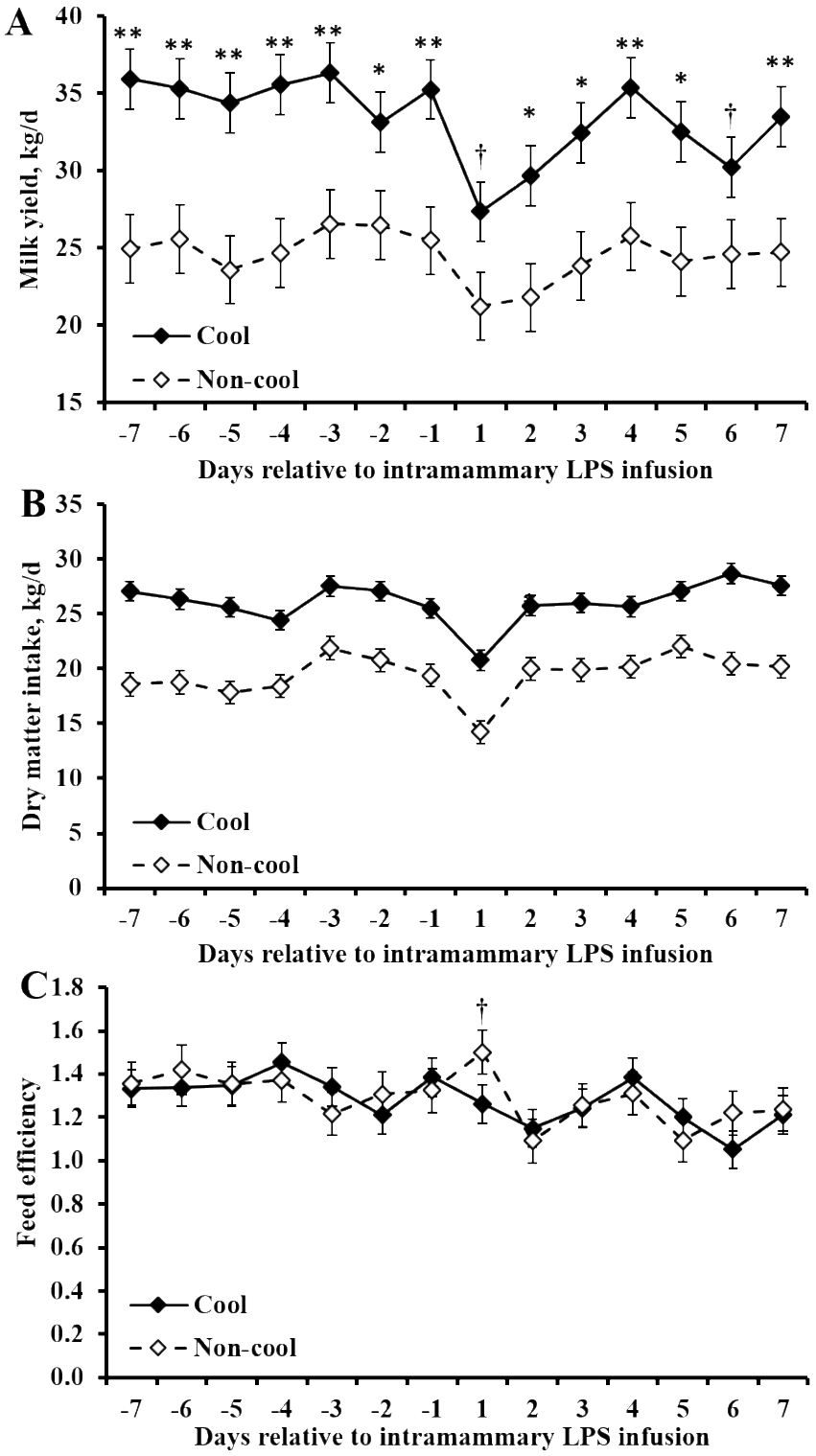

Figure 4.2: Milk yield (A), dry matter intake (B) and feed efficiency ([milk yield/dry matter intake], C) of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) in response to an intramammary lipopolysaccharide (LPS) infusion. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $\dagger P \leq 0.10$.

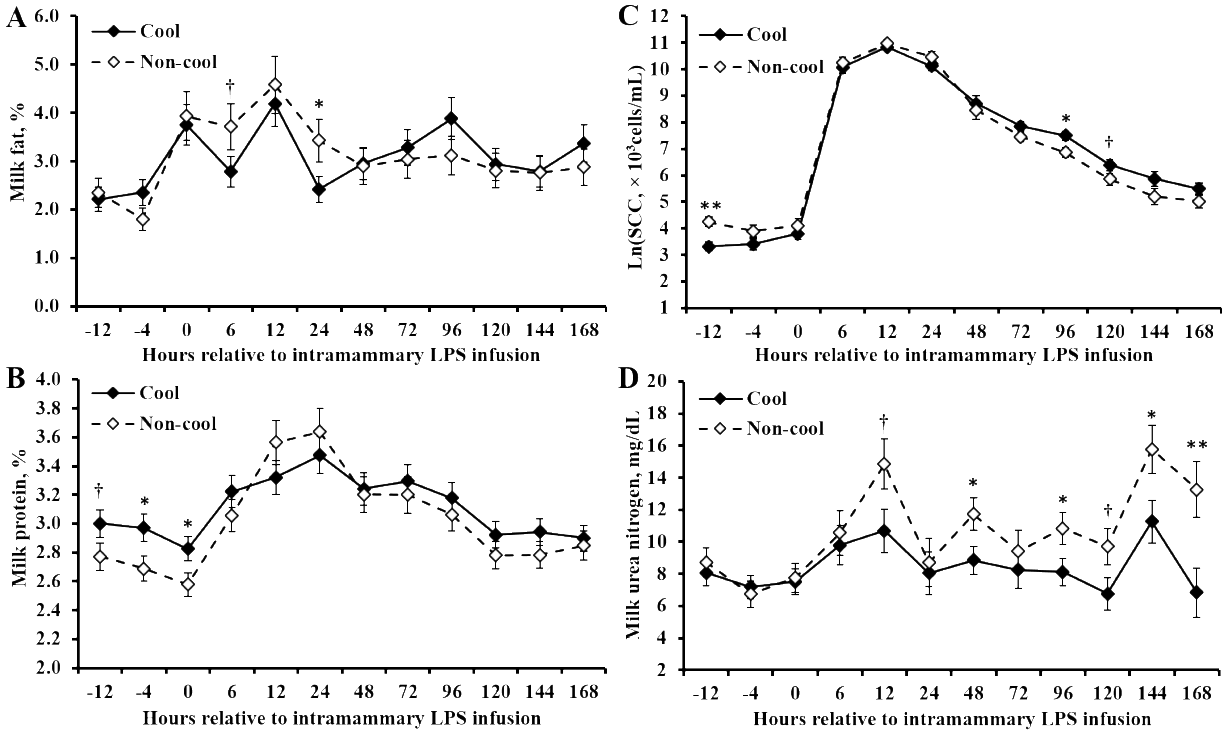

Figure 4.3: Milk concentrations of fat (A), protein (B), ln(somatic cell count [SCC]) (C) and urea nitrogen (D) in lipopolysaccharide (LPS) infused quarter of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]). $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, †$P \leq 0.10$

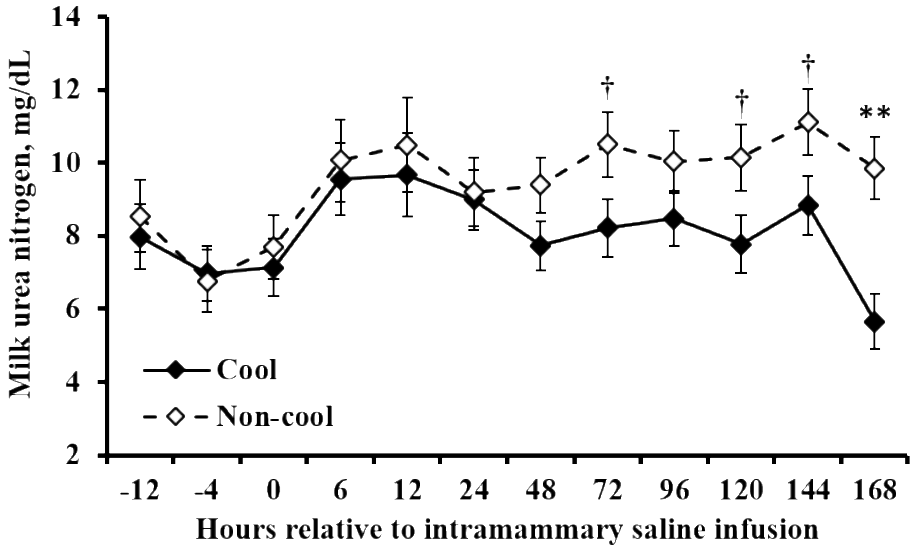

Figure 4.4: Milk concentration of urea nitrogen in saline infused quarter of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]). $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, †$P \leq 0.10$

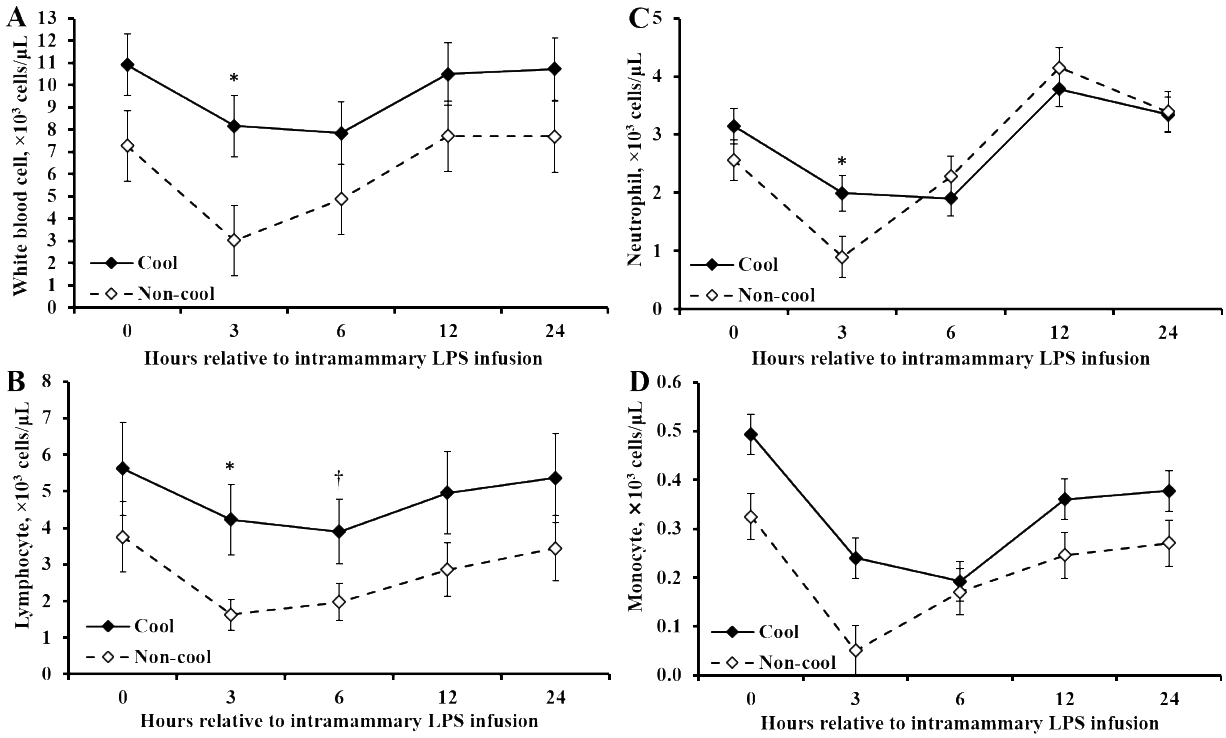

Figure 4.5: The blood concentrations of white blood cells (A), lymphocytes (B), neutrophils (C), and monocytes (D) of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) after intramammary lipopolysaccharide (LPS) infusion. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, †$P \leq 0.10$.

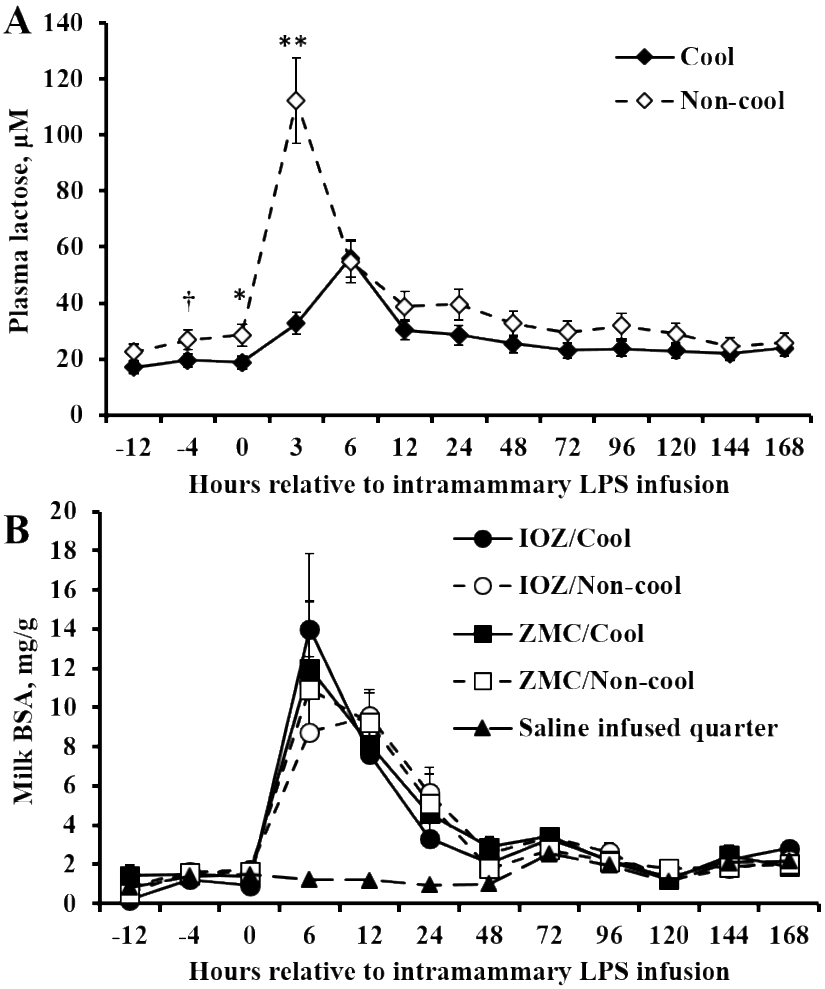

Figure 4.6: The plasma concentration of lactose (A) of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) in response to intramammary lipopolysaccharide (LPS) infusion. Milk concentration of bovine serum albumin (BSA) (Figure 3.6B) in LPS infused quarter of cows under cooling or not, and fed diets supplemented with 75 mg/kg Zn hydroxychloride (IOZ), or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (ZMC) (cooled cows fed IOZ: solid circle [●]; non-cooled cows fed IOZ: open circle [○]; cooled cows fed ZMC: solid square [■]; non-cooled cows fed ZMC: open square [□]). The solid triangle [▲] in B represents the milk BSA concentration in saline infused quarters. For plasma lactose concentration, effect of Zn source ($P = 0.76$), cooling ($P = 0.06$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.99$), Zn source by time interaction ($P = 0.72$), cooling by time interaction ($P < 0.01$), cooling by Zn source by time interaction ($P = 0.52$). For milk BSA concentration in LPS infused quarter, effect of Zn source ($P = 0.94$), cooling ($P = 0.95$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.87$), Zn source by time interaction ($P = 0.57$), cooling by time interaction ($P = 0.28$), cooling by Zn source by time interaction ($P = 0.44$). **$P \leq 0.01$, *$P \leq 0.05$, †$P \leq 0.10$.

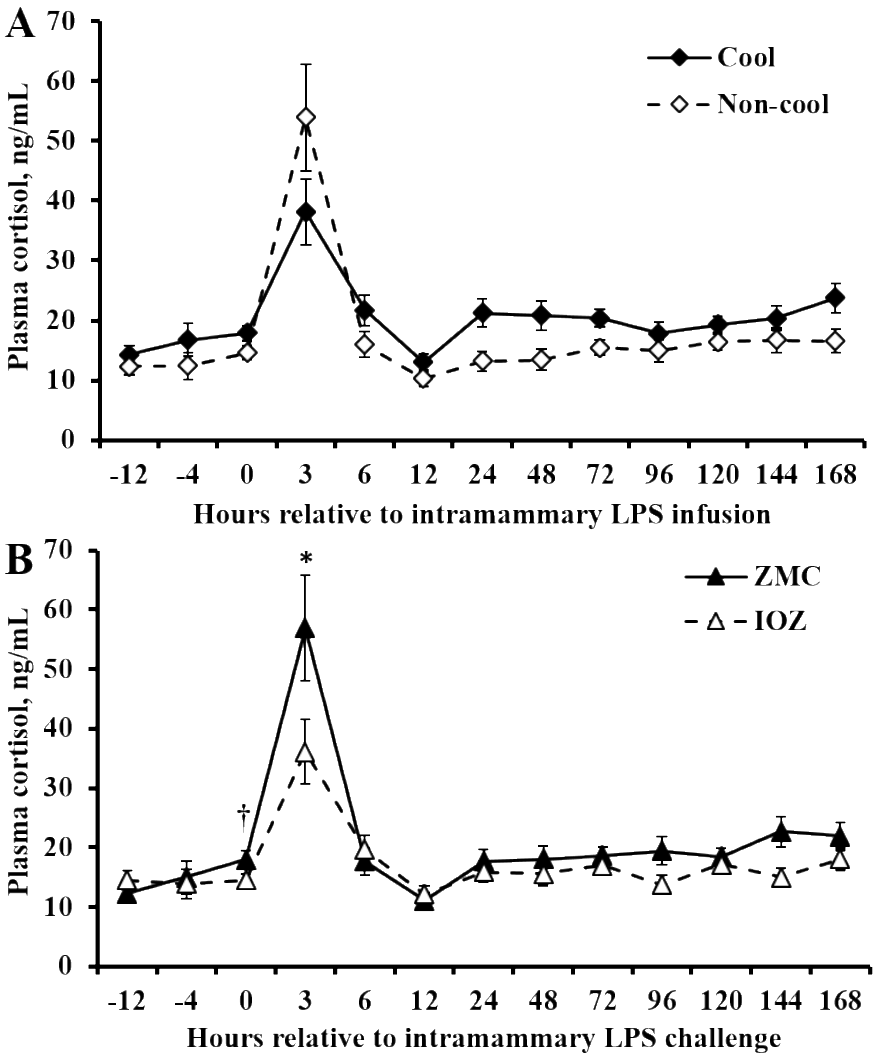

Figure 4.7: The effects of evaporative cooling (A) and dietary Zn source (B) on plasma concentrations of cortisol of lactating dairy cows in response to intramammary lipopolysaccharide (LPS) infusion. Cows were exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]), and fed diets supplemented with 75 mg/kg Zn hydroxychloride (IOZ, open triangle [Δ]), or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (ZMC, solid triangle [▲]). Effect of Zn source ($P = 0.18$), cooling ($P = 0.03$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.82$), Zn source by time interaction ($P = 0.10$), cooling by time interaction ($P = 0.24$), cooling by Zn source by time interaction ($P = 0.27$). $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, †$P \leq 0.10$.

CHAPTER 5

RESPONSE OF LACTATING DAIRY COWS FED DIFFERENT SUPPLEMENTAL ZINC SOURCES WITH AND WITHOUT EVAPORATIVE COOLING TO INTRAMAMMARY LIPOPOLYSACCHARIDE INFUSION: METABOLITE AND MINERAL PROFILES IN BLOOD AND MILK[3]

[3] Marins, T. N., A. P. A. Monteiro, X. Weng, J. Guo, R. M. Orellana Rivas, J. K. Bernard, D. J. Tomlinson, J. M. DeFrain, and S. Tao. 2020. *Journal of Animal Science*, 98(10):skaa323. Reprinted here with permission of the publisher.

ABSTRACT

The objective of this study was to determine the effect of evaporative cooling and dietary supplemental Zn source on blood metabolites, insulin and mineral concentrations and milk mineral concentrations following intramammary lipopolysaccharide (LPS) infusion. Seventy-two multiparous Holstein cows were assigned to one of 4 treatments with a 2 × 2 factorial arrangement. Treatments included two environments: with or without evaporative cooling using fans and misters over the freestall and feedbunk, and two dietary sources of supplemental Zn: 75 mg/kg of dry matter (DM) supplied by Zn hydroxychloride (inorganic Zn, IOZ), or Zn hydroxychloride (35 mg of Zn/kg of DM) + Zn-Met complex (ZMC, 40 mg of Zn/kg of DM). A subset of cows (n = 16; 263 ± 63 d in milk) were infused with 10 μg of LPS or a saline control in the left or right rear quarters on d 34 of the environmental treatment. Individual milk samples collected from LPS infused quarters at -4, 0, 6, 12, 24, 48, 72, 96, and 144 h relative to infusion were analyzed for minerals. Blood samples were collected at the same time with an additional sample collected at 3 h post infusion to analyze glucose, non-esterified fatty acids (NEFA), insulin, and minerals. Cooling by time interactions ($P \leq 0.07$) were observed for plasma glucose and NEFA, and serum insulin. Compared with cooled cows, non-cooled cows had lower concentrations of plasma glucose except at 3 h following intramammary LPS infusion, greater serum insulin at 3 and 12 h, and lower plasma NEFA at 24 and 48 h after infusion. Relative to cooled cows, non-cooled cows tended ($P = 0.07$) to have lower serum K concentration, and had lower ($P < 0.01$) serum Zn 6 h following infusion (cooling by time interaction: $P < 0.01$). Relative to ZMC cows, IOZ cows had greater ($P \leq 0.09$) concentrations of plasma Se, skim milk Na and Se, and skim milk Na to K ratio. Regardless of treatment, intramammary LPS infusion reduced ($P < 0.01$) serum or plasma concentrations of Ca, Mg, Zn, Fe and Se, but increased ($P < 0.01$) their concentration in skim milk. In conclusion,

deprivation of cooling resulted in more rapid and prolonged insulin release, and influenced the systemic and mammary mineral metabolism during mammary inflammation induced by LPS of lactating dairy cows. Dietary supplementation of Zn-Met complex reduced blood and milk Se concentrations compared with cows fed Zn from inorganic source.

Key words: cooling, dairy cow, intramammary lipopolysaccharide infusion, zinc negative effects

INTRODUCTION

Heat stress not only negatively affects animals' production and reproduction, but also compromises their health (West, 2003). During heat stress, the incidences of disease, especially mastitis, increase presumably because of the favorable environment for survival and growth of pathogens coupled with the impaired immune function of the cow (Kadzere et al., 2002). Escherichia coli (***E. coli***) is an environmental pathogen that causes mastitis in dairy herds (Wenz et al., 2001; Bradley, 2002). Lipopolysaccharide (**LPS**), a component of the structural membrane of gram-negative bacteria, is an endotoxin responsible for disease pathogenesis caused by *E. coli*. Its presence within the mammary gland induces a series of immune cascades from local inflammation to systemic immune responses (Burvenich et al., 2003). These LPS induced responses subsequently alter energy and mineral metabolism of a dairy cow. Immunoactivation caused by intravenous or intramammary LPS infusion is related to increased insulin secretion and significant glucose consumption by the immune system (Waldron et al., 2006; Kvidera et al., 2017). Further, mammary inflammation induced by LPS is associated with reduced blood mineral concentrations, including Zn and Fe, in dairy cows, suggesting altered mineral metabolism (Lohuis et al., 1988). However, the impact of heat stress on energy and mineral metabolism during mastitis or LPS induced mammary inflammation is still unclear.

Mineral nutrition affects the incidence, duration and severity of diseases. Zinc is an essential micro mineral for dairy cattle and adequate Zn supplementation is positively related to disease resistance (Weiss, 2002; Spears and Weiss, 2008). The dietary source of supplemental Zn also influences mammary health, but inconsistent results have been reported. Kellogg et al. (2004) reported a positive impact of supplementation of Zn-Met complex compared with $ZnSO_4$ or Zn oxide for reducing milk somatic cell count (**SCC**). In contrast, Whitaker et al. (1997) reported no effect of dietary Zn source on udder health. Although most of those studies focus on milk SCC and disease incidence, there is still a lack of understanding how different sources of supplemental Zn affects metabolic and mineral responses during mammary inflammation or mastitis in lactating dairy cows. Therefore, we hypothesized that heat stress and dietary source of supplemental Zn (Zn-Met complex vs. Zn hydroxychloride) alter metabolic responses and mineral metabolism after LPS infusion into lactating quarters of dairy cows. The objective of the current study was to evaluate the effects of deprivation of evaporative cooling and replacing a portion of the dietary inorganic source of supplemental Zn with Zn-Met complex on blood metabolites, insulin and mineral concentrations, and milk mineral concentrations of lactating dairy cows to an intramammary LPS infusion during summer.

MATERIALS AND METHODS

This study was conducted during the summer at the Dairy Research Center, the University of Georgia - Tifton campus. Prior to beginning the trial, experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Georgia.

The cows used for the intramammary LPS infusion were a subset of cows from an experiment previously reported by Weng et al. (2018). Briefly, 72 multiparous Holstein cows were

randomly assigned to four treatments with a 2×2 factorial arrangement. Treatments included two environmental conditions: evaporatively cooled or not cooled, and two dietary supplemental Zn sources: 75 mg of supplemental Zn/kg of DM supplied by Zn hydroxychloride (**IOZ** [inorganic Zn], Micronutrients, Indianapolis, IN), or 75 mg of supplemental Zn/kg of DM supplied by Zn hydroxychloride (35 mg/kg of DM) + Zn-Met complex (40 mg/kg of DM, **ZMC**, Zinpro Corporation, Eden Prairie, MN). Before the initiation of the environmental treatments, all cows were pre-fed their respective dietary treatments for 12 wk. Cows were fed the same total mixed ration once daily at 1300 h and each Zn supplement was mixed with the total mixed ration diet before feeding. Daily intake was measured using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH). The ingredient composition and chemical composition of the diets were reported by Weng et al. (2018). After the initiation of the environmental treatment, non-cooled cows were deprived of evaporative cooling for 12 wk, and cooled cows remained under evaporative cooling. The evaporative cooling system consisted of misters attached to the front face of fans over feedbunks and freestalls. Cows were housed in adjacent pens according to their environmental treatments. The average temperature humidity index was 77.6 ± 3.8 and 77.8 ± 3.8 (mean ± SD) in cooled and non-cooled pens, respectively. Cows were milked three times each day at 0800, 1600, and 2400 h. Milk yield of individual cows was recorded at each milking (Alpro, DeLaval, Kansas City, MO).

The procedures of intramammary LPS infusion, and milk yield, dry matter intake (**DMI**), and hematological profiles during intramammary LPS infusion were described in a companion paper (Marins et al., 2019). Briefly, the intramammary infusion of LPS or saline was performed on d 34 of the environmental treatment. Twenty cows (n = 5/treatment) with negative milk sample cultures and SCC below 200,000 cells/mL from both rear quarters collected 3 d before infusion

were selected for the infusion study. The infusion procedure started 2 h after 0800 milking. The left rear quarter was infused with 10 μg of *E coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO, Greco et al., 2015) dissolved in 5 ml pyrogen-free saline solution, and the right rear quarter was infused with 5 ml pyrogen-free saline solution. Individual quarter milk samples (50 mL) were collected at -12, -4, 0, 6, 12, 24, 48, 72, 96, and 144 h relative to infusion for milk composition analysis or stored at -20 °C for mineral analysis. Blood samples were collected to obtain serum and plasma at the same time points with an additional sample collected at 3 h post-infusion. There were four cows with SCC above 200,000 cells/mL in at least one of the rear quarters at -12, -4, or 0 h relative to infusion of LPS or saline. Thus, the data and sample collected from these cows were eliminated from final analyses, resulting in n = 4, 4, 5, and 3 cows for cooled cows fed IOZ, non-cooled cows fed IOZ, cooled cows supplemented with ZMC, and non-cooled cows supplemented with ZMC, respectively. The remaining 16 cows averaged 263 ± 63 d in milk, 3.2 ± 1.4 parity, and 66.7 ± 55.5 × 10^3 cells/mL for SCC of milk collected at -12, -4, and 0 h before infusion (± SD).

Plasma concentrations of glucose (Autokit Glucose, Wako Chemicals USA, Inc., Richmond, VA), non-esterified fatty acids (**NEFA;** HR Series NEFA-HR(2), Wako Chemicals USA, Inc.) and insulin (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden) from samples collected at -4, 0, 3, 6, 12, 24, 48, 96, and 144 h relative to intramammary LPS infusion were determined by colorimetric or enzyme-linked immunosorbent assay according to manufacturers' procedures. The intra- and interassay CV were, 3.5 and 4.9% for glucose, 1.3 and 3.6% for NEFA and 3.4 and 4.1% for insulin. Concentrations of Zn, Fe and Cu of serum samples collected at -4, 0, 3, 6, 12, 24, 48, and 72 h relative to intramammary LPS infusion were measured by atomic absorption (AAnalyst 100, Perkin Elmer, Waltham, MA), and the intra-assay CV were 1.3, 2.0, and 4.5%, respectively. Concentrations of Na, K, Ca, and Mg of serum samples collected

at -4, 0, 3, 6, 12, 24, 48, and 72 h relative to intramammary LPS infusion were determined at the Veterinary Diagnostic Laboratories of the University of Georgia (Tifton, GA) using an Advia 1800 Chemistry System (Siemens Medical Solutions USA, Inc., Malvern, PA).

Milk samples collected from LPS infused quarters were thawed at room temperature and then centrifuged at 3,000 × g for 15 min at 4 °C to obtain skim milk. Analyses to measure skim milk mineral concentrations (Na, K, Ca, Mg, Fe, Cu, Zn, and Se) and plasma Se concentration of samples collected between -4 and 72 h relative to intramammary LPS infusion were performed at Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI) using an Agilent ICP/MS according to the method described by Wahlen et al. (2005). The Cu concentration of skim milk in most of samples was below detection limit (0.02 μg/mL), thus not included in the statistical analysis.

Plasma or serum concentrations of metabolites, insulin and minerals, and skim milk concentrations of minerals were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). The SAS models included cooling, supplemental Zn source, time relative to infusion, and their respective interactions. Cow nested within treatment was included as a random variable and time was included as a repeated measure. Least squares means ± SEM are reported and significance and tendency were declared when $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

RESULTS

Weng et al. (2018) reported the performance data of cows fed different dietary treatments under different environments in detail. Relative to cooled cows, non-cooled cows carried more heat load, as suggested by greater ($P < 0.01$) respiration rates (64.8 vs. 73.9 breath/min) and vaginal temperature (39.03 vs. 39.94 ℃). As a result, non-cooled cows had lower ($P < 0.01$) milk yield

(25.9 vs. 35.4 kg/d) and DMI (21.6 vs. 26.7 kg/d) compared with cooled cows. Supplemental Zn source did not affect these measures ($P > 0.25$). Milk yield and composition, and hematological profiles during intramammary LPS infusion were reported previously (Marins et al., 2019). Cooled cows produced more ($P \leq 0.05$) milk from -7 to -1 d and at 2, 3, 4, 5, and 7 d relative to intramammary LPS infusion, but tended ($P \leq 0.08$) to have greater milk yield at 1 and 6 d after intramammary LPS infusion compared with non-cooled cows. Treatments had no apparent impact on milk SCC. Relative to cooled cows, non-cooled cows had lower ($P < 0.03$) blood concentrations of neutrophils and lymphocytes at 3 h after LPS infusion. Supplemental Zn source had no ($P \geq 0.13$) impact on these measurements (Marins et al., 2019).

In the current study, supplemental Zn source and its interactions with cooling and/or time had no impact on plasma concentrations of glucose, NEFA and insulin ($P > 0.15$, Table 5.1). Compared with cooled cows, non-cooled cows had lower ($P < 0.01$) plasma glucose concentrations except at 3 h following intramammary LPS infusion (cooling × time interaction: $P = 0.07$, Figure 5.1). There was also a cooling by time interaction ($P < 0.01$) for serum insulin concentration. Non-cooled cows tended ($P = 0.09$) to have a greater serum insulin concentration immediately before infusion; and circulating insulin increased in both groups of cows following intramammary LPS infusion with greater ($P < 0.05$) serum insulin concentration at 3 and 12 h after infusion for non-cooled compared with cooled cows (Figure 5.1). Relative to cooled cows, non-cooled cows had lower ($P < 0.05$) plasma NEFA concentrations at 24 and 48 h after intramammary LPS infusion (cooling × time interaction: $P = 0.02$, Figure 5.1).

Regardless of treatment, intramammary LPS infusion reduced ($P < 0.01$) serum or plasma concentrations of K, Ca, Mg, Zn, Fe and Se, and their concentrations reached nadirs around 3-12 h following infusion (Table 5.1 and Figure 5.2). In contrast, LPS infusion had no ($P > 0.10$) impact

on serum concentrations of Na and Cu (Table 5.1 and Figure 5.2). Supplemental Zn source, and its interaction with cooling or time had no impacts on blood mineral concentrations, except that cows supplemented with IOZ tended ($P = 0.09$) to have greater plasma concentration of Se relative to those supplemented with ZMC (Table 5.1). Regardless of the time, cooled cows tended to have greater serum K concentrations ($P = 0.07$) compared with non-cooled cows (Table 5.1). There was also a cooling by time interaction ($P < 0.01$) for serum Zn concentration because of the higher ($P < 0.01$) serum Zn concentrations for cooled cows compared with non-cooled cows at 6 h following intramammary LPS infusion (Figure 5.3). Although there was a cooling by time interaction ($P = 0.02$) for serum Fe concentrations, no treatment effect was identified at any time points by PDIFF or SLICE function of SAS.

Intramammary LPS infusion dramatically affected skim milk concentrations of minerals. Following infusion, concentrations of Na, Ca, Mg, Zn, Fe and Se increased (time effect: $P < 0.01$) and peaked approximately 6-24 h after infusion (Table 5.2 and Figure 5.4). In contrast, the skim milk K concentration decreased (time effect: $P < 0.01$) and reached the nadir at 12 h after intramammary LPS infusion, which resulted in an increased (time effect: $P < 0.01$) ratio of Na to K (Table 5.2 and Figure 5.4). No interactions between supplemental Zn source and time were observed, but cows fed IOZ tended ($P \leq 0.07$) to have greater skim milk concentrations of Na and Se, and greater ($P = 0.04$) skim milk Na to K ratio compared with cows supplemented with ZMC (Table 4.2). Regardless of the supplemental Zn source, non-cooled cows tended ($P = 0.07$) to have greater skim milk K concentration compared with cooled cows (Table 5.2). Further, there was a cooling by time interactions ($P = 0.05$) for skim milk Fe concentration, because Fe concentration in skim milk of cooled cows tended ($P = 0.08$) to be greater at 6 h but lower at 12 ($P = 0.09$) and

24 (P = 0.05) h following intramammary LPS infusion compared with non-cooled cows (Figure 5.3).

DISCUSSION

In the current study, no cooling by supplemental Zn source interactions were observed for any measurements. Due to the occurrence of subclinical mastitis (SCC > 200,000 cells/mL) prior to the LPS infusion, several cows were eliminated from the final analysis which reduced the number of animals per treatment. Therefore, it is important to recognize this limitation that may impede our ability to detect any potential environment by diet interactions. Despite significant glucose consumption by mammary inflammation, plasma glucose concentration is transiently increased following intramammary LPS challenge (Waldron et al., 2006; Vernay et al., 2012; Gross et al., 2018). A similar increase in blood glucose concentration was observed at 12 h following intramammary LPS infusion in the current study. This likely is due to the enhanced hepatic glycogenolysis and gluconeogenesis (reviewed by Moyes, 2015). In the present study, plasma glucose concentration of non-cooled cows remained lower before and after intramammary LPS infusion except at 3 h after infusion compared with cooled cows, which may reflect the lower DMI resulting from heat stress.

Serum insulin concentration also increased following intramammary LPS infusion, consistent with previous studies (Waldron et al., 2006; Vernay et al., 2012; Gross et al., 2018). The mechanism of the increased insulin secretion following mammary inflammation by LPS is unknown, but may in response to increased glucose and enhanced pancreatic sensitivity to glucose stimulation. Compared with cooled cows, non-cooled cows had a more rapid and prolonged increase in serum insulin concentration, suggesting a more sensitive pancreatic insulin release

during inflammation. During lactation, heat-stressed cows have reduced glucose concentration but have constant or increased plasma insulin concentration (Wheelock et al., 2010; Koch et al., 2016), indicating that factors rather than glucose concentration influence insulin secretion under heat stress conditions. The data from the current study suggest that the elevated insulin secretion of heat-stressed lactating dairy cows is related to inflammatory responses and immune activation. Regardless of treatments, intramammary LPS infusion resulted in reduced plasma NEFA concentration at 12 h post infusion, which may be a result of the increased circulating insulin. The increased NEFA concentration at 24 h following LPS infusion of cooled cows suggests adipose tissue mobilization (Moyes, 2015), which likely is a secondary response because of the reduced DMI following infusion. In contrast, a similar response was not observed in non-cooled cows potentially because of the inhibitory effects of heat stress on body fat mobilization. In lactating dairy cows, heat stress is reported to inhibit adipose tissue mobilization despite the substantially decreased DMI (Wheelock et al., 2010; Lamp et al., 2015).

Interestingly, deprivation of evaporative cooling tended to decrease K concentration in serum but tended to increase skim milk K concentration. In cattle, sweating rate increases as ambient temperature increases, and there is a greater loss of K than of Na during sweating (Jenkinson and Mabon, 1973). This may be one mechanism that explains the lower plasma K concentration of non-cooled cows than cooled cows observed in the current study. The mammary gland is believed to have evolved from apocrine-like skin gland associated with hair follicles (Capuco and Akers, 2009), similar to apocrine sweat gland of cattle. Thus, the increased milk K concentration of non-cooled cows than cooled cows may resemble the greater K loss during sweating of heat-stressed cattle. Because mammary epithelial integrity is not significantly altered by deprivation of evaporative cooling (Weng et al., 2018), this phenomenon may also indicate

greater Na^+-K^+-ATPase activity of the basolateral membrane of mammary epithelial cells (Shennan and Peaker, 2000).

The Na to K ratio in milk is a reliable marker for mammary epithelial integrity (Stelwagen et al., 1999). In the current study, supplementation of ZMC lowers the Na to K ratio in skim milk compared with IOZ, suggesting lower mammary epithelial permeability. This is consistent with previous studies that reported replacing a portion of inorganic sources of Zn with amino acids chelated Zn improved epithelial integrity of mammary gland in lactating dairy cows (Weng et al., 2018) and ileum of growing pigs (Pearce et al., 2015). The advantage of amino acids chelated Zn on epithelial integrity may be due to the greater bioavailability over inorganic source of Zn (Spears, 2003). It is also reported that micro minerals influence immunity partly by their involvement in the antioxidant system (Andrieu, 2008; Spears and Weiss, 2008). For example, both Zn and Se act as cofactors in anti-oxidative enzymes to neutralize free radicals. Zinc serves as a structural cofactor of Cu/Zn superoxide dismutase and promotes synthesis of metallothionein (Spears and Weiss, 2008); and Se is an integral part of Se containing glutathione peroxidases (Brigelius-Flohe and Maiorino, 2013). In the present study, feeding ZMC resulted in lower Se concentrations in both plasma and skim milk relative to IOZ, potentially suggesting that supplementation of amino acids chelated Zn reduces Se requirement because of the greater bioavailability compared with inorganic source of Zn. If this phenomenon will alter activities of anti-oxidative enzymes is unknown but deserves further investigation.

Regardless of the dietary or environmental treatments, intramammary LPS infusion reduced the serum concentrations of K without changing the Na concentration. Similarly, Ohtsuka et al. (1997) reported that cows with severe coliform mastitis had lower serum K concentrations compared with healthy animals. Intramammary LPS infusion also reduced concentrations of macro

(Ca, Mg), and micro mineral (Zn, Fe, Se) in the blood but simultaneously increased their concentrations in skim milk. Mammary inflammation induced by LPS is associated with disrupted mammary epithelial junction, as indicated by the increased skim milk Na to K ratio following LPS infusion. The impaired mammary epithelial integrity enables the paracellular transport between blood and milk based on the concentration gradient, which may partially explain the altered milk and blood mineral concentrations (Oliver and Calvinho, 1995). However, since the skim milk concentrations of Ca and Mg were 2-3 fold greater than serum before infusion, macro mineral transport from blood to milk through paracellular route is unlikely. Because intravenous LPS infusion also decreases serum macro (Waldron et al., 2003) and micro mineral concentrations (Lohuis et al., 1988; Erskine and Bartlett, 1993; Hennigar and McClung, 2016), reduced serum or plasma mineral concentrations observed in the current study may result from the systemic inflammation induced by intramammary LPS infusion. Lipopolysaccharide induced inflammation is related to decreased gut motility, therefore the reduced intestinal absorption of mineral may be a potential mechanism for the lower blood macro and micro mineral concentration following intramammary LPS infusion (Lohuis et al., 1988; Waldron et al., 2003). Additionally, an endotoxin shock is associated with hepatic Ca sequestration and increased intracellular Ca concentration in liver cells, which is suggested to be associated with various endotoxin induced metabolic disorders but also partly explains the decrease in blood Ca concentrations (Sakaguchi and Furusawa, 2006).

In the current study, because the blood micro mineral concentrations were greater than skim milk prior to infusion, the paracellular transport from blood to milk may contribute to the decreased blood concentrations and increased skim milk concentrations of micro minerals. Relative to cooled cows, non-cooled cows had greater reduction in serum Zn concentrations following intramammary LPS infusion, potentially suggesting greater tissue sequestration of blood

Zn to cope with inflammation related oxidative stress under heat stress. However, this hypothesis has never been tested and deserves further investigation. Intramammary LPS infusion significantly reduced the milk yield of dairy cattle (Marins et al., 2019), which may be the primary mechanism of the increased concentrations of macro and micro mineral in skim milk collected from the LPS infused quarter due to a concentrating effect. After LPS infusion, the increase in skim milk Fe is delayed in non-cooled cows compared with cooled cows. The mechanism of this delay is not clear but may be associated with altered lactoferrin production. Future study needs to focus on the impact of heat stress on mammary gland immune responses to mammary infection or inflammation.

CONCLUSION

Following intramammary LPS infusion, non-cooled cows maintained lower plasma glucose concentrations, but had a more rapid and prolonged increase in serum insulin concentration compared with cooled cows. These data may indicate that heat-stressed lactating dairy cows have more sensitive pancreatic insulin release induced by inflammation. Compared with cooled cows, non-cooled cows had greater reduction in serum Zn concentration and a delayed increase in skim milk Fe concentration after intramammary LPS infusion. Because both Zn and Fe are related to immune responses, these data potentially suggest that heat stress affects the systemic and mammary immune responses during mammary inflammation. Future studies in this area are required. The results of our current trial suggest that dietary sources of supplemental Zn has no apparent impact on metabolic and mineral response of cows to intramammary LPS infusion. However, the reduced plasma and milk concentrations of Se in cows supplemented with Zn-Met

complex compared with IOZ suggest a potential interaction between the requirements of Zn and Se in lactating dairy cows.

ACKNOWLEDGEMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection. Partial funding provided by Zinpro Corporation (Eden Prairie, MN).

DECLARATION OF INTEREST

D.J.T and J.M.D are employed by Zinpro Corporation. The remaining authors are associated with The University of Georgia and have no actual or potential conflicts of interest.

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Table 5.1: Plasma concentrations of glucose and NEFA, and serum concentrations of insulin and minerals of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either evaporative cooling (CL) or not (NC), subjected to an intramammary lipopolysaccharide infusion.

	IOZ		ZMC		SEM	*P* value						
	CL	NC	CL	NC		Z[1]	C	Z×C	T	Z×T	C×T	Z×C×T
Glucose, mg/dL	57.9	53.5	57.2	52.7	1.1	0.53	< 0.01	0.95	< 0.01	0.54	0.07	0.39
NEFA, µEq/L	132.8	127.2	134.9	105.4	8.9	0.26	0.07	0.18	< 0.01	0.82	0.02	0.63
Insulin, µg/L	0.41	0.60	0.42	0.60	0.10	0.92	0.08	0.94	< 0.01	0.21	< 0.01	0.45
Na, mEq/L	139.1	138.3	136.4	138.5	2.7	0.66	0.80	0.59	0.70	0.88	0.91	0.80
K, mEq/L	4.53	4.28	4.51	4.46	0.07	0.31	0.07	0.19	<0.01	0.40	0.17	0.67
Ca, mg/dL	9.20	9.13	9.28	9.08	0.23	0.94	0.57	0.78	<0.01	0.48	0.15	0.18
Mg, mg/dL	2.37	2.33	2.42	2.26	0.09	0.92	0.29	0.49	<0.01	0.51	0.18	0.99
Cu, µg/mL	0.81	0.72	0.69	0.73	0.07	0.48	0.77	0.39	0.18	0.60	0.54	0.91
Fe, µg/mL	2.89	2.60	2.58	2.88	0.24	0.94	0.98	0.25	<0.01	0.48	0.02	0.92
Zn, µg/mL	1.19	1.09	1.17	1.12	0.08	0.97	0.34	0.80	<0.01	0.97	<0.01	0.25
Se, ng/mL	109.2	103.0	102.3	95.4	4.0	0.09	0.13	0.94	<0.01	0.79	0.34	0.83

[1]Z: zinc effect; C: cooling effect; T: time effect.

Table 5.2: Minerals concentrations of skim milk of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), exposed to either evaporative cooling (CL) or not (NC), and subjected to an intramammary lipopolysaccharide infusion.

	IOZ		ZMC		SEM	*P* value						
	CL	NC	CL	NC		Z[1]	C	Z×C	T	Z×T	C×T	Z×C×T
Na, µg/mL	1081	982	864	789	101	0.07	0.41	0.91	<0.01	0.86	0.61	0.44
K, µg/mL	1091	1290	1269	1407	82	0.11	0.07	0.72	<0.01	0.98	0.92	0.25
Na/K ratio	0.91	0.70	0.61	0.48	0.12	0.04	0.18	0.98	<0.01	0.97	0.72	0.54
Ca, µg/mL	426.3	470.7	465.3	407.8	39.2	0.76	0.87	0.21	<0.01	0.65	0.86	0.77
Mg, µg/mL	75.9	80.1	84.9	77.3	4.9	0.54	0.73	0.25	<0.01	0.58	0.31	0.08
Fe, µg/mL	0.16	0.18	0.15	0.16	0.02	0.49	0.46	0.85	<0.01	0.95	0.05	0.99
Zn, µg/mL	0.93	0.93	0.92	0.85	0.12	0.74	0.75	0.80	<0.01	0.54	0.21	0.35
Se, µg/mL	0.031	0.035	0.025	0.028	0.003	0.06	0.33	0.84	<0.01	0.70	0.32	1.00

[1]Z: zinc effect; C: cooling effect; T: time effect.

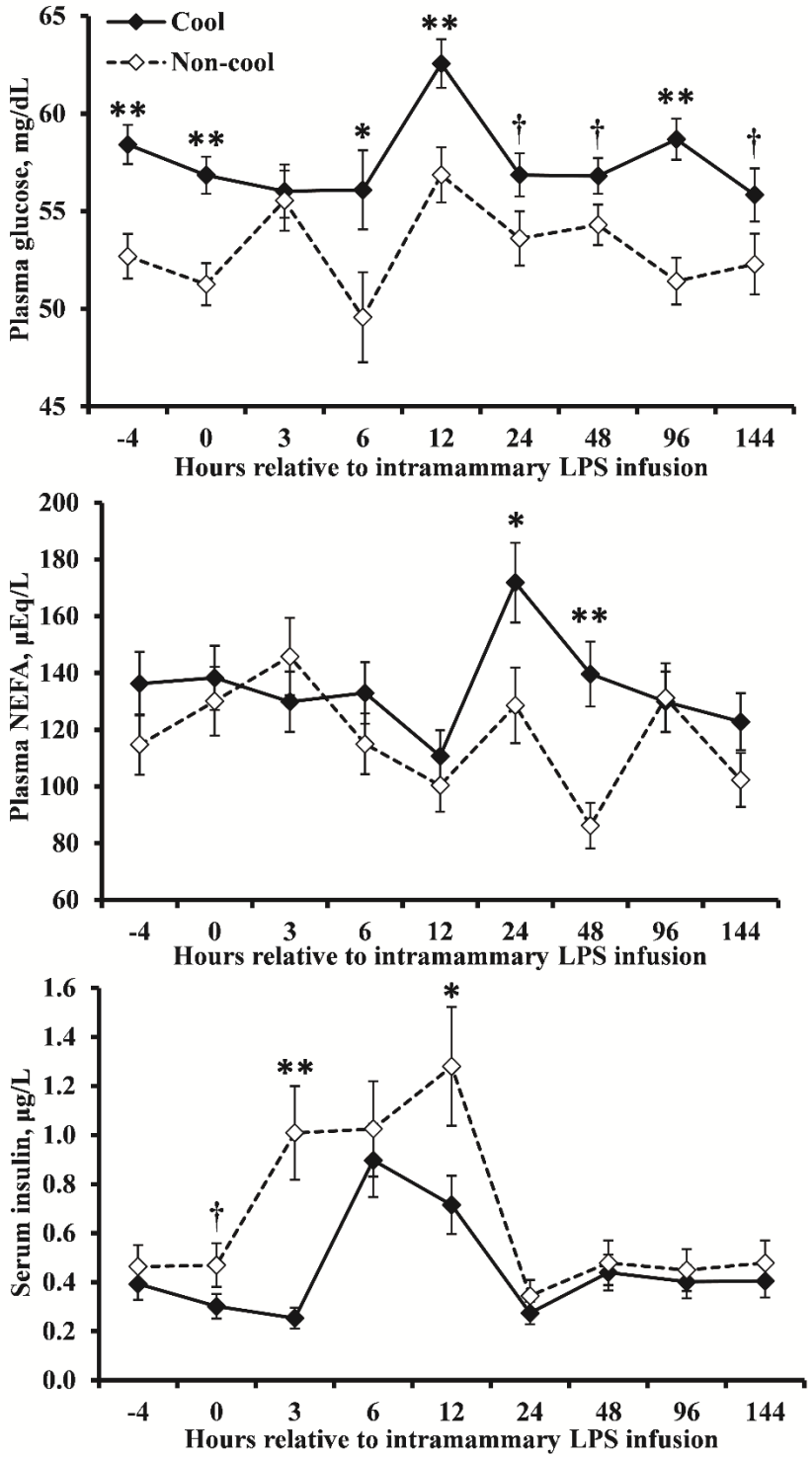

Figure 5.1: The plasma concentrations of glucose and NEFA, and serum concentration of insulin of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) after intramammary lipopolysaccharide (LPS) infusion. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $\dagger P \leq 0.10$.

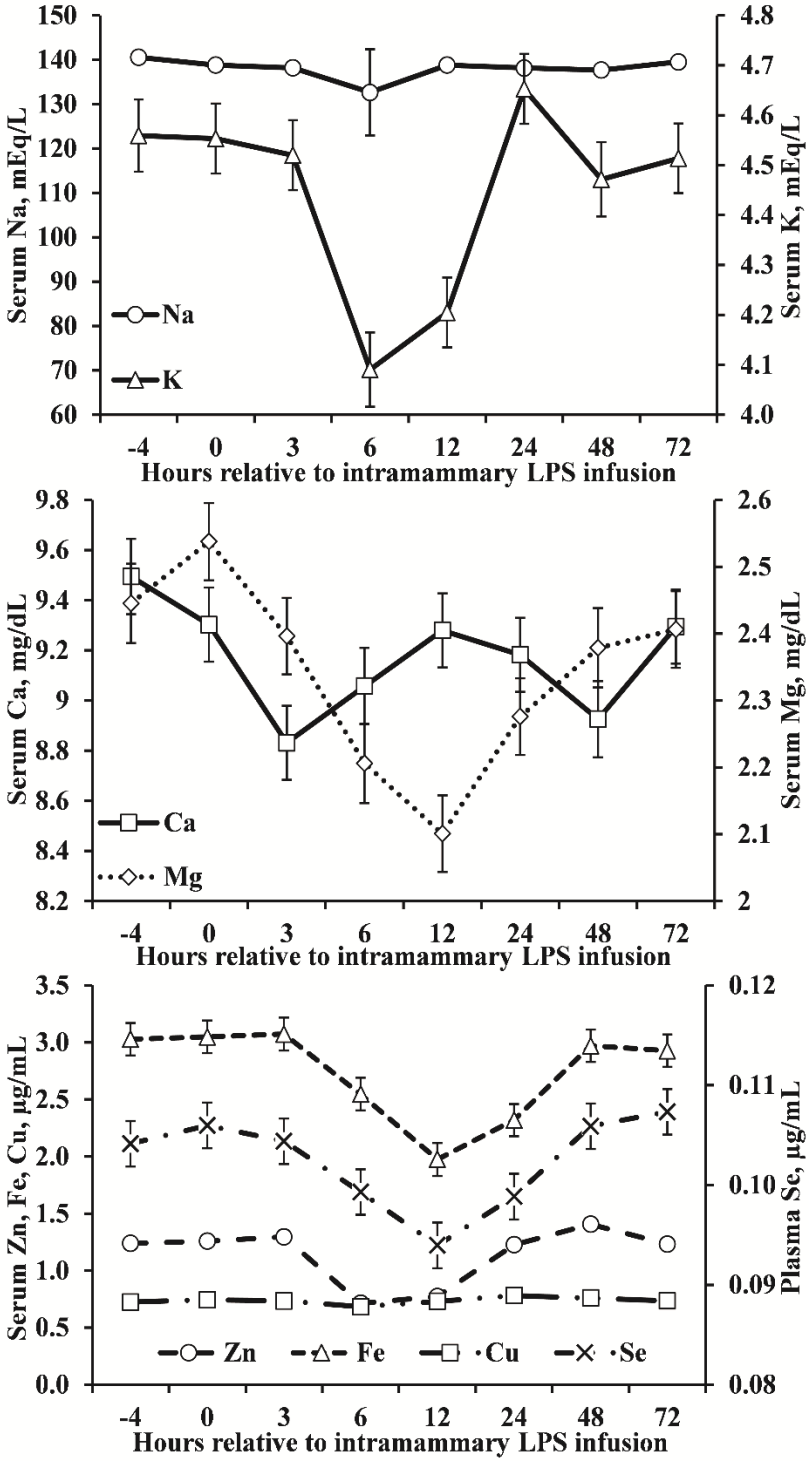

Figure 5.2: Serum concentration of Na (open circle [○] with solid line), K (open triangle [Δ] with solid line), Ca (open square [□] with solid line), Mg (open diamond [◊] with round dot line), Zn (open circle [○] with dash line), Fe (open triangle [Δ] with square dot line), Cu (open square [□] with long dash dot dot line) and Se (cross [×] with dash dot line) after intramammary lipopolysaccharide (LPS) infusion.

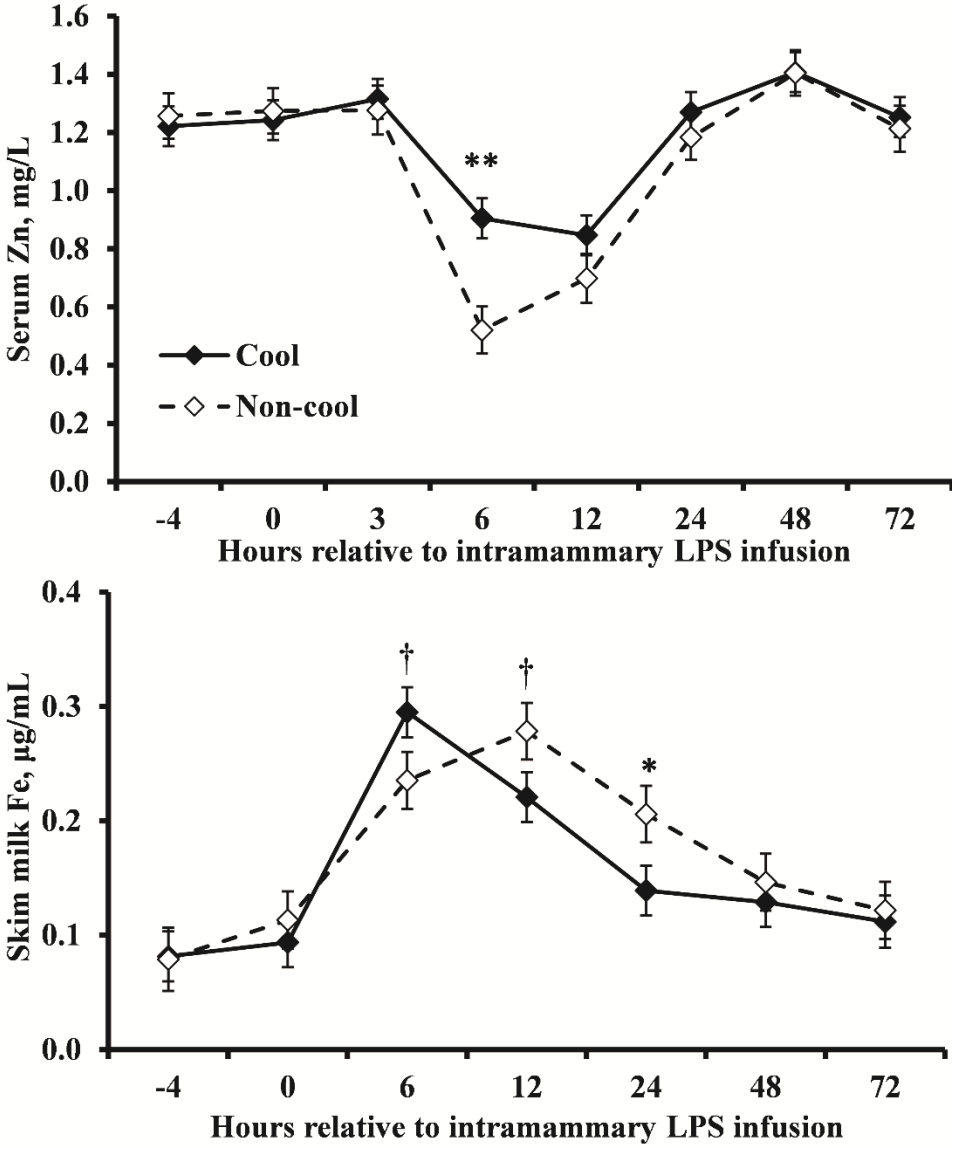

Figure 5.3: The serum concentration of Zn and skim milk concentration of Fe of cows exposed to evaporative cooling (solid diamonds [♦]) or not (open diamonds [◊]) after intramammary lipopolysaccharide (LPS) infusion. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, $\dagger P \leq 0.10$.

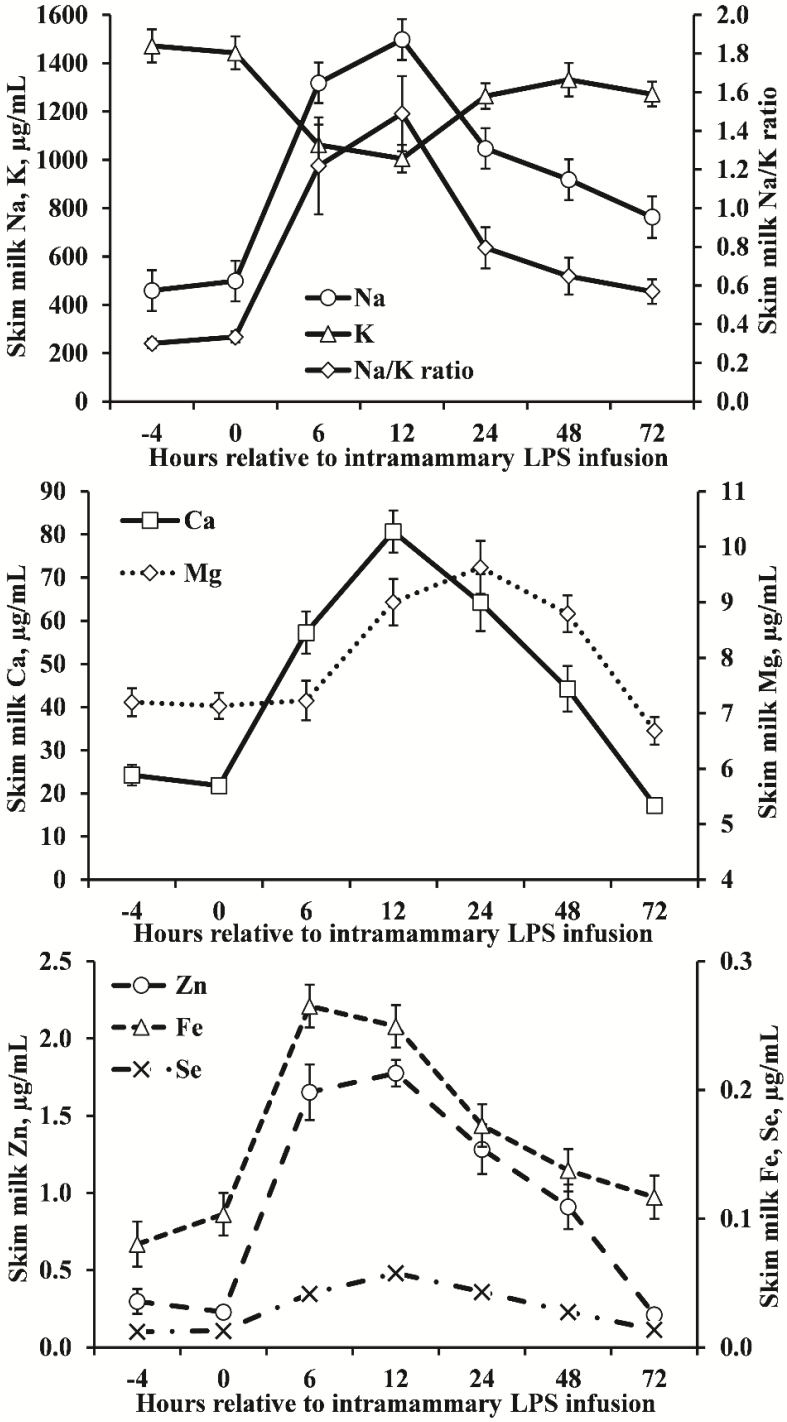

Figure 5.4: Skim milk concentration of Na (open circle [○] with solid line), K (open triangle [Δ] with solid line), Na/K ratio (open diamond [◊] with solid line), Ca (open square [□] with solid line), Mg (open diamond [◊] with round dot line), Zn (open circle [○] with dash line), Fe (open triangle [Δ] with square dot line) and Se (cross [×] with dash dot line) after intramammary lipopolysaccharide (LPS) infusion.

CHAPTER 6

IMPACT OF HEAT STRESS AND A FEED SUPPLEMENT ON HORMONAL AND INFLAMMATORY RESPONSES OF DAIRY COWS[4]

[4] Marins, T.N., J. Gao, Q. Yang, R.M. Binda, C.M.B. Pessoa, R.M. Orellana Rivas, M. Garrick, V.H.L.R. Melo, Y.C. Chen, J.K. Bernard, M. Garcia, J.D. Chapman, D.J. Kirk, and S. Tao. 2021. *Journal of Dairy Science*, 104:8276–8289. Reprinted here with permission of the publisher.

ABSTRACT

The aim of this trial was to evaluate the impact of an immunomodulatory supplement (OmniGen® AF, OG) and heat stress on hormonal, inflammatory and immunological responses of lactating dairy cows. Sixty multiparous Holstein cows were randomly assigned to 4 treatments in a 2×2 factorial arrangement using 2 environments: cooled using fans and misters, or non-cooled, and 2 top-dressed feed supplements (56 g/d): OG or a placebo (CTL). Temperature-humidity index averaged 78 during the 8 wk trial. Blood was drawn to analyze cortisol, prolactin, and circulating tumor necrosis factor (TNF)-α and interleukin-10 (IL10). Peripheral blood mononuclear cells (PBMC) were isolated and stimulated with hydrocortisone, prolactin, lipopolysaccharide (LPS) individually, or in several combinations, to assess induced proliferation and cytokine production. At d 52, 6 cows/treatment were injected i.v. with a LPS bolus (ivLPS) to assess hormone and cytokine responses. For cooled cows, feeding OG increased plasma cortisol concentration relative to CTL. Non-cooled cows fed CTL had lower circulating TNF-α concentrations than non-cooled-OG and cooled-CTL cows, with cooled-OG intermediate. Hydrocortisone+LPS stimulated PBMC from OG cows tended to proliferate more than CTL. Relative to cooled cows, PBMC from non-cooled cows produced more TNF-α and IL10 when stimulated with LPS. Following ivLPS, cooled-OG cows had a greater cortisol response than the other treatments. In conclusion, OG supplementation enhanced cortisol release under basal condition and induced inflammation with cooling compared with CTL. This suggests that heat stress inhibits OG mediated cortisol release. Heat stress seemed to enhance the inflammatory responses of PBMC from lactating cows. Yet, OG supplementation promoted PBMC proliferation under stress, or in the presence of hydrocortisone.

Key words: heat stress, immune stimulator, hormonal responses, inflammatory responses

INTRODUCTION

In dairy cattle, cortisol is a well-recognized stress hormone. In addition to the major role in lactogenesis to initiate lactation and galactopoiesis to maintain lactation (Tucker, 2000; Lacasse et al., 2019), prolactin (**PRL**) is a robust stress hormone in dairy cattle (Tucker, 1971). Both cortisol and PRL also contribute to the immune responses of the cows. Prolactin enhances proliferation and triggers pro-inflammatory responses of peripheral lymphocytes (Auchtung and Dahl, 2004; Brand et al., 2004; Díaz et al., 2013). In contrast, cortisol exerts anti-proliferative and anti-inflammatory effects on peripheral immune cells (Ashwell et al., 2000; Coutinho and Chapman, 2011). It is important to note that prolonged elevation of circulating PRL minimizes its stimulating effect on peripheral blood mononuclear cells (**PBMC**), measured as ex vivo proliferation, by reducing the expression of PRL receptor (**PRLR**) and associated PRL signaling (Auchtung et al., 2003; Auchtung and Dahl, 2004). Emerging evidence from human and rodent studies also indicate that long term exposure to stress is associated with reduced expression of glucocorticoid receptor (**GR**) and diminished anti-proliferative and anti-inflammatory effects of glucocorticoids on circulating immune cells (Silverman and Sternberg, 2012). Therefore, PRL and cortisol appear to have opposite regulatory functions on the immune responses. The secretion of PRL and cortisol, and their modulatory effects on immune cells, are likely to impact the immune and inflammatory responses of the animal during the course of disease and stress.

Both environment and nutrition affect hormonal responses in animals and alter their functional immune responses. For instance, heat stress impairs the immune function of dairy cattle (do Amaral et al., 2010, 2011), and increases both PRL and cortisol secretion (Tucker, 1971; Christison and Johnson, 1972; Tucker et al., 1991) in lactating dairy cattle as parts of the stress responses. However, it is unknown if the negative effects of heat stress on immunity are mediated

through the altered PRL or cortisol secretion and/or their immunomodulatory effects on the proliferative and inflammatory responses of immune cells. It has also been reported that feeding an immunomodulatory supplement (OmniGen® AF [**OG**], Phibro Animal Health Corp., Teaneck, NJ) to dairy cows boosts innate immune function and alters circulating cortisol concentration (McBride et al., 2016; Fabris et al., 2018; Hall et al., 2018). These studies suggest that there are reproducible effects of OG on the stress response, regulation of glucocorticoid biology, and the innate immune response in cattle. In contrast, the impact of OG supplementation on PRL secretion has not been well studied. It is unclear at this time how dietary supplementation of OG will modulate the proliferation and production of inflammatory products by circulating immune cells in the presence or absence of stress hormones ex vivo. Additionally, it is not clear if heat stress, or feeding this immunomodulatory supplement, will influence PRL and cortisol secretion leading to the associated alterations in inflammatory responses of cows in the presence of an infection. Therefore, we hypothesize that both heat stress and this immunomodulatory feed supplement (OG) will alter PRL and cortisol secretion under basal condition and during a period of acute inflammation. Further, heat stress and OG supplementation will affect the proliferation of lymphocytes and the production of inflammatory products by peripheral mononuclear cells from lactating dairy cows in the presence of PRL or cortisol ex vivo. Our primary objective was to examine the impact of deprivation of evaporative cooling, with or without supplementation with OG, on secretion of stress hormones and circulating inflammatory cytokines under basal condition and during induced acute systemic inflammation. We also addressed the effects of these in vivo conditions on ex vivo PBMC proliferation and cytokine production using cells from lactating dairy cows during summer heat stress conditions.

MATERIALS AND METHODS

The experiment was conducted at Dairy Research Center on The University of Georgia-Tifton campus during the summer of 2018. The University of Georgia Institutional Animal Care and Use Committee approved the procedures and animal handling prior to trial initiation (AUP#: A2017 10-014-Y3-A2). Sixty multiparous lactating Holstein dairy cows blocked by parity (2.5 ± 0.7) and DIM (189.3 ± 47.8 d) were randomly assigned to 1 of 4 treatments in a 2 × 2 factorial arrangement within each block. The randomization was achieved by drawing 4 balls, which represent 4 treatments, successively without replacement. Treatments included two environments: cooled (**CL**) by an evaporative cooling system or not cooled (**NC**), and two dietary supplements (56 g/d/cow, as fed basis): OG, or a biologically inert bentonite absorbent as placebo (AB20, Phibro Animal Health Corp, control, **CTL**). Although the impact of supplementing OG on milk yield was not consistently reported, two recent studies (Brandão et al., 2016; Fabris et al., 2017) reported that supplementing OG from the dry period to early lactation improved milk yield of cows under heat stress. The milk yield data from cows supplemented with OG or not from these two experiments were used to calculate power. Using a level of significance of 0.05 and 80% power, 30 experimental units per dietary treatment was calculated based on data published by Fabris et al. (2017) (40.5 vs. 35.9 kg/d for cows supplemented with OG or not, respectively, SD = 6.3 kg/d), and 25 animals per dietary treatment was calculated based on data published by Brandão et al. (2016) (30.3 vs. 27.1 kg/d for cows supplemented with OG or not, respectively, SD = 3.9 kg/d). Therefore, a sample size of 30 cows per dietary treatment was used.

Cows were housed in adjacent pens in the same barn according to their environmental treatments and managed in the same manner. The barn is an open sided free-stall barn with open ridge vent. During the entire experiment, cows were housed under the shade of the barn without

exposure to sunlight except for traveling between the barn and the milking parlor. Prior to the initiation of the environmental treatments, all cows were provided with evaporative cooling and received their respective dietary treatments for a period of 2 mo. This feeding length is recommended by the manufacturer to achieve an optimum immune response resulting from OG supplementation. The evaporative cooling system consisted of misters attached to the front face of fans over the free stalls and feed bunks. The fans were automatically switched on when ambient temperature exceeded 20 °C and provided a minimum 9.7 km/h air speed at the cow level. Continuous misters were activated when ambient relative humidity was below 85%. After the initiation of the environmental treatments, the cows continued to receive their respective dietary treatments, but the evaporative cooling was deprived in the pen housing the NC cows for 8 wk.

The air temperature and relative humidity in the barn were monitored every 15 min during the experiment by Hobo Pro Series Temp probes (Onset Computer Corporation, Pocasset, MA). The temperature-humidity index (**THI**) was calculated based on THI = (1.8×T+32) - ((0.55-0.0055×RH) × (1.8×T - 26)), where T = air temperature (°C), RH = relative humidity (%) (NRC, 1971). The vaginal temperature of all cows was measured every 5 min for 4 continuous day each week using an ibutton (Mouser Electronics, Mansfield, TX) attached to a blank controlled internal drug release device, and respiration rate was counted (1500 h) three times each week.

All cows were individually fed the same diet as a TMR once each day (1600 h). The OG or CTL supplements were mixed with 2 kg of the TMR and delivered to cows to ensure total consumption. The remaining TMR was delivered after the supplement was consumed. Individual feed intake was recorded daily using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH). The OG and CTL supplements were color coded and the treatment information were not disclosed to all research personnel until the end of the experiment. Samples of TMR and

individual ingredients were collected three times a week and DM was determined by placing samples in a forced air oven for 48 h at 55 °C. Ration ingredients were adjusted for any changes in DM content. The TMR samples were composited by month and grounded to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) for chemical analysis at Cumberland Valley Analytical Services (Waynesboro, PA). The ingredient composition and nutrient content of the diet are reported in Table 6.1 and 6.2, respectively. Cows were milked three times (0800, 1600, and 2400 h) each day with yield recorded at each milking (Delpro, DeLaval, Kansas City, MO). Milk samples were collected from 3 consecutive milkings once each week, preserved with bronopol-B-14 as a preservative, and analyzed for milk components (fat, protein, lactose, SNF, MUN, and SCC, Dairy One Cooperative, Ithaca, NY). The BW and BCS (Wildman et al., 1982) were assessed weekly.

Blood samples were collected from coccygeal vessel of the same subset of cows (n = 8/treatment) on d 1, 3, 5, 7, 15, 28, 44, and 56 of environmental treatments into sodium-heparinized vacutainers (Becton Dickinson, Franklin Lakes, NJ) and immediately put in ice. Samples were centrifuged at 1,700 × g for 30 min at 4 °C to collect plasma for PRL and cortisol analyses. Serum samples were also collected at d 1, 3, 8, 15, and 28 of the environmental treatment for cytokine analyses.

To isolate PBMC, whole blood samples (~30 mL) were collected from the same subset of cows (n = 8/treatment) into sodium-heparinized vacutainers (Becton Dickinson) at d 3, 14 and 42 and immediately transported to the lab at ambient temperature. Additional blood samples were collected into vacutainers containing K3 EDTA (Becton Dickinson) at the same time points for whole blood hematology analysis at the University of Georgia Tifton Veterinary Diagnostic Laboratory (Tifton, GA). The procedures for PBMC isolation and proliferation and cytokine

production assays followed those described in do Amaral et al. (2010) with some modifications. Briefly, PBMC were isolated and purified by density gradient centrifugation using Fico/Lite LymphoH (Atlanta Biologicals, Lawrenceville, GA). Cell viability was assessed using trypan blue and was always above 95%. Part of the isolated cells were stored in TRI-reagent solution (Sigma-Aldrich, St. Louis, MO) at -80 °C for total RNA extraction and gene expression analysis. The rest of the cells were washed in complete RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (GE Healthcare, Chicago, IL), 200 IU/mL penicillin and 0.2 mg/mL streptomycin (MP Biomedicals, Solon, OH). The concentration of PBMC was adjusted to 1×10^6 cells/mL in complete RPMI and added (100 µL/well) to a 96 well, flat-bottom sterile plate (Corning Inc., Corning, NY). Then, 100 µL of complete RPMI containing PRL purified from bovine pituitary gland (100 ng/mL, National hormone & peptide program, UCLA, Torrance, CA), hydrocortisone (0.1 µM, Sigma-Aldrich), LPS from *Escherichia coli* O111:B4 (20 µg/mL, Sigma-Aldrich), hydrocortisone and LPS, PRL and LPS, or without LPS or either hormone (as control), were added into wells in triplicate. The final concentrations of hydrocortisone (0.05 µM of hydrocortisone ~18 ng/mL of cortisol) and PRL (50 ng/mL) were within, but on the high end, of the published normal range to mimic the circulating concentrations under stress conditions (Koprowski and Tucker, 1973; do Amaral et al., 2010; Hall et al., 2018). Plates were incubated 68 h at 37°C with 5% CO_2. Then, 30 µL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, CellTiter 96@ non-radioactive cell proliferation assay, Promega, Madison, WI) was added to each well followed by an additional 4 h incubation. One-hundred microliters of stabilization solution (Promega) was added to each well followed by 1 h incubation at room temperature before reading at 570 nm on a plate reader. The PBMC proliferation was assessed as stimulation index calculated as the ratio of the mean optical density of treated wells to the mean optical density of control wells.

To examine the cytokine production, PBMC concentration was adjusted to 2×10^6 cells/mL in complete RPMI, and 1 mL/well PBMC suspension was added to a 6 well, flat-bottom sterile plate (Corning Inc.). One milliliter of complete RPMI containing LPS (20 μg/mL), hydrocortisone (0.1 μM) with LPS, or PRL (100 ng/mL) with LPS were added into wells in triplicate, and cultured at 37 °C in 5% CO_2 for 48 h. Following incubation, plates were centrifuged for 2 min to collect supernatant for cytokine analyses. Culture supernatants were stored at -80ºC until assessed using commercial ELISA assays in a batch.

Total RNA was extracted from PBMC using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) with an on-column DNase (PureLink™ DNase Set, Invitrogen). Following extraction, the second DNase treatment was performed on the RNA extract using a Turbo DNA-free kit (Invitrogen) according to manufacturer's instructions. A High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) was used for cDNA synthesis. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) and have PCR efficiencies above 90% (Table 6.3). All primers were blasted against the bovine genome to ensure primers were aligned only on the target genes. The qRT-PCR was performed on a SteponePlus Real-Time PCR System (Applied Biosystems) using the Power SYBR green PCR Master Mix (Applied Biosystems) and cDNA from 10 ng of purified RNA per reaction under following conditions: 95 °C for 10 min for initial denaturation and enzyme activation, 40 cycles of 95 °C for 15 seconds and 57 °C for 1 min for amplification. For each reaction, a melt curve was generated to ensure a single product. The *YWHAZ* was used as a housekeeping gene and target genes included prolactin receptor (*PRLR*), glucocorticoid receptor (*NR3C1*), interleukin-10 (*IL10*), and tumor necrosis factor (*TNF*) (Table 6.3). In all plates, "no template controls" were included to ensure reactions were free of contamination, primer dimers and misprimed products. The relative

gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle. The same pool sample was included in all plates and used as the calibrator sample.

To test the physiological and inflammatory responses of cows to an in vivo immune challenge, a bolus of LPS was intravenously infused to a subset of cows (n = 24, 6 per treatment) on d 52-54 of the experiment. An indwelling jugular vein catheter was inserted 2 d before the challenge as previously described (Tao et al., 2012). Three hours after the morning milking (~ 1100 h), a LPS bolus (0.20 μg/kg of BW, prepared from *Escherichia coli* O111:B4, Sigma-Aldrich), dissolved in isotonic saline solution, was infused intravenously into the jugular vein through the catheter followed by 10 mL of sterile saline solution to flush the catheter. Blood samples were collected through the catheter at -1, -0.5, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h relative to LPS infusion to harvest plasma and serum. The catheter was flushed with 3 mL sterile saline containing sodium heparin between samplings to avoid clotting, and the first 2-3 mL of blood collection was discarded before each subsequent sample. Milk samples were collected from 3 milkings before and 6 milkings after the LPS administration for milk composition analyses, and milk yield was recorded. Daily DMI were recorded from d -1 to 2 relative to LPS infusion. The vaginal temperature was monitored every 5 min from d -1 to 2 relative to LPS infusion, and respiration rates were counted at -1, -0.5, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h relative to LPS infusion. During and after the intravenous infusion of the LPS bolus, all cows remained in their respective pens.

The concentrations of PRL (ELISA Kit for Prolactin SEA846Bo, Cloud-Clone Corp., Katy, TX), cortisol (Cortisol EIA kit, Arbor Assay, Ann Arbor, MI), tumor necrosis factor-α (**TNF-α**, Bovine TNF-α DIY ELISA, Kingfisher Biotech, Inc., Saint Paul, MN), and interleukin 10 (**IL10**, Bovine IL-10 DIY ELISA, Kingfisher Biotech, Inc.) of the plasma, serum or culture

supernatant were measured using commercially available kits following the manufacturers' procedures. All kits were validated for bovine. The intra- and inter-assay CV were 5.5 and 15.1% for IL10 assay, 3.7 and 13.0% for TNF-α assay, 4.9 and 8.1% for cortisol assay, and 3.6 and 16.7% for PRL assay, respectively. The same block of cows, where all treatments were represented, was always placed in the same 96 well plate.

Calculation and Statistical Analyses

The SAS 9.4 (SAS Institute, Cary, NC) was used to perform all statistical analyses. The UNIVARIATE procedure was used to calculate parity and days after parturition, and their means ± standard deviation are reported. Repeated measure data, including DMI, milk yield and composition, plasma concentrations of hormones, serum concentrations of cytokines, and stimulation index and cytokine production of PBMC after in vitro culture were analyzed using the MIXED procedure. The statistical model included fixed effects of block, diet, environment, time and their interactions, and cow nested within treatment (diet × environment) was the random effect. Separated analyses were performed to examine the hormonal effect alone on PBMC proliferation and cytokine production stimulated by LPS regardless of dietary or environmental treatments using PROC MIXED procedure. The statistical model include the main effects of block and hormone (no hormone vs. PRL vs. hydrocortisone) and cow, diet, environment and diet by environment interaction were treated as the random variables. The least squares means ± standard error of the means were reported.

For data collected during intravenous LPS infusion, the repeated measures were analyzed using the MIXED procedure with a similar statistical model mentioned above. The increments of hormones and cytokines were calculated by subtracting the average value before infusion from the maximal value after infusion. The area under curve (**AUC**) between times was calculated based

on the trapezoidal method, in which the value of hormone and cytokine concentration was calculated by subtracting the average value before infusion from the actual value. The accumulated AUC of hormones and cytokines were calculated from 0-3 h, 0-6 h and 0-24 h as proxy for hormone and cytokine responses to acute inflammation. The increment and accumulated AUC were analyzed using the MIXED procedure. The statistical model included fixed effects of block, diet, environment, and diet by environment interaction, and cow nested within treatment (diet × environment) was the random effect. When the interaction between diet and environment was significant or tended to be significant, the PDIFF option requesting pairwise differences adjusted with Tukey method was used for mean separation. The least squares means ± standard error of the means were reported. Significance and tendency were declared when $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

RESULTS

During the experiment, ambient temperature, relative humidity and THI averaged 26.8 ± 3.4 °C, 85.4 ± 13.9%, and 78.1 ± 3.8 in the pen where NC cows were housed, and 26.5 ± 3.1 °C, 85.9 ± 12.9%, and 77.8 ± 3.5 in the pen for CL cows. These data indicate that all cows were exposed to similar degree of heat stress. Compared with CL cows, NC cows had higher ($P < 0.01$) vaginal temperature and respiration rate (Table 6.4). Dietary treatments did not affect the vaginal temperature, but supplementation of OG slightly reduced (4 breath/min, $P < 0.05$) the respiration rate compared with CTL for CL cows (diet × environment: $P = 0.07$, Table 6.4). Neither dietary nor environmental treatment affected BW, but cows supplemented with OG maintained a greater (2.81 vs. 2.68, SEM = 0.04, respectively; $P = 0.03$) BCS than those fed CTL (Table 6.4).

Regardless of the dietary treatment, deprivation of evaporative cooling reduced ($P < 0.01$) DMI and milk yield, but did not ($P > 0.20$) affect milk concentrations of lactose, protein, fat, SNF, and SCC (Table 6.4). Due to the lower milk yield, NC cows had lower ($P \leq 0.02$) yield of milk fat, protein, lactose and SNF than CL cows (Table 6.4). Compared with CL cows, NC cows had greater ($P = 0.03$) MUN concentration (12.0 vs. 13.1 mg/dL, SEM = 0.3 mg/dL, respectively; Table 6.4). Neither dietary treatment nor diet by environment interaction affected ($P > 0.15$) DMI, milk yield, or yield and concentration of milk components, except cows fed OG tended ($P = 0.07$) to have greater milk protein concentrations (2.81 vs. 2.73%, SEM = 0.03%, respectively) compared with those supplemented with CTL (Table 6.4).

Diet, environment and their interaction had no ($P > 0.15$) effect on circulating PRL and IL10 concentrations. An interaction of diet and environment ($P = 0.02$) was observed for plasma cortisol concentration. This is because, for CL cows, those fed OG had greater ($P = 0.04$) plasma cortisol concentration than CTL. Plasma cortisol concentration for NC cows fed CTL was similar to NC cows fed OG, but was greater ($P \leq 0.04$) than CL cows fed either OG or CTL (Table 6.4). Additionally, NC cows had greater ($P < 0.01$) plasma cortisol concentrations at 1 and 5 d of the environmental treatment relative to CL cows (environment × time: $P < 0.01$, Figure 6.1). A tendency for a diet by environment interaction ($P = 0.07$) was observed for serum concentration of TNF-α. This was because NC cows supplemented with CTL tended to have lower ($P \leq 0.06$) serum TNF-α concentration than NC-OG and CL-CTL cows, while CL cows fed OG tended to have intermediate concentrations (Table 6.4).

Blood concentrations of white blood cells, lymphocytes or monocytes were not affected by environment, diet or their interactions ($P \geq 0.22$, Table 6.5). A diet by environment interaction ($P = 0.04$) was observed for blood neutrophil concentration, because CL cows fed CTL had the

highest ($P < 0.02$) concentration compared with other treatments (Table 6.5). Regardless of the environmental and dietary treatment, addition of hydrocortisone to the cell culture reduced ($P < 0.01$) LPS stimulated PBMC proliferation and production of TNF-α but did not ($P = 0.16$) affect IL10 production (Figure 6.2). Addition of PRL did not ($P \geq 0.25$) affect cell proliferation or TNF-α production, but reduced ($P = 0.01$) IL10 production of PBMC stimulated by LPS (Figure 6.2). When stimulated by LPS alone, NC cows fed OG tended to have a higher ($P = 0.09$) level of PBMC proliferation than CL cows fed OG. Neither was different from CTL cows under either CL or NC (diet × environment interaction: $P = 0.10$; Table 6.6). It is notable that when incubated with LPS and hydrocortisone, PBMC isolated from cows supplemented with OG tended ($P = 0.09$) to have a stronger proliferative response compared with CTL without regard to the environment (Table 6.6). Diet, or diet by environment interaction, had no ($P > 0.35$) impact on cytokine production by PBMC stimulated by LPS, or LPS with either hormone (Table 6.6). When stimulated by LPS, with or without PRL, PBMC isolated from NC cows produced more ($P \leq 0.01$) TNF-α compared with CL cows without regard to the dietary treatments. However, addition of hydrocortisone suppressed TNF-α production to a similar amount across all treatments ($P \geq 0.16$). The PBMC isolated from NC cows incubated with LPS alone tended ($P = 0.09$) to produce more IL10 than PBMC from CL cows. However, when PRL or hydrocortisone were added to the cell culture, the production of IL10 was reduced to a similar amount for all treatments ($P \geq 0.12$, Table 6.6). Diet, environment, or their interaction did not ($P \geq 0.20$) affect the mRNA expression of genes examined in the current experiment (Table 6.7).

Regardless of treatments, intravenous infusion of a LPS bolus reduced DMI, milk yield, and milk concentration of lactose (Time effect: $P < 0.01$; Table 6.8, Figure 6.3). However, infusion of the LPS bolus increased concentrations of milk protein, MUN, and somatic cell count (Time

effect: $P < 0.01$; Table 6.8, Figure 6.3). Milk fat concentration was initially reduced and then increased following infusion of the LPS bolus (Time effect: $P < 0.01$; Figure 6.3). There were no ($P > 0.30$; Table 6.8) treatment by time interactions for DMI or milk yield and composition. These suggested that the experimental treatments did not affect production following acute systemic inflammation. After the infusion of the LPS bolus, vaginal temperature increased sharply, then decreased below the temperature prior to infusion (Time effect: $P < 0.01$; Figure 6.4). Compared with CL cows, NC cows had similar peak vaginal temperature, but vaginal temperature remained higher throughout the assessment period (environment × time: $P < 0.01$; Figure 6.4).

Circulating cortisol, PRL, TNF-α, and IL10 concentrations increased significantly following LPS infusion. Peak concentrations of PRL and cytokines occurred 1 h after infusion, but the peak of cortisol concentration occurred at 3 h after infusion (Time effect: $P < 0.01$; Figure 6.5). There was a tendency ($P = 0.07$) for a diet by environment interaction in cortisol production (as AUC) from 0-3 h following LPS infusion. This is because CL cows fed OG had the greatest AUC among the treatments (Table 6.9). Diet and environment interactions ($P \leqslant 0.04$) were also observed for PRL increment, and AUC from 0-3 and 0-6 h following infusion. Cooled cows fed CTL had higher values compared with NC cows fed CTL, and neither of them were different from the other two treatments (Table 6.9). Treatment did not influence TNF-α response to LPS infusion; however, cows supplemented with OG tended ($P = 0.09$) to have greater IL10 AUC from 0-3 h following infusion of the LPS bolus compared with those fed CTL (Table 6.9).

DISCUSSION

In this experiment, daily THI averaged 78 which is above the THI threshold (68) for reduced milk yield by heat stress in lactating dairy cows (Zimbelman et al., 2009). The reduced

DMI and higher vaginal temperature and respiration rate indicate that NC cows had greater heat load compared with CL cows. Thus, the environmental treatment was effective in representing heat stress responses, and a suitable experimental model for studying the impact of heat stress on lactating dairy cows. Although feeding OG slightly reduced respiration rate in CL cows, diet had no impact on vaginal temperature. This indicates that OG supplementation did not affect the primary physiological response to the heat load carried by cows.

Consistent with previous studies (Weng et al., 2018; Safa et al., 2019), deprivation of evaporative cooling dramatically reduced milk yield indicating impaired lactation performance by heat stress. Compared with CL cows, NC cows had similar concentrations of milk fat and protein. Previous studies suggested that heat stress increases, does not change, or decreases milk fat and protein concentrations in lactating dairy cows (Knapp and Grummer, 1991; Garner et al., 2016; Weng et al., 2018). These conflicting results are explained in part by the use of different experimental models, differences in dietary compositions, cows being in different stages of lactation, the severity and degree of heat stress exposure or the efficacy of the cooling process, and the duration of the experiment (Tao et al., 2018). Deprivation of evaporative cooling increased MUN concentration. This suggests an alteration in ruminal nitrogen metabolism and/or an increased hepatic AA deamination mediated by heat stress (Ríus, 2019). In this experiment, the dietary treatment had no effect on production performance of cows. Similarly, previous studies reported that supplemental OG during lactation did not affect milk yield or composition in dairy cows housed under either thermal neutral or heat stress conditions (Leiva et al., 2017; Hall et al., 2018; Marins et al., 2019). In contrast, Brandão et al. (2016) and Fabris et al. (2017) reported higher milk yield when OG was supplemented during the dry and early lactation periods under heat stress conditions.

In this experiment, acute, but not prolonged, exposure to heat stress increases cortisol secretion, consistent with others (Christison and Johnson, 1972; Alvarez and Johnson, 1973; Hall et al., 2018) who reported that acute heat stress stimulated cortisol release, but that chronic heat stress either decreased, or had no impact on, circulating cortisol concentration in dairy cattle. Because heat stress does not affect the cortisol clearance rate (Christison and Johnson, 1972), these data suggest that chronic heat stress suppressed cortisol release. The impact of OG supplementation on cortisol release has been variable across reported studies. During lactation, circulating cortisol concentration was either reduced or unchanged, in cows supplemented with OG relative to controls under both thermal neutral and heat stress conditions (McBride et al., 2016; Leiva et al., 2017; Hall et al., 2018). In contrast, Fabris et al. (2018) reported that cows fed OG during the dry period had greater plasma cortisol concentration compared with those without supplementation during summer. In our current experiment, supplemental OG increased plasma cortisol concentrations only in CL cows. This suggests that the greater heat load with NC cows may diminish the impact of OG on cortisol secretion.

Prolactin secretion is sensitive to environmental cues. It is impacted by heat stress, day length and milking in dairy cattle (Tucker, 1971; Auchtung et al., 2005; Tucker et al., 1991). Increasing ambient temperature decreases dopaminergic neuron activity, which in turn increases pituitary PRL secretion (Tucker et al., 1991; do Amaral et al., 2010). In this current experiment, PRL concentration for NC cows was numerically greater than CL cows but not statistically different. Among all treatment groups, CTL-NC cows had the lowest basal serum TNF-α concentration. This, coupled with similar serum IL10 concentrations among the treatments, indicates that deprivation of evaporative cooling might reduce basal inflammation in CTL cows, and suggests that feeding OG might maintain the inflammation status of the cows in the face of

increased heat load. Similarly, it has been reported that there was a lower circulating TNF-α concentration in early lactating dairy cows without evaporative cooling than in cooled cows (Safa et al., 2019). In early lactation, infusion of recombinant bovine TNF-α reduces feed intake and milk yield of dairy cows (Yuan et al., 2013). In contrast, administration of nonsteroidal anti-inflammatory drug after calving increases subsequent milk yield (Carpenter et al., 2016). These data suggest that upregulated inflammation in early lactation is associated with reduced milk production. However, heat stress decreases milk yield (Tao et al., 2020) and supplementation of OG reduces mortality and morbidity rates without negative effect on milk production (Chapman et al., 2016). Therefore, the significance of altered basal inflammation as measured by circulating TNF-α is not clear. Additionally, because we only measured two inflammatory cytokines in this experiment, a more comprehensive examination into the inflammatory status of lactating cows exposed to heat stress and fed OG is required.

Following intravenous LPS administration, all cows reached a similar peak (40.6 °C) of vaginal temperature around 4 h after infusion of the LPS bolus. This indicated similar fever development in response to LPS. A reduced vaginal temperature following LPS induced fever response may be the result of reduced heat production mediated by reduced DMI and milk yield. The fact that no environmental effects were observed for TNF-α and IL10 production (as AUC) following infusion of the LPS bolus suggests that heat stress had no specific impact on the inflammatory response. However, for CL cows, supplementation with OG induced a stronger cortisol response to LPS than was observed in CTL. This was indicated by a larger cortisol AUC from 0-3 h after infusion of the LPS bolus. No similar dietary effect was observed for NC cows. This may suggest that heat stress diminished the effect of OG supplementation on cortisol release. It is worth noting that deprivation of evaporative cooling reduced PRL responses to intravenous

infusion of the LPS bolus in cows fed CTL, but not supplemented with OG. This suggests that heat stress may reduce PRL responses to intravenous infusion of the LPS bolus, but that supplementation with OG maintains the PRL responses during acute systemic inflammation under heat stress condition. However, there was no apparent association between the altered PRL responses and any of the cytokine responses after LPS infusion. The biological significance of the altered PRL response under heat stress and with OG supplementation during inflammation, or diseases, is not yet clear. This relationship deserves further investigation.

Without regard to treatment, cell proliferation and TNF-α production by PBMC in response to LPS were inhibited by addition of hydrocortisone in cell culture. This confirmed the immune inhibitory role of glucocorticoid (Coutinho and Chapman, 2011). Supplementation with PRL in the culture system did not influence PBMC proliferation or production of TNF-α, but did inhibit IL10 production. This suggests that PRL might affect some inflammatory responses of PBMC, possibly by inhibiting the production of anti-inflammatory cytokines in dairy cattle. In contrast, others (Auchtung and Dahl, 2004) reported increased PBMC proliferation when PRL was added to an in vitro culture model. One difference compared with the current experiment, which was conducted using lactating cows and added purified PRL from bovine pituriary to the culture system at a physiologically relavant concentration (50 ng/mL), Auchtung and Dahl (2004) used recombinant methionyl bovine PRL at a supraphysiological concentration (1 μg/mL) in their cultures of PBMC collected from dairy steers.

Heat stress has been shown to influence immune cell function of dairy cows (Dahl et al., 2020). Under in vitro conditions, PBMC isolated from thermal neutral dairy cows have lower mitogen-induced proliferative capacity when culture temperatures exceed 41°C (Lacetera et al., 2006). In contrast, in the current experiment, no difference was observed in PBMC proliferation

between the environmental treatments employed. The inconsistent PBMC proliferative responses under in vitro and in vivo heat stress are also observed in other species. Exposure PBMC collected from healthy humans, to elevated ambient temperature (40 or 38.5 vs. 37 °C) in vitro after mitogen stimulation, increased cell proliferation (Roberts and Steigbigel, 1977; Smith et al., 1978). When whole body hyperthermia was induced in healthy humans by immersion in a water bath, the proliferative response of isolated PBMC to mitogen stimulation was not altered (Kappal et al., 1991). Of note, PBMC isolated from NC cows had greater production of both pro- (TNF-α) and anti-inflammatory (IL10) cytokines after LPS stimulation compared with CL cows in our experiment. Similarly, in mice, peritoneal macrophages isolated from heat-stressed animals produced more TNF-α after in vitro LPS stimulation compared with mice under thermal neutrality (Lee et al., 2012). These results suggest that heat stress enhances the inflammatory responses of mononuclear cells of dairy cows in vitro, which, however, were lost in the presence of hydrocortisone. It is important to note that the basal cytokine production by cultured PBMC without LPS stimulation was not measured in this current experiment. The similar gene expression of TNF-α and IL10 of PBMC may indicate similar basal cytokine production by PBMC among treatments. However, the possibility that the TNF-α and IL10 secretion by PBMC into the culture media without mitogen stimulation is affected by diets or environment cannot be ignored.

No impact of the dietary treatments was observed for PBMC cytokine production. This suggests that OG supplementation did not affect the inflammatory response of blood mononuclear cells isolated from lactating dairy cows. However, it is important to note that only one pro-inflammatory cytokine (TNF-a) was measured in this experiment, and the possibility that OG affects the secretion of other pro-inflammatory cytokines cannot be ruled out. Nevertheless, OG supplementation increased cell proliferation to LPS in PBMC isolated from NC cows, and PBMC

isolated from cows fed OG displayed more proliferation in the presence of hydrocortisone compared with cows fed CTL under either environmental treatment. These data suggests that OG supplementation improved immune function during stress conditions, especially when cortisol secretion was increased. Similarly, during immune supression induced by dexamethasone, neutrophils collected from sheep fed OG had higher L-selectin expression, and improved phagocytic activity and oxidative burst than those without OG supplementation (Wang et al., 2007; Garcia et al., 2020). The cellular mechanisms responsible for these phenonmenon are not yet understood, but do not appear to be due to altered glucocorticoid signaling. This is because similar gene expression of GR in PBMC was measured for both dietary treatments. A previous experiment, conducted in mice, reported that improved immune cell function in OG supplementated animals, under a dexamethasone induced immune supression protocol, was mediated through the TLR-MyD88 pathway (Ortiz-Marty et al., 2017).

Previous studies have reported that heat stress during the prepartum period reduced *PRLR* gene expression in PBMC (do Amaral et al., 2010). Preisler et al. (2000a, b) also reported that stress associated with parturition reduced GR expression on blood immune cells during early lactation. The reduced expression of hormone receptors might dampen the immune-modulatory function of PRL and cortisol on immune cells. In the current experiment, neither the environment nor dietary treatment affected the gene expression of GR and PRLR genes in PBMC, despite that plasma cortisol concentrations were affected by the interaction between diet and environment. Similarly, Bubolz (2010) reported that heat stress elevated circulating PRL but did not alter gene or protein expression of PRLR in PBMC relative to thermal neutral dairy heifers. Our results support that heat stress and OG supplementation does not affect PBMC function by altering expression of hormone receptors. In this experiment, addition of hydrocortisone to PBMC culture

suppressed cytokine production to similar but low levels among all treatments. This suggests an overwhelming anti-inflammatory effect of glucocorticoid under any set of environment or diet employed. It is important to note that the selected hormone concentrations in the cell culture are similar to or higher than the plasma concentrations observed in stressed dairy cattle. It is possible that heat stress or OG supplementation alters the inflammatory responses of PBMC to an in vitro LPS stimulation differently if lower hormone concentrations were employed in the cell culture. This requires additional investigation.

CONCLUSIONS AND IMPLICATIONS

Environment, nutrition, hormones and immunity are interrelated. In the current experiment, we confirmed that acute heat stress increased cortisol release, and revealed that blood mononuclear cells, isolated from heat-stressed cows, produce more pro- and anti-inflammatory cytokines after LPS stimulation in vitro. These results indicated that heat stress enhanced the inflammatory responses of circulating immune cells from lactating dairy cows, but does not alter PBMC proliferation. In contrast, this enhanced inflammatory response was not observed in NC cows under basal condition, or during acute systemic inflammation induced by intravenous LPS infusion, compared with CL cows. Indeed, the data collected in this experiment indicated that heat stress reduced the basal level of inflammation, by reducing the level of circulating pro-inflammatory cytokine (e.g. TNF-α) concentration. These data suggested that immune cells from heat-stressed cows may mount a different set of inflammatory responses to mitogens under in vitro than in vivo conditions. This is possibly due to the presence of anti-inflammatory mediators (including cortisol) in the blood.

Previous research has shown that OG boost immune function of dairy cattle. In this experiment, we demonstrated that OG supplementation improved cortisol release in cows exposed to evaporative cooling under basal condition (without infusion of the LPS bolus), and during acute inflammation stimulated by intravenous infusion of the LPS bolus. However, these effects were not observed in NC cows. These data suggest that the stimulating effect of OG on cortisol release is diminished by severe heat stress. It is notable that supplementation with OG resulted in stronger proliferative responses by PBMC collected from NC cows than CL cows, or when in the presence of hydrocortisone regardless of the environmental treatment. This further documents the benefits of OG on immunity in dairy cattle during periods of stress. The greater IL10 response of cows fed OG compared with CTL during acute systemic inflammation is also of interest. This, coupled with an increased cortisol response, seems to suggest an enhanced capacity for inflammatory resolution that followed intravenous LPS infusion in OG fed cows. More research is required to verify the impact of OG supplementation on both the immune defense during other stressful events and its potential role in disease recovery (e.g., following a bacteria challenge or infection).

ACKNOWLEDGEMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection. Authors also thank Dr. David J. Hurley for his comments and review of the manuscript. This project was supported by Phibro Animal Health Corp. (Teaneck, NJ) and the National Institute of Food and Agriculture (Washington, DC) Hatch project (GEO00916).

DECLARATION OF INTEREST

Miriam Garcia, James D. Chapman, and David J. Kirk are employees of Phibro Animal Health Corp. The remaining authors are associated with the University of Georgia and declare no conflicts of interests.

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Table 6.1: Ingredient composition of the experimental diet

Ingredient	**% of DM**
Corn silage	29.38
Oat silage	10.68
Brewers grains, wet	10.68
Ground corn	9.35
Molasses	4.90
Corn Gluten Feed	5.34
Soybean hulls	7.12
Citrus pulp	7.57
Energy booster 100[1]	1.78
Soybean meal	5.79
Amino plus[2]	1.78
Prolak[3]	2.40
Salt	0.36
Calcium carbonate	0.89
Sodium sesquinate	0.80
Magnesium oxide	0.45
Rumensin[4]	0.26
DCAD plus[5]	0.22
Trace mineral-vitamin premix[6]	0.25

[1]Hydrogenatted fatty acid supplement, MilkSepcialties Global Inc., Eden Prairie, MN.
[2]Expeller soybean meal, Ag Processing Inc., Omaha, NE.
[3]Marine and animal RUP supplement, H. J. Baker & Bros. Inc., Westport, CT.
[4]3% Rumensin mix, Elanco Animal Health, Indianapolis, IN.
[5]Potassium carbonate, Arm Hammer Animal Nutrition, Church & Dwight Co., Inc. Princeton, NY
[6]Mineral-vitamin premix contained (DM basis): 29.5% Ca; 0.06% P, 0.42% Mg; 0.31% S; 377 ppm Co; 3,472 ppm Cu; 530 ppm Fe; 388 ppm I; 23,882 ppm Mn; 110 ppm Se; 13,313 ppm Zn; 1,221,966 IU/kg Vitamin A; 129,456 IU/kg Vitamin D; 2,817 IU/kg Vitamin E

Table 6.2: Chemical composition of the experimental diet.

	DM basis
CP, %	15.1 ± 1.3[1]
SP[2], % of CP	31.7 ± 4.1
aNDFom[3], %	32.9 ± 0.3
ADF, %	21.9 ± 1.3
Starch, %	19.2 ± 1.8
Sugar, %	3.7 ± 0.4
EE[4], %	4.6 ± 0.3
NFC, %	38.4 ± 1.5
Ash, %	10.7 ± 0.7
NE_L, Mcal/kg	1.64 ± 0.02
Calcium, %	1.0 ± 0.3
Phosphorus, %	0.5 ± 0.0
Magnesium, %	0.4 ± 0.1
Potassium, %	1.7 ± 0.2
Sodium, %	0.5 ± 0.1
Manganese, mg/kg	102.4 ± 12.6
Zinc, mg/kg	75.2 ± 7.5
Copper, mg/kg	18.2 ± 1.6
Iron, mg/kg	846.8 ± 83.9

[1]Mean ± Standard Deviation
[2]SP = Soluble protein
[3]aNDFom = ash free aNDF
[4]EE = Ether extract

Table 6.3: Names, symbols, GenBank accession numbers, primer sequences, amplicon sizes and primer efficiency of genes examined.

Protein name	Gene symbol	GenBank accession#	Primer[1]	Sequence (5' - 3')	Amplicon size, bp	Efficiency, %
Prolactin receptor	*PRLR*	NM_001039726	F	AGAGAGCTCCATCCAGATAC	109	94.4
			R	CACTGCCCAGACCATAATC		
Glucocorticoid receptor	*NR3C1*	NM_001206634	F	ACCTTACTGCTCCTCTCTTC	125	93.4
			R	CTGGAGTTTCCTTCCCTCT		
Interleukin-10	*IL10*	NM_174088	F	CCTTGTCGGAAATGATCCAG	148	91.5
			R	GGCAGAAAGCGATGACAG		
Tumor necrosis factor	*TNF*	NM_173966	F	ACACTCAGGTCCTCTTCTC	142	91.0
			R	GGTTGTCTTCCAGCTTCAC		
14-3-3 protein zeta/delta	*YWHAZ*	NM_174814	F	CTGGTACAGAAGGCCAAAC	127	90.7
			R	ACGGGCTCCTACAACAT		

1F = forward; R = reverse

Table 6.4: Vaginal temperature (VT), respiration rate (RR), BW, BCS, DMI, milk yield, milk component concentration and yield, plasma cortisol and prolactin, and serum TNF-α and IL10 concentrations of cows fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Parameter	CL		NC			P-value		
	OG	CTL	OG	CTL	SEM	D[1]	E	D×E
VT, °C	38.99	38.97	39.98	40.06	0.06	0.66	< 0.01	0.41
RR, breath/min	62.02[a]	66.13[b]	84.88[c]	83.73[c]	1.42	0.30	< 0.01	0.07
BW, kg	678.8	673.5	661.4	654.6	17.9	0.73	0.31	0.96
BCS	2.83	2.71	2.80	2.65	0.06	0.03	0.42	0.74
DMI, kg/d	29.03	28.51	24.22	23.92	0.57	0.47	< 0.01	0.84
Milk yield, kg/d	36.96	37.91	29.52	31.27	1.02	0.19	< 0.01	0.70
3.5% FCM[2], kg/d	37.04	38.23	31.30	32.65	1.32	0.28	< 0.01	0.86
ECM[3], kg/d	36.2	37.76	30.85	32.12	1.18	0.31	< 0.01	0.95
Milk composition								
Lactose, %	4.79	4.76	4.69	4.69	0.08	0.81	0.28	0.85
Lactose, kg/d	1.75	1.78	1.39	1.47	0.06	0.38	< 0.01	0.69
Protein, %	2.80	2.78	2.83	2.68	0.05	0.07	0.48	0.16
Protein, kg/d	1.01	1.02	0.83	0.83	0.03	0.88	< 0.01	0.73
Fat, %	3.53	3.63	3.76	3.78	0.16	0.71	0.25	0.80
Fat, kg/d	1.25	1.33	1.10	1.15	0.07	0.34	0.02	0.90
SNF, %	8.52	8.51	8.51	8.36	0.07	0.28	0.29	0.36
SNF, kg/d	3.10	3.15	2.52	2.61	0.09	0.44	< 0.01	0.84
MUN, mg/dL	12.25	11.81	13.12	13.00	0.46	0.55	0.03	0.73
Ln(SCC, 10^3cells/mL)	4.67	4.20	4.26	4.30	0.29	0.47	0.60	0.38
Cortisol, ng/mL	14.24[b]	11.83[c]	15.20[ab]	17.14[a]	0.88	0.59	< 0.01	0.02
Prolactin, ng/mL	27.77	25.41	37.08	29.55	7.30	0.52	0.38	0.76
TNF-α, ng/mL	1.06[xy]	1.36[x]	1.48[x]	0.44[y]	0.37	0.27	0.40	0.07
IL10, pg/mL	343.8	338.0	1019.4	474.1	273.3	0.41	0.18	0.42

[1]D = Diet; E = Environment

[2]3.5% FCM = (0.4324 × kg of milk yield) + (16.216 × kg of milk fat yield).

[3]ECM = (0.327 × kg of milk yield) + (12.95 × kg of milk fat yield) + (7.65 × kg of milk protein yield)

[abc]Means within the same row with different superscripts differ ($P \leq 0.05$)

[xy]Means within the same row with different superscripts tend to differ ($P \leq 0.10$)

Table 6.5: Hematological profile of cows fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Cell type	CL		NC			*P*-value		
	OG	CTL	OG	CTL	SEM	D[1]	E	D×E
White blood cells	8.67	10.45	9.81	9.27	1.07	0.56	0.98	0.29
Lymphocytes	5.20	6.16	6.42	6.02	0.95	0.77	0.57	0.48
Neutrophils	2.63[b]	3.42[a]	2.57[b]	2.36[b]	0.23	0.22	0.02	0.04
Monocytes	0.38	0.40	0.36	0.42	0.03	0.22	0.94	0.48

[1]D = Diet; E = Environment

*ab*Means within a row with different superscripts differ ($P \leq 0.05$)

Table 6.6: Cell proliferation and cytokine production of peripheral blood mononuclear cells isolated from cows fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Parameter	CL		NC			*P*-value		
	OG	CTL	OG	CTL	SEM	D[1]	E	D×E
Proliferation, stimulation index								
LPS	1.58^{x}	2.28^{xy}	2.69^{y}	1.85^{xy}	0.45	0.93	0.46	0.10
LPS + HDC[2]	1.04	0.96	1.42	0.99	0.14	0.09	0.17	0.24
LPS + PRL[3]	1.52	1.96	2.68	1.72	0.41	0.65	0.31	0.11
TNF-α, ng/mL								
LPS	0.38	0.33	0.50	0.57	0.06	0.96	0.01	0.36
LPS + HDC	0.15	0.14	0.18	0.16	0.02	0.54	0.16	0.86
LPS + PRL	0.40	0.36	0.59	0.63	0.06	0.99	< 0.01	0.49
IL10, pg/mL								
LPS	95.3	106.0	145.6	139.3	24.0	0.90	0.09	0.71
LPS + HDC	87.8	82.3	127.0	107.1	19.6	0.53	0.12	0.75
LPS + PRL	81.9	68.4	98.1	94.4	21.1	0.69	0.34	0.80

[1]D = Diet; E = Environment

[2]HDC = Hydrocortisone

[2]PRL = Prolactin

[xy]Means within the same row with differ superscripts tend to differ ($P \leq 0.10$).

Table 6.7: Relative expression of genes of peripheral blood mononuclear cells isolated from cows fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Gene	CL		NC			*P*-value		
	OG	CTL	OG	CTL	SEM	D[1]	E	D×E
NR3C1	2.23	1.60	1.95	1.88	0.48	0.48	0.97	0.57
PRLR	0.74	0.73	1.12	0.80	0.20	0.47	0.29	0.50
TNF	1.77	1.29	0.65	1.34	0.55	0.64	0.28	0.24
IL10	7.70	23.68	11.29	10.88	8.32	0.39	0.75	0.36

[1]D = Diet; E = Environment

Table 6.8: Vaginal temperature (VT), respiration rate (RR), DMI, milk yield and composition of cows given an intravenous LPS bolus. Cows were fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Parameter	CL		NC			*P*-value						
	OG	CTL	OG	CTL	SEM	D[1]	E	T	D×E	D×T	E×T	D×E×T
VT[2], °C	38.82	38.97	39.42	39.35	0.13	0.92	0.01	<0.01	0.31	0.39	<0.01	0.24
RR[3], breath/min	56.20	55.32	71.25	71.87	3.47	0.96	<0.01	<0.01	0.83	0.85	0.20	0.55
Dry matter intake, kg/d	23.03	23.11	18.39	18.04	1.38	0.92	<0.01	<0.01	0.88	0.93	0.98	0.70
Milk yield, kg/milking	7.14	9.00	6.21	6.49	0.61	0.10	0.01	<0.01	0.21	0.99	0.62	0.81
Milk composition												
Lactose, %	4.05	4.26	4.08	4.01	0.25	0.80	0.67	<0.01	0.58	0.91	0.93	0.68
Protein, %	3.14	2.88	2.91	2.86	0.07	0.05	0.13	<0.01	0.18	0.93	0.69	0.72
Fat, %	4.07	3.90	4.27	4.34	0.23	0.82	0.17	<0.01	0.60	0.98	0.57	0.95
Solid not fat, %	8.57	8.37	8.27	8.22	0.16	0.45	0.18	<0.01	0.66	0.71	0.43	0.30
Urea nitrogen, mg/dL	13.00	11.68	11.24	12.4	0.61	0.90	0.41	<0.01	0.06	0.35	0.32	0.78
Ln(SCC, 10^3cells/mL)	5.53	5.63	5.10	4.52	0.43	0.58	0.09	<0.01	0.45	0.88	0.92	0.99

[1]D = Diet; E = Environment; T = Time

Table 6.9: Increments and area under the curve (AUC) of circulating cortisol, prolactin, TNF-α and IL10 following intravenous infusion of a LPS bolus of lactating cows fed diets supplemented with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

Parameter	CL		NC			*P*-value		
	OG	CTL	OG	CTL	SEM	D[1]	E	D×E
Cortisol, ng/mL								
Increment	70.09	58.28	62.76	59.18	5.29	0.17	0.55	0.45
AUC, 0-3 h, ×h	160.6[a]	129.1[b]	123.2[b]	132.0[b]	10.5	0.30	0.12	0.07
AUC, 0-6 h, ×h	290.5	254.5	244.5	243.4	21.6	0.41	0.21	0.43
AUC, 0-24 h, ×h	374.5	351.8	272.0	360.1	57.7	0.58	0.43	0.35
Prolactin, ng/mL								
Increment	42.70[ab]	70.93[a]	54.91[ab]	26.38[b]	10.76	0.99	0.15	0.02
AUC, 0-3 h, ×h	66.8[ab]	125.8[a]	88.4[ab]	35.0[b]	20.6	0.89	0.11	0.02
AUC, 0-6 h, ×h	86.2[ab]	165.3[a]	113.1[ab]	34.6[b]	35.6	0.99	0.17	0.04
AUC, 0-24 h, ×h	-95.9	-61.8	-115.9	-102.7	67.0	0.73	0.66	0.88
TNF-α, ng/mL								
Increment	39.34	29.15	38.91	39.33	8.16	0.56	0.56	0.53
AUC, 0-3 h, ×h	61.42	60.67	69.35	70.13	12.59	0.99	0.50	0.95
AUC, 0-6 h, ×h	72.67	85.78	85.73	90.13	15.50	0.58	0.58	0.78
AUC, 0-24 h, ×h	74.32	87.21	97.16	91.46	14.84	0.81	0.38	0.54
IL10, pg/mL								
Increment	2686[ab]	2475[b]	4308[a]	1897[b]	576	0.04	0.38	0.08
AUC, 0-3 h, ×h	3657	3423	5623	2698	868	0.09	0.49	0.14
AUC, 0-6 h, ×h	5194	4985	6549	4439	1066	0.29	0.71	0.39
AUC, 0-24 h, ×h	5513	8098	7345	6682	2309	0.68	0.93	0.49

[1]D = Diet; E = Environment

[ab]Means within a row with different superscripts differ ($P \leq 0.05$)

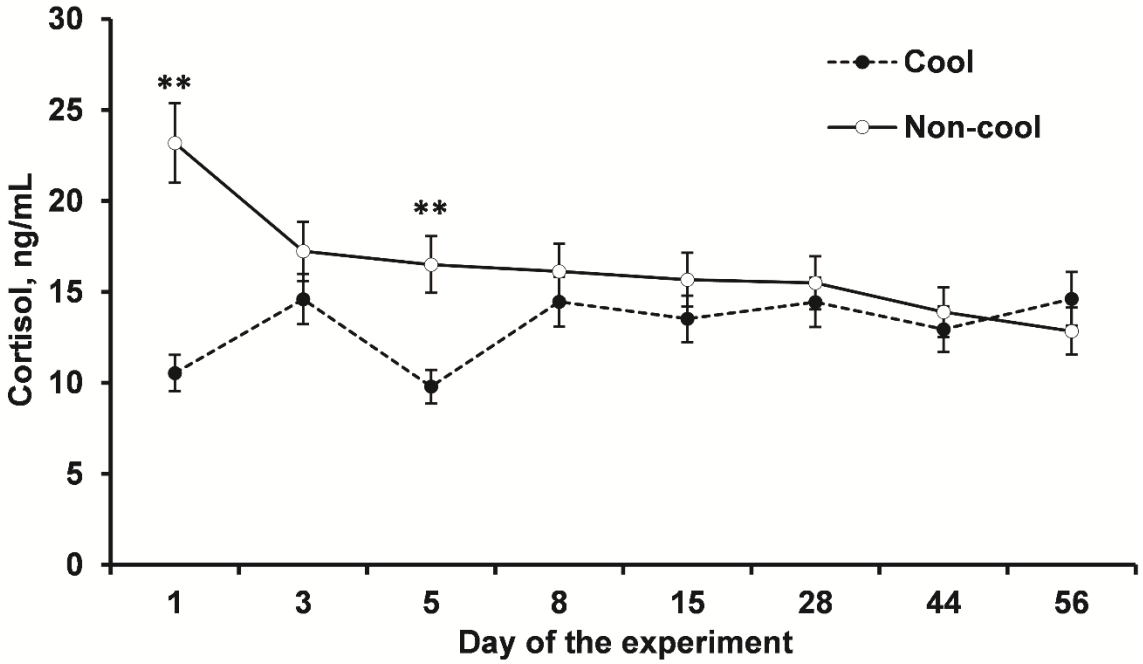

Figure 6.1: Plasma concentrations of cortisol in cows with (solid circles [●]) or without evaporative cooling treatment (open circles [○]). $^{**}P < 0.01$, $^{*}P < 0.05$, $\dagger P \leq 0.10$.

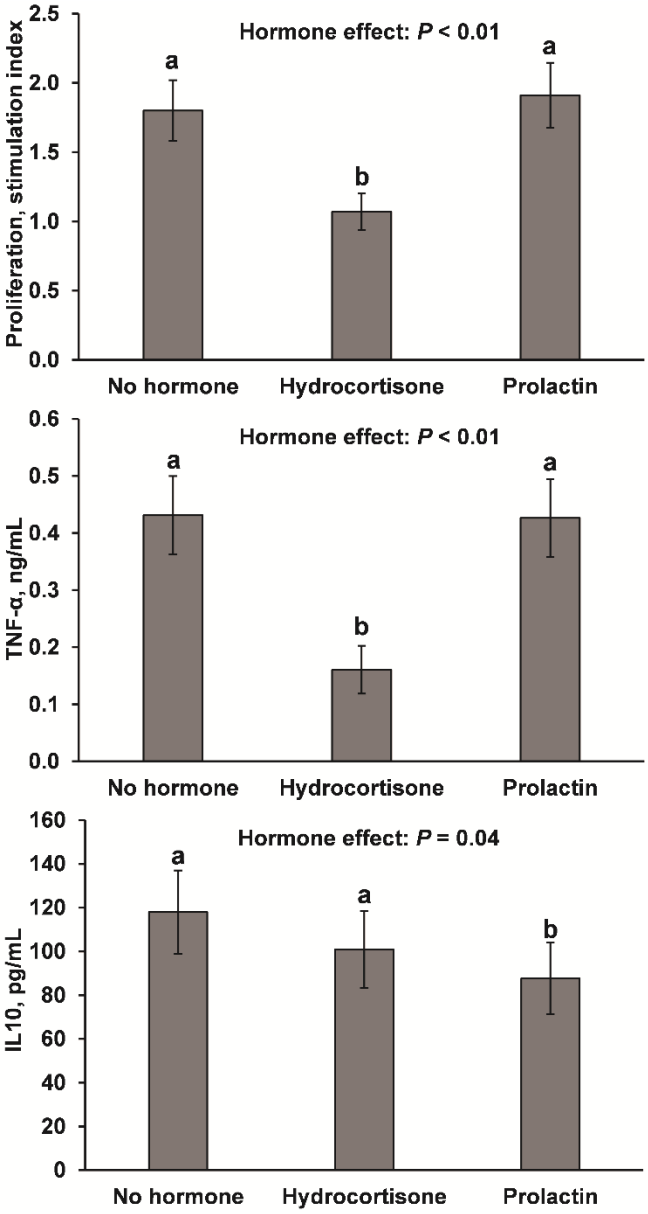

Figure 6.2: Cell proliferation and cytokine production of peripheral blood mononuclear cells stimulated by LPS and cultured with prolactin, hydrocortisone or without hormones. a vs. b: $P \leq 0.01$.

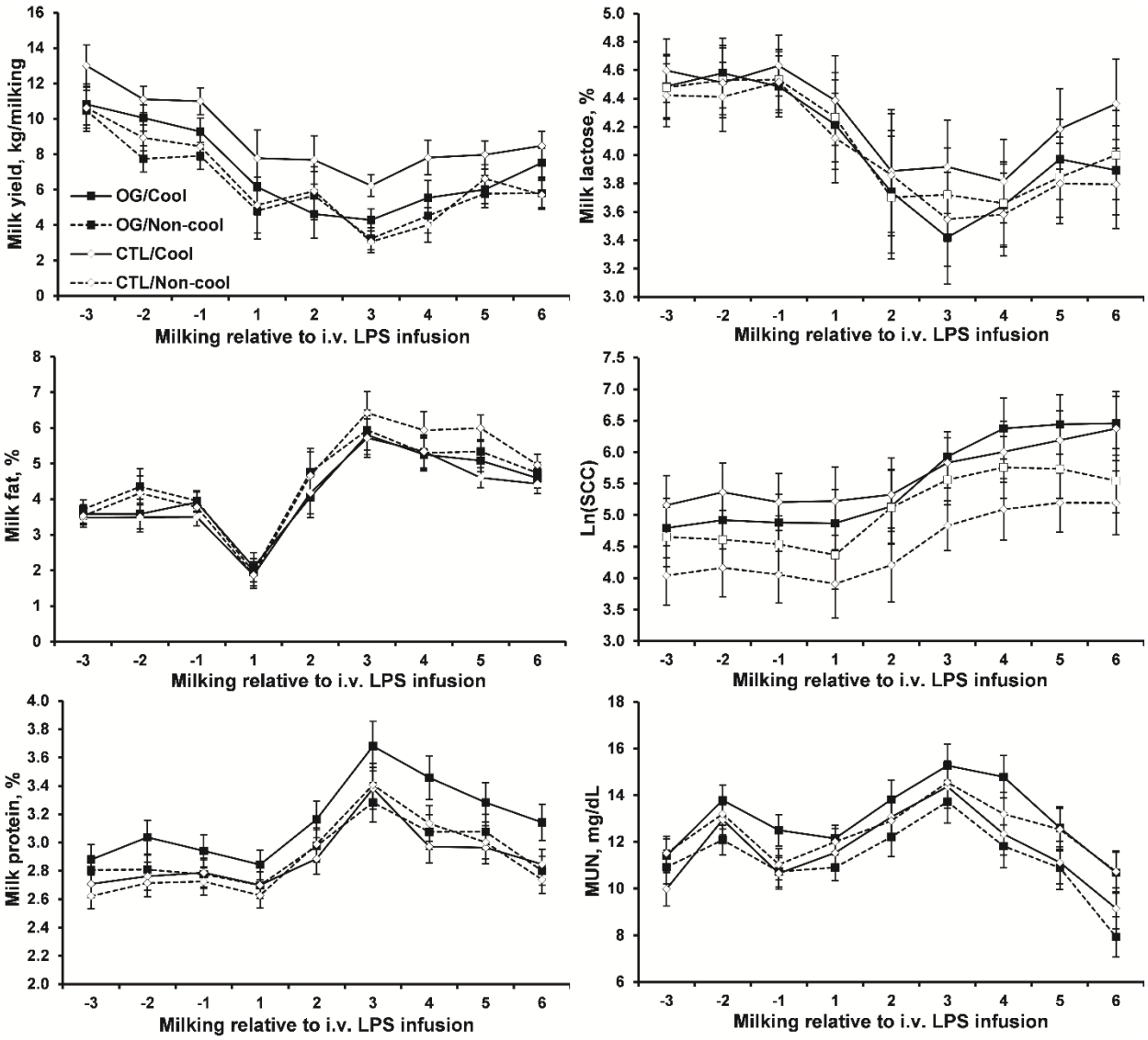

Figure 6.3: Milk yield and component concentrations of cows given an intravenous LPS bolus (i.v. LPS). Cows were fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling (CL) or not (NC).

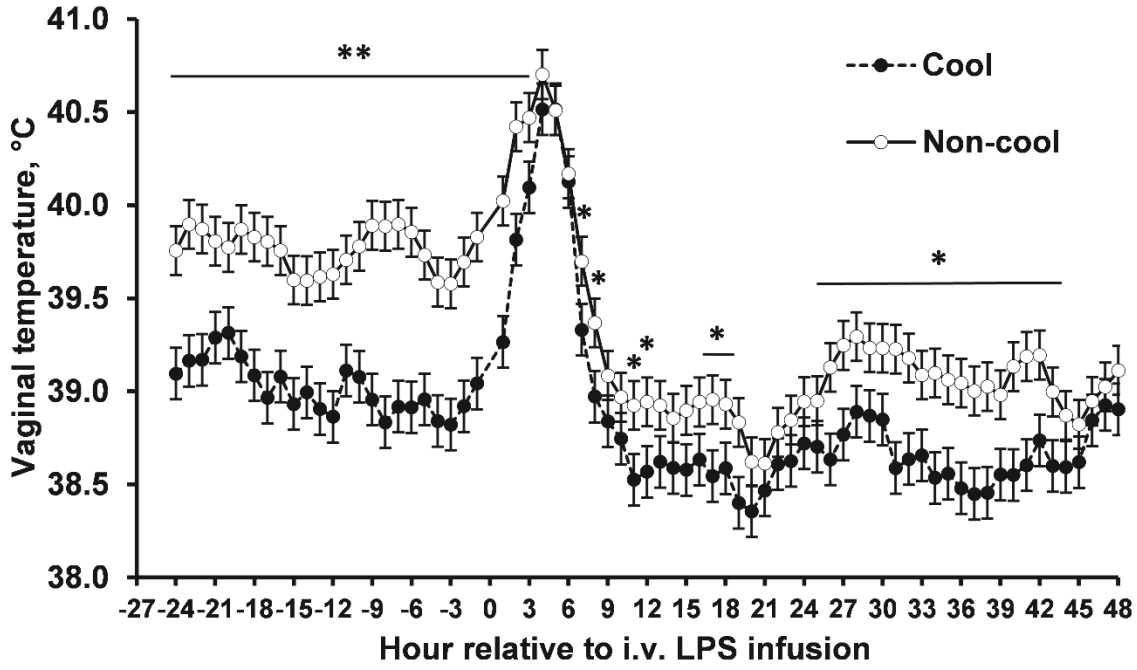

Figure 6.4: Vaginal temperature of cows with (solid circles [●]) or without evaporative cooling treatment (open circles [○]) before and after intravenous infusion of a LPS bolus (i.v. LPS). ** $P < 0.01$, * $P < 0.05$, † $P \leq 0.10$.

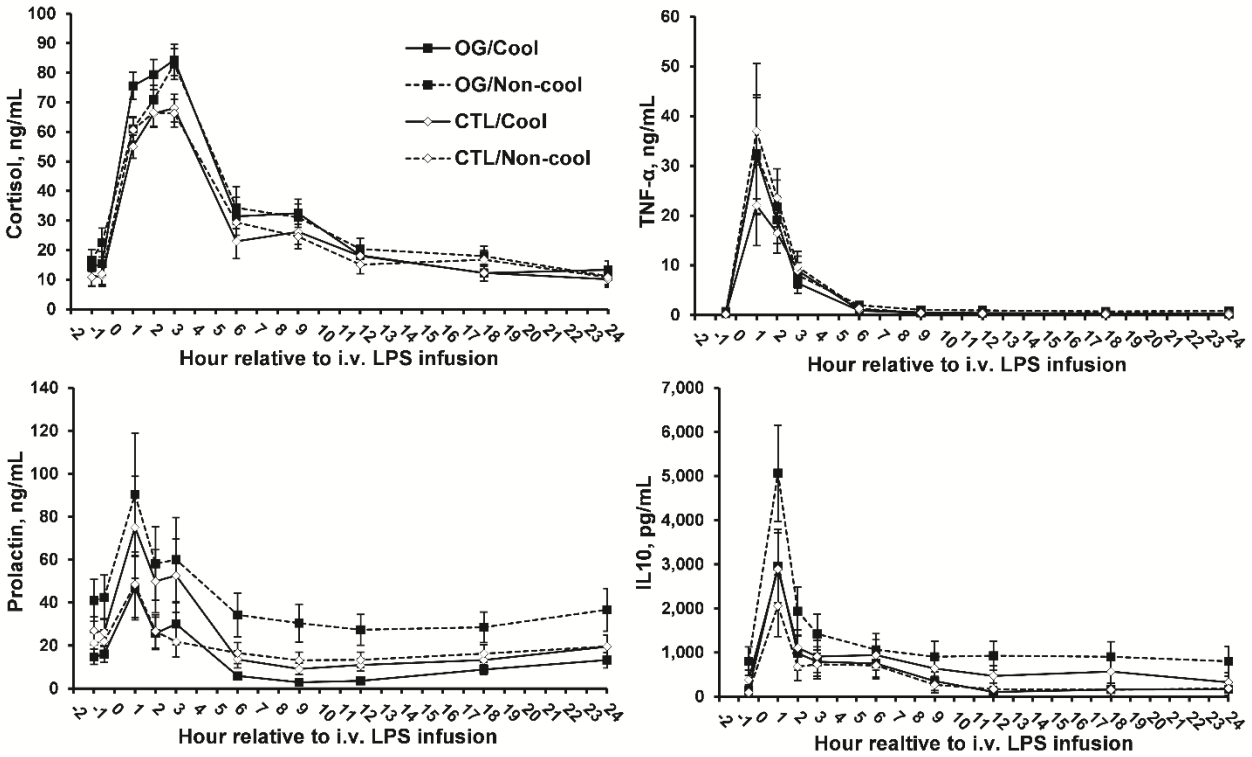

Figure 6.5: Circulating cortisol, prolactin, TNF-α and IL10 concentrations of cows given an intravenous LPS bolus (i.v. LPS). Cows were fed diets with OmniGen® AF (OG) or placebo (CTL) exposed to either cooling or not.

CHAPTER 7

EFFECTS OF HEAT STRESS ABATEMENT ON BEHAVIORAL RESPONSE IN LACTATING DAIRY COWS PRIOR TO AND FOLLOWING AN INTRAMAMMARY LIPOPOLYSACCHARIDE INFUSION[5]

[5] Marins, T.N., R.M. Orellana Rivas, Y.-C. Chen, V.H.L.R. Melo, Z. Wang, H. Liu, J. Gao, C.G. Savegnago, A.M. Roper, J.K. Bernard, P. Melendez, and S. Tao. 2025. *Journal of Dairy Science*, 108:1882–1895. Reprinted here with permission of the publisher.

ABSTRACT

The assessment of animal behavior serves as a valuable approach to identify illness and animal responses to environmental stimuli. Both heat stress and mastitis are reported to impact the behavioral responses of dairy cattle. However, little is known about the effects of heat stress on the lactating cow's behavioral responses to mastitis. The aims of the current study were to evaluate the effects of deprivation of evaporative cooling on behavioral responses of lactating dairy cows prior to and following mammary inflammation induced by intramammary lipopolysaccharide (IM-LPS) infusion and to characterize the impact of deprivation of evaporative cooling on feed sorting in lactating dairy cows during summer. Multiparous mid-lactating Holstein dairy cows (n = 18, parity = 2.4 ± 0.6, DIM = 136 ± 61 d) were randomly assigned to: evaporatively cooled (CL, n = 9) or not cooled (NC, n = 9) for 36 d (average temperature-humidity index = 78.4). The evaporative cooling system included misters and fans. Misters were installed on the front face of each fan, which was placed over both feed bunk and free stalls. On d 30, the left rear quarters of a subset of cows (n = 14, 7/treatment) were infused with a bolus of LPS (10 µg of *Escherichia coli* O111:B4 LPS). Feeding and resting behaviors were recorded throughout the experiment using automated sensor devices (NEDAP). Sorting activity based on particle size separation using a Penn State Particle Separator was assessed at d 3, 16, and 26 of the experiment. Prior to IM-LPS, NC cows had lower ruminating and eating time, and consequently greater inactive time compared with CL cows. Relative to CL cows, NC cows had reduced lying time, and greater standing and walking time. The NC cows also had greater standing bouts, only at the beginning of the experiment, relative to CL cows. Additionally, NC cows sorted more for long particles (NC: 99.4% vs. CL: 94.4%) and sorted against medium and short particles on d 3 of the experiment compared with CL cows. However, following IM-LPS, no significant differences in behavioral responses were

observed between NC and CL cows. In conclusion, deprivation of evaporative cooling negatively affected the behavioral responses of lactating dairy cows during summer. Further, heat-stressed cows without evaporative cooling sorted more for long particles of the diet potentially as a response to cope with the reduced rumen pH. However, deprivation of evaporative cooling does not have a significant impact on the lactating cow's behavioral responses to LPS induced mammary inflammation.

Key words: behavior, lipopolysaccharide infusion, mastitis, heat stress

INTRODUCTION

Optimal welfare can only be achieved when animals are healthy, express natural behavior, and are maintained in affective states (von Keyserlingk and Weary, 2017; Beaver et al., 2020). Therefore, the assessment of animal behavior is a valuable approach to detect not only illness but also the immediate animal responses to environmental stimuli (Weary et al., 2009; von Keyserlingk and Weary, 2017; Eckelkamp and Bewley, 2020). Adequate lying time is considered an important aspect of animal welfare, which is affected by intrinsic (e.g., reproductive status, days since calving, age, and disease) and extrinsic (e.g., environment and management) factors (Tucker et al., 2021). Feeding behavior, including feed sorting, also provides useful information regarding the cow's health and welfare (Schirmann et al., 2016; Beauchemin, 2018; Llonch et al., 2018). Thus, studying lying and feeding behaviors provides useful information to assess the dairy cow's welfare under diseases or environmental insults.

Heat stress poses a consistent and significant concern due to its detrimental impacts on productivity, reproduction, and health in livestock animals (Lacetera, 2019). In dairy cattle, heat stress is reported to affect the cow's behavioral responses. There is a positive correlation between

the increased temperature-humidity index (**THI**) and greater standing time with a reduction in lying time in lactating dairy cows (Cook et al., 2007; Herbut and Angrecka, 2018; Tullo et al., 2019). Reduced intake is a hallmark of the heat-stressed lactating dairy cow, and there is an unfavorable correlation between elevated ambient temperature and the duration of rumination (Soriani et al., 2013; Abeni and Galli, 2017; Moretti et al., 2017). This suggests an altered digestion by heat stress. Changes in feed sorting are also observed in heat-stressed lactating dairy cows. Miller-Cushon et al. (2019) found that lactating cows exposed to heat stress sorted for long particles from the TMR, which suggests a preference for forage over concentrate and is speculated to be a response associated with the reduced rumen pH caused by heat stress. However, these data are in contrast with the traditional notion that heat-stressed lactating dairy cows sort against longer forage particles and prefer smaller and nutrient-dense grain particles due to the lower heat increment of concentrate over forage (Beede and Collier, 1986; West, 2003). Thus, additional studies are needed to confirm the impact of heat stress on feed sorting in lactating dairy cows.

In addition to environmental insults, diseases such as mastitis strongly affect cow behavior leading to impaired welfare. When mastitis was experimentally induced by either *Escherichia coli* (***E. coli***) or lipopolysaccharide (**LPS**) in lactating dairy cows, decreases in feed intake, eating time, and lying time were observed (Siivonen et al., 2011; Cyples et al., 2012; Fogsgaard et al., 2012). Heat stress negatively influences animal health (Sejian et al., 2018; Dahl et al., 2020). Vitali et al. (2020) highlighted a greater risk of developing clinical mastitis under heat stress conditions; presumably because of the favorable environment for survival and growth of pathogens coupled with the impaired immune function of the cow (Kadzere et al., 2002). In a study evaluating 22,212 Holstein cows from 15 large dairy herds, Gernand et al. (2019) reported a positive correlation between THI and the incidence of clinical mastitis. Further, mastitis cases occurred during summer

are commonly caused by environmental pathogens, such as *E. coli* (Olde Riekerink et al., 2007; Hogan and Smith, 2012). However, studies examining the behavioral changes of mastitic dairy cows under heat stress condition are not available.

Thus, we hypothesized that heat stress alters the lactating dairy cow's behavioral response prior to and following experimentally induced mastitis by intramammary LPS infusion (IM-LPS). The objectives of the current study were to evaluate the effects of deprivation of evaporative cooling on behavioral responses of lactating dairy cows prior to and following an IM-LPS, and to characterize the impact of deprivation of evaporative cooling on feed sorting in lactating dairy cows during summer.

MATERIALS AND METHODS

Animals, housing, environment, and experimental design

All experimental procedures and animal handling were approved by the University of Georgia Institutional Animal Care and Use Committee (AUP#: A2017 10-014-Y3-A2) before the initiation of the experiment. The experiment was conducted at the Dairy Research Center on the University of Georgia Tifton campus (Tifton, GA) during the summer (June to August).

This current study was part of a larger trial reported by Chen et al. (2023). Briefly, multiparous lactating Holstein dairy cows (n = 30, parity = 2.4 ± 0.6, DIM = 156 ± 57 d [means ± SD]) were randomly assigned to 1 of 2 treatments, evaporatively cooled (CL, n = 15) or without cooling (NC, n = 15). All cows were housed in two adjacent pens under the same free stall barn with sand-bedding. Both pens had the same size and distance to the milking parlor. Except for their specific environmental treatments, cows were managed in the same manner throughout the experiment. The experiment was divided into a 10-d acclimation (baseline), followed by a 36-d

environmental challenge. During the acclimation period, all cows received evaporative cooling including fans and misters. Fans were placed over both the feed bunk and free stalls, and misters were installed on the front face of each fan. During the environmental challenge, evaporative cooling was provided continuously to CL cows but not to NC cows. The initiation of the environmental challenge was considered as d 1 of the experiment.

Cows were milked twice daily (0500 and 1700 h) and individual milk yields were recorded at each milking (Delpro, DeLaval USA, Gurnee, IL). Cows were fed individually the same diet as a TMR once daily (1600 h) throughout the experiment in a Calan Broadbent feeding system (American Calan Inc., Northwood, NH, USA). The diet contained (DM basis) 28.3% corn silage, 14.1% oat silage, 3.5% Bermuda grass hay and 54.1% concentrates, and provided 44.4% aNDFom, 17.7% ADF, 19.3% starch, 16.6% CP, 4.7% sugar, 4.4% EE and 8.7% ash. Cows had ad libitum access to fresh water and feed was pushed up at 0500, 0800, 1000, 1200, 1400, 1800, 2000, and 2200 h. Air temperature and relative humidity were monitored every 15 min throughout the experiment using the Hobo Pro Series Temp probes (Onset Computer Corp., Pocasset, MA). The THI was calculated based on THI = (1.8 × T + 32) – ([0.55 – 0.0055 × RH] × [1.8 × T – 26]), where T = air temperature (°C) and RH = relative humidity (%) (NRC, 1971). The vaginal temperature was measured using an iButton (Mouser Electronics, Mansfield, TX) attached to a blank (progesterone-free) intravaginal implant (CIDR - Zoetis Animal Health), as described by Dikmen et al. (2014) and Kaufman et al. (2018), every 5 min for 4 consecutive d each week for all cows.

Six cows per treatment were subjected to mammary biopsy collection to study gene expression (Chen et al., 2023) and were not used to assess the behavioral responses. The remaining cows (n = 18; 9/treatment, parity = 2.4 ± 0.6, DIM = 136 ± 61 d [means ± SD]) were fitted with

automated sensor devices (NEDAP SmartTag Neck and Smarttag Leg sensors, NEDAP Livestock Management, Groenlo, the Netherlands) at d -7 of the experiment. The sample size for the assessment of behavior was determined based on the availability of cows and power tests. Two power tests were performed. In the first analysis, the lying time of Holstein cows in their first week of lactation raised during winter and summer (820 vs. 670 min/d, SD = 82 min/d, respectively) reported by Toledo et al. (2023) was used to calculate animal number. Eight cows per treatment was calculated using a level of significance of 0.05 and 90% power. In the second analysis, the lying time of Holstein cows in their mid-lactation housed under thermal neutral and heat stress (548 vs. 758 min/d, SD = 95 min/d, respectively, Wang et al., 2024) was used to calculate animal number using a level of significance of 0.05 and 90% power. A sample size of 6/treatment was determined. Thus, 9 cows/treatment will provide sufficient sample size. SmartTag Leg was attached to the right front leg to record lying, standing, and walking time, number of standing bouts (moving from lying to standing), and walking activity (step counting) every 15 minutes (Van Erp-Van der Kooij et al., 2016; Nielsen et al., 2018). SmartTag Neck measured eating, ruminating, and inactive (not eating and not ruminating) time every minute (Borchers et al., 2021). SmartTags have a G-sensor, which measures accelerated movements in a particular direction, based on a three-dimensional (x-, y-, and z-axes) accelerometer. Data was transferred via an Ethernet connection from a local receiver station to NEDAP servers, and then datasets were generated. Data obtained from NEDAP contained observations for each cow in 1-min periods for SmartTag Neck and 15-min periods for Smarttag Leg.

To calculate feed sorting, individual fresh TMR and ort samples from the same subset of cows (n = 18, 9/treatment) were collected at d -1, -2, -3, 3, 4, 5, 16, 17, 26, and 27 of the experiment. To ensure feed availability and prevent the absence of feed leftovers, daily feed allowance for each

cow was adjusted to maintain 10% residual feed. Diets were mixed in a high-capacity mixer wagon mounted on load cells and TMR was off-loaded into a lower capacity Calan Data Ranger mixer which delivered the correct amount of feed to each individual bunk. Representative TMR samples were collected daily from each load. On the following day, orts from individual cows were well mixed using a shovel and a representative sample collected. The TMR and orts samples were analyzed for particle size separation using a Penn State Particle Separator (PSPS) within 3 h of collection by a single trained person to ensure consistency. The PSPS consists in three-screen (19.0, 8.0 and 1.18 mm) that separates the particles into 4 fractions: long (particle size [PS] > 19 mm), medium (19 < PS > 8 mm), short (8 < PS > 1.18 mm), and fine (< 1.18 mm) particles (Kononoff et al., 2003). After separation, the dry matter content of each PS fraction from TMR and orts was determined by drying in a forced-air oven at 55°C for 48 h.

At d 30 of the experiment, an IM-LPS was performed on a subset of cows (n = 7/treatment). Cows without clinical mastitis during the experimental period were initially selected. Then, milk samples were collected from individual quarters of initially selected cows on d 28 of the experiment to measure SCC. Only cows with individual quarter milk SCC below 200,000 cells/mL were eligible for IM-LPS. Four cows (n = 2/treatment) did not meet the selection criterion. Thus, a total of 14 cows (n = 7/treatment) were enrolled in the IM-LPS. Two hours after the morning milking (0800 h), the left rear quarter of each cow was infused with 10 μg of *E. coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO) dissolved in 5 mL pyrogen-free saline solution and massaged throughout to move the inoculum into the gland cistern. The vaginal temperature was monitored from -24 to 48 h relative to LPS infusion.

Calculations and statistical analysis

The behavior recordings used in the analyses started on d -5 of the experiment and behavioral data collected from NEDAP servers were visually checked for error and were summed by day (minute/day, bout/day, or step/day) using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Sorting activity for each PS fraction of the PSPS was determined by expressing the actual intake of each PS fraction as a percentage of the predicted intake of this fraction (DM basis) (Leonardi and Armentano, 2003; Miller-Cushon et al., 2019). The predicted intake for each PS fraction was computed by multiplying the total DMI by the percentage of this PS fraction (DM basis) in the fresh TMR offered. Thus, sorting activity = 100% indicate no sorting, < 100% indicate selective refusals (sorting against), and > 100% indicate preferential consumption (sorting for). The results were averaged by day to form 4 periods: baseline (daily average of data collected from d -1, -2, and -3), d 3 (daily average of data collected from d 3, 4 and 5), d 16 (daily average of data collected from d 16 and 17), and d 26 (daily average of data collected from d 26 and 27).

All statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC). The PROC UNIVARIATE procedure was used to calculate the means ± SD of the THI, DIM, and parity. Repeated measures data collected during the experimental challenge and prior to the IM-LPS, including behavior measures and feed sorting, were analyzed using the PROC MIXED procedure. The statistical models included fixed effects (cooling, time, and their interaction) with cow nested within the treatment (cooling) as the random variable. The data collected at the baseline were included in the statistical models as covariates.

Behavioral data collected following IM-LPS were analyzed using PROC MIXED procedure as repeated measures data. The model included cooling, time, and cooling by time interaction, with cow nested within cooling as the random variable. Data were reported as least

squares means ± standard error of the means. Significance and tendency were declared when $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively. When a significance or tendency for the time effect was observed, multiple comparisons were performed using PDIFF function adjusted by Tukey-Kramer method.

RESULTS

During the environmental challenge, THI averaged 78.6 ± 4.5 and 78.1 ± 4.5 (mean ± SD) in the pens housing NC and CL cows, respectively, and the average daily THI was greater than 72 throughout the entire study and the daily minimum THI only dropped below 68 between d 12 and 16 of the experiment (Figure 7.1 A). During IM-LPS, the daily average THI was consistently higher than 79, and the minimum THI was higher than 75 (Figure 7.1 B).

Overall performance

The performance data were reported in detail by Chen et al. (2023). Briefly, NC cows had greater vaginal temperature (CL: 38.9 vs. NC: 39.6 ℃, $P < 0.01$) during the environmental challenge compared with CL cows. Both DMI (CL: 25.7 vs. NC: 22.5 kg/d, $P < 0.01$) and milk yield (CL: 33.1 vs. NC: 29 kg/d, $P < 0.01$) were lower for NC cows compared with CL cows. Following IM-LPS, a treatment by time interaction ($P < 0.01$) was observed for vaginal temperature, such that the vaginal temperature for NC cows reached a similar peak at 6 h after IM-LPS compared with CL cows (CL: 41.2 vs. NC: 41.0 °C, $P > 0.10$ at 6 h following IM-LPS) but remained higher before and after the peak. Regardless of treatment, IM-LPS reduced DMI and milk yield (Time effect: $P < 0.01$). Compared with CL cows, milk yield (CL: 31.7 vs. NC: 23.5 kg/d) and DMI (CL: 22.4 vs. NC: 18.2 kg/d) for NC cows remained lower ($P < 0.01$) after IM-LPS. However, relative to the data collected before IM-LPS, NC cows had greater ($P = 0.01$)

reduction in DMI on d 4 and tended ($P = 0.09$) to have greater reduction in DMI on d 2 following IM-LPS compared with CL cows (Treatment × Time: $P = 0.08$). Both NC and CL cows had similar ($P \geq 0.78$) changes in milk yield relative to pre-infusion level.

Behavioral responses during the environmental challenge

During the environmental challenge, treatment by time interactions were observed for ruminating ($P < 0.01$), eating ($P = 0.05$), and inactive time ($P < 0.01$). Compared with CL cows, NC cows had lower ($P < 0.01$) ruminating time on d 1, 3, 4, 5, 6, 8, 9, 10, 12, 24, 26, and 29 and tended ($P \leq 0.08$) to have lower rumination time at d 2, 14, and 28 of the experiment (Figure 7.2 A). Relative to CL cows, the eating time for NC cows was lower ($P < 0.01$) on d 1, 3, 4, 5, 6, 7, 8, 9, 10, 15, 17, 19, 20, 21, 23, 24, 26, and 29, and tended to be lower ($P \leq 0.07$) on d 12, 14, and 27 of the experiment (Figure 7.2 B). Inversely, NC cows displayed greater ($P < 0.01$) inactive time on d 1, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 17, 18, 20, 21, 24, 26, and 29, and tended ($P \leq 0.09$) to have greater inactive time on d 19, 22, and 27 of the experiment compared than CL cows (Figure 7.2 C).

Additionally, there was a tendency of a treatment by time interaction ($P = 0.09$) for lying time during the environmental challenge. This is because the lying time for NC cows was lower on d 1, 2, 3, 6, 8, 9, 10, 11, 15, 16, 17, 20, 22, 23, 24, 25, and 27, and tended to be lower on d 7 and 14 of the experiment compared with CL (Figure 7.3 A). No treatment by time interaction ($P = 0.14$) was observed for standing time, but NC cows had greater standing time compared with CL cows (811.4 vs 726.7 min/d, SEM = 10.5 min/d, Treatment effect: $P < 0.01$) (Figure 7.3 B). Compared with CL cows, walking time on d 1, 2, 8, 9, 10, 14, 16, 21, 24, 27, and 29 was greater ($P \leq 0.05$) and tended ($P = 0.10$) to be greater on d 22 of the experiment (Treatment × Time interaction: $P < 0.01$) for NC cows (Figure 7.3 C). Further, we observed that NC cows had greater

($P < 0.03$) standing bouts only on d 3, 4, and 5 of the experiment relative to CL cows (Treatment × Time interaction, $P = 0.01$, Figure 7.3 D). A treatment by time interaction ($P < 0.01$) was observed for walking activity, such that the step count for NC cows was greater on d 1, 2, 3, 8, 9, 10, 16, 21, and 27 and tended to be greater on d 14 and 25 of the experiment compared with CL cows (Figure 7.3 E).

Changes in feed sorting during the environmental challenge

There was a treatment effect ($P = 0.05$) for the sorting activity towards long particles (Figure 7.4 A) because NC cows sorted more for long particle compared with CL (NC: 99.4% vs. CL: 94.4%). A tendency for treatment by time interaction ($P = 0.09$) suggested that NC cows sorted against ($P = 0.02$) medium particle on d 3 of the experiment compared with CL cows (Figure 7.4 B). Compared with CL cows, NC cows sorted against ($P < 0.01$) short particle on d 3 but sorted in favor ($P = 0.03$) on d 16 of the experiment (Treatment × Time interaction: $P < 0.01$, Figure 7.4 C). No ($P \geq 0.40$) treatment by time interaction and treatment effect were observed for the sorting behavior for fine particles (Figure 7.4 D).

Behavioral responses following IM-LPS challenge

Regardless of treatment, cows spent less time eating ($P < 0.05$) on d 1 and 2 and ruminating ($P < 0.01$) on d 2 following IM-LPS, resulting in a greater ($P < 0.01$) inactive time on d 2 and a tendency ($P = 0.07$) for greater inactive time on d 1 after IM-LPS compared with pre-infusion values (min/d, Time effect: $P < 0.01$, Figure 7.5 A, C, E). Compared with CL cows, NC cows had lower ($P < 0.01$) daily rumination time (CL: 525 vs. NC: 391min/d), greater ($P < 0.01$) inactive time (CL: 787 vs. NC: 960 min/d) and tended ($P = 0.09$) to have lower eating time (CL: 125 vs. NC: 76 min/d) after IM-LPS (Figure 7.5 A, C, E). However, no treatment effect ($P \geq 0.14$) was observed for the changes in rumination, eating, or inactive time relative to data collected prior to

IM-LPS (Figure 7.5 B, D, F). We observed treatment by time interactions ($P < 0.01$) for the changes in rumination time and inactive time relative to values collected before IM-LPS because NC cows spent less time ruminating (CL: 110 vs. NC: 86 %, $P < 0.01$) and more inactive time (CL: 94 vs. NC: 109 %, $P < 0.01$) on d 5 following IM-LPS compared with CL (Figure 7.5 B, F).

Cows spent more ($P = 0.01$) time lying but less ($P = 0.02$) time standing up on d 4 after IM-LPS compared with data collected prior to infusion. However, no treatment or treatment by time interactions were observed for lying and standing time expressed as the actual minutes per day or changes (%) relative to data collected prior to infusion ($P \geq 0.14$, Figure 7.6 A, B, C, D). Regardless of treatment, time relative to IM-LPS infusion ($P < 0.01$) affected the walking time (min/d) and activity (step/d) of cows following IM-LPS (Figure 7.6 E, G). Compared with data collected prior to infusion, we observed reduced walking time (min/d) and activity (step/d) (Time: $P < 0.01$) on d 4 after IM-LPS (Figure 7.6 E, G). Compared with CL cows, NC cows tended ($P = 0.10$) to walk more (CL: 34.5 vs. NC: 40.9 min/d) prior to IM-LPS (Treatment × Time: $P = 0.01$, Figure 7.6 E) and tended ($P = 0.09$) to have more steps per day on d 4 (CL: 2923 vs. NC: 3495 step/d) following IM-LPS (Treatment × Time: $P = 0.09$, Figure 7.6 G). When expressed as change (%) relative to pre-infusion, CL cows had greater ($P < 0.01$) walking time (CL = 126 vs. NC = 99%) and tended ($P = 0.07$) to have greater activity (CL = 113 vs. NC = 98%) on d 3 following IM-LPS than NC cows (Treatment × Time: $P < 0.01$, Figure 7.6 F, H). Treatment, time or their interactions did not affect the standing bout ($P \geq 0.28$) following IM-LPS.

DISCUSSION

Environmental challenge

Heat stress is a significant concern for the well-being of dairy cows. The adoption of cost-effective heat abatement strategies is critical to maintain animal productivity, ensure the producer's economic returns, and achieve the optimal welfare of dairy cows during summer (Polsky and von Keyserlingk, 2017; Gunn et al., 2019). In this current study, pens housing CL and NC cows had similar high THI values throughout the entire experiment, indicating both CL and NC cows were exposed to consistent and intensified heat stress. However, NC cows had greater vaginal temperature and respiration rate (Chen et al., 2023), indicating greater heat load carried by NC compared with CL cows. Further, NC cows had a 12.4% reduction in milk yield and 12.5% decrease in DMI relative to CL cows. These observations are hallmarks of the heat-stressed lactating dairy cows. Thus, the experimental model used in this study is suitable for examining the influence of heat stress on lactating dairy cows' behavioral responses. It is recognized that the milk yield of a lactating dairy cow starts to decline when daily average THI exceeds 68 (Zimbelman et al., 2009), but the THI threshold for altered behavior by heat stress has not been identified. Allen et al. (2015) reported fewer cows standing when THI was below 68 compared with cows exposed to higher THI. Similarly, Heinicke et al. (2018) observed in high-yielding Holstein-Friesian dairy cows in the moderate climatic zone of Germany that lying time decreased more significantly when THI was above 67. In our current study, the average daily THI was always above 68, indicating the behavior traits of cows were affected by heat stress.

Animal behavior provides information of how animals interpret surroundings and their corresponding reactions. Understanding the behavioral responses of the cow will facilitate the environmental adjustment strategies and management practices that promote both animal

productivity and well-being (Ratnakaran et al., 2016; Orihuela, 2021). During the environmental challenge, we found that the absence of heat abatement decreased daily ruminating and eating times, consequently increasing the inactive time that reflects the idle time when animals are not ruminating or eating. The reduced eating time for NC cows compared with CL cows agrees with previous reports suggesting that heat stress poses direct negative effects on eating behavior (Bernabucci et al., 2010; Ramón‐Moragues et al., 2021). It is known that dairy cows vary their daily eating time when exposed to different THI, and specifically the eating time decreases as the mean daily THI increases (Hut et al., 2022). Eating time is positively associated with DMI (Johnston and DeVries, 2018) and heat stress leads to a significant decrease in DMI (West, 2003). Thus, the lower eating time for NC cows is likely associated with their reduced intake compared with CL cows.

Climate factors also affect the daily diurnal pattern and total time of rumination. Soriani et al. (2013) reported that, when THI exceeds 76, daily rumination time decreases by 2.2 min per unit increase in THI in dairy cows housed in a free-stall barn with conventional milking. Similarly, Moretti et al. (2017) reported that the rumination time of lactating dairy cows decreases as THI increases, and high-producing cows had the greatest reduction compared with low producing cows. Corazzin et al. (2021) reported that late-lactating Italian Holstein Friesian cows exposed to THI above 72 had reduced daily rumination time (330 vs. 510.9 min/d) compared with THI below 72. However, during summer, providing heat abatement using fans and sprinklers largely improves the lactating dairy cow's daily rumination time (493.9 vs. 397.9 min/d, for cows with and without heat abatement) (Corazzin et al., 2021). Similarly, in our study cows cooled with mister and fans had greater daily rumination time compared with cows without cooling (515.6 vs. 466.4 min/d).

Feed sorting compromises nutrition and feeding management, resulting in undesired rumen fermentation and digestive efficiency (Miller-Cushon and DeVries, 2017). It was stated that dairy cows under heat stress sort in favor of concentrate over forage as an adaptive response to enhance energy intake and minimize the heat increment through digestion (Beede and Collier, 1986; West, 1999). In contrast, Miller-Cushon et al. (2019) reported that dairy cows exposed to heat stress sorted for long forage particles, speculating that cows are motivated to maintain forage intake in response to the reduced rumen pH caused by heat stress (Burhans et al., 2022). In our current study, we observed that both CL and NC cows had similar preference in sorting (> 100%) for fine particles prior to the environmental challenge (baseline). However, during the environmental challenge, NC cows sorted in favor of long particles indicating a preference for forage compared with CL cows. In addition, NC cows had a more pronounced selective refusal of medium and short particles only at d 3 of the environmental challenge. Dairy cows have reduced ruminal pH and are at risk of sub-acute ruminal acidosis during heat stress (Zhao et al., 2019; Burhans et al., 2022). Thus, the preference of long particle for NC cows than CL cows may be a response to prevent a further reduction in rumen pH by heat stress, and the unfavorable sorting for medium and short particles on d 3 of the environmental challenge for NC cows indicates that this response is more pronounced during acute heat stress exposure. Our results are consistent with Miller-Cushon et al. (2019) but contradict others (Beede and Collier, 1986; West, 1999); thereby, additional research is still warranted to elucidate or confirm the impact of heat stress on feed sorting in lactating dairy cows during the short and long-term exposure to heat stress.

Adequate lying time is an important aspect of animal welfare and associated with the productivity of dairy cows (Fregonesi and Leaver, 2001; Polsky and von Keyserlingk, 2017; Tucker et al., 2021). Indeed, it has been reported that more productive cows lay longer than their

less productive herdmates (Lovarelli et al., 2020). Measures of lying behavior provides insightful data on cows' interactions with their surrounding environment. Cook et al. (2007) and Allen et al. (2015) reported that increasing THI was associated with reduced lying time and consequently increased standing time. In agreement, we observed that NC cows had lower lying time (NC: 592 vs. CL: 679 min/d) and greater standing time (NC: 811 vs. CL: 727 min/d) compared with CL cows. However, it is important to note that both NC and CL cows had lower lying time and greater standing time than previously reported under non-heat stress conditions. Cook et al. (2007) reported that a high-producing dairy cow housed in a free-stall barn spent 12 to 13 h/d (720 to 780 min/d) lying down under non-heat stressed condition. Similarly, Toledo et al. (2023) reported that early lactating multiparous Holstein cows in a sand-bedded free-stall barn equipped with fans and soakers over the feed line spent 814 ± 23.9 min/d lying during winter, which is greater than the lying time (717 ± 21.1 min/d) of early lactating cows during summer. These reports may suggest that, in our present study, both CL and NC cows had reduced lying time and increased standing time due to different mechanisms. Cows without evaporative cooling decrease their lying time while simultaneously increasing standing time to expose more body surface area to cooler air for heat dissipation (Allen et al., 2015; Nordlund et al., 2019). In contrast, CL cows may spend more time standing in alleys near the water system and fans to receive cooling (Cook et al., 2007). In either case, the extended periods of standing may contribute to a high risk of lameness in the late summer (Galindo et al., 2000; Cook et al., 2007; Proudfoot et al., 2010). In this current study, no cows experienced lameness during the study period.

In addition to total lying and standing time per day, lying or standing bout frequency in a day is also changed in response to environmental factors (Tucker et al., 2021). However, the impact of heat stress on standing bout is inconsistently reported. Pate et al. (2020) observed that lactating

Holstein cows exposed to a heat stress challenge using electric heat blankets had about 1 more standing bout within a 24 h period compared with cows without heat challenge. Toledo et al. (2023) reported early lactating cows during a hot season had greater numbers of standing bouts compared with cows during the cool season (15 ± 0.7 vs. 12 ± 0.7 bout/d, respectively). In contrast, Nordlund et al. (2019) reported that the number of daily lying bouts of lactating cows remained similar (between 11.1 and 12.2 bout/d) when THI increased from 68.5 to 79.0 while the total lying time decreased from 9.5 to 6.2 h/d. Similarly, Heinicke et al. (2018) reported that Holstein-Friesian cows had constant standing bouts (between 9 and 10 bout/d) at various ambient THI ranging from 42 to 77. In the present study, NC cows had greater number of standing bouts only at the beginning of the environmental challenge, possibly suggesting that the increased standing bout is a response to acute but not to prolonged exposure to heat stress.

Decreased activity and movement are described as behavioral strategies adopted by lactating cows in response to environmental changes (West, 2003; Van Os, 2019). Hut et al. (2022) reported that dairy cows housed in a barn with access to pasture and milked in a conventional milking parlor spent less time walking as daily temperature and THI increased in the temperate climate of Netherlands. In another study, cows' locomotor activity showed a negative correlation with increasing THI during summer (Herbut and Angrecka, 2018). In contrast, Antanaitis et al. (2023) reported that cows exposed to THI above 72 had greater walking activity compared with cows exposed to THI below 72. Similarly, Endres and Barberg (2007) evaluated 12 herds with bedded-pack housing system in Minnesota and reported that the cow's average number of steps was greater when THI was ≥72 compared with when THI below 72. Consistently, we observed increased walking time (min/d) and activity (step/d) for NC cows compared with CL cows. Because both NC and CL cows traveled a similar distance to the milking parlor and were housed

in pens with similar areas, the different walking time and activity resulted from the presence of heat abatement. The increased walking and activity of NC cows may be an effort to seek a climatically favorable (or cooled) area in the pen. Additionally, because the frequency of drinking bouts of dairy cows increases under heat stress conditions (McDonald et al., 2020; Tsai et al., 2020), cows without evaporative cooling may visit water troughs more frequently, which also increases the walking time and activity.

IM-LPS challenge

Mastitis poses detrimental effects on animal welfare and the profitability of dairy farms (Ruegg, 2017), and summer conditions are associated with increased incidences of clinical mastitis (Olde Riekerink et al., 2007; Gernand et al., 2019; Vitali et al., 2020)). The occurrence of mastitis alters the activity patterns of a cow due to inflammation and pain (Mainau et al., 2022). However, there is a lack of studies examining the behavior of cows with mastitis or mammary gland inflammation under heat stress conditions.

Changes in feeding behavior and intake are frequently associated with the development of disease in animals (Weary et al., 2009). Regardless of treatment, after IM-LPS, ruminating and eating times decreased with a corresponding increase in inactive time. These results support the findings from previous studies with experimentally induced mastitis using intramammary infusion of live *E. coli* or LPS (Siivonen et al., 2011; Zimov et al., 2011; Fogsgaard et al., 2012; Fitzpatrick et al., 2013; Ginger et al., 2023) or with naturally occurring mastitis cases (Stangaferro et al., 2016). Chen et al. (2023) reported that, regardless of treatment, cows in this study had reduced DMI after IM-LPS infusion which partially explains the reduced eating and rumination time (Aditya et al., 2017; Beauchemin, 2018). The changes in cow behavior are also proposed to be driven by the presence of systemic inflammation. During diseases, the release of cytokines could

directly affect appetite centers in the brain, alter gastric motility, and modulate other endocrine factors that in turn influence rumination and rumen motility (Brown and Bradford, 2021). Chen et al. (2023) reported that IM-LPS induced a significant feverish response but did not affect circulating TNF-α in lactating cows. Relative to baseline, we observed that CL cows had greater rumination time on d 5 following IM-LPS than NC. Non-cooled cows had lower reduction in DMI on d 4 after IM-LPS (Chen et al., 2023), which provides a potential explanation for the lower rumination time compared with CL cows given that the diet can physically stimulate rumination (Beauchemin, 2018).

Lying behavior changes during diseases, and sick cows are more motivated to lie down to cope with infection by minimizing the consumption of body energy reserves (Weary et al., 2009). However, lying time may be negatively altered in mastitic cows due to the pain resulting from swollen and inflamed udder (Fogsgaard et al., 2012; Medrano-Galarza et al., 2012; Fitzpatrick et al., 2013). Siivonen et al. (2011) reported that cows spent less time lying during the day following intramammary LPS infusion compared with the day before infusion. Similarly, Cyples et al. (2012) reported that early lactating cows spent less time lying down on the day of the mastitis challenge compared with 2 d before the challenge, but no differences were found in the following 2 days. Furthermore, Medrano-Galarza et al. (2012) and Fogsgaard et al. (2015) reported that cows with naturally occurring mastitis spent less time lying down on the day of mastitis detection compared with healthy herdmates. In contrast, we did not observe changes in lying and standing time of cows during the first days following IM-LPS compared with the data collected prior to infusion. In contrast with previous studies, the heat stress environment and associated reduction (or increase) in lying (or standing) time may mask the potential effect of IM-LPS on lying behaviors. Indeed, we did not observe changes in lying or standing responses relative to pre-infusion level between

CL and NC cows, further suggesting that providing heat abatement did not significantly affect the lying behavior of cows with mammary inflammation.

In the present study, the standing bouts remained unchanged after IM-LPS. Similar to our results, Cyples et al. (2012) reported no differences in the number and the average duration of lying bouts between the day before and the day after intramammary LPS infusion. In contrast, Fogsgaard et al. (2015) reported that cows with naturally occurring mastitis and milked in automatic milking systems had a higher number of daily lying bouts compared with healthy herdmates. Additionally, Siivonen et al. (2011) observed that cows walked more steps on the day following intramammary LPS infusion compared with the day before infusion. However, Medrano-Galarza et al. (2012) reported that mastitic cows walked similar steps per day compared with healthy herdmates. Similarly, we did not observe significant changes in walking time or activity following IM-LPS. These data may suggest the presence or absence of heat abatement had little impact on the lactating cow's behavioral responses following experimentally induced mastitis. However, it is important to note that LPS induced mammary inflammation is transient and cannot completely represent the continuous stimulation caused by live bacteria during mastitis. Thus, additional research is still warranted to examine the impact of heat stress or heat stress abatement on behavioral responses of cows with naturally occurring mastitis or experimentally induced mastitis using live bacteria.

In this study, we reported that the absence of evaporative cooling reduced lying time but increased standing time, walking time, and activity of lactating dairy cows during summer. In addition, deprivation of heat abatement decreased rumination and eating time with a consequent increase in inactive time. Cows without evaporative cooling sorted for long particles of the diet, potentially suggesting a preference for forage as a response to cope with the reduced rumen pH.

However, deprivation of evaporative cooling does not seem to have an impact on the lactating cow's behavioral responses to LPS induced mammary inflammation.

CONCLUSION

Our data confirmed that, during summer, deprivation of evaporative cooling negatively affects the behavioral responses of lactating dairy cows. In this current study, the absence and presence of evaporative cooling induced distinct hyperthermic responses, which in turn resulted in different behavioral changes of lactating dairy cows. These data suggest that close associations among environmental parameters, body temperature and behavioral responses of lactating cows under different cooling settings exist. However, with the limited animal numbers, we were unable to examine these correlations. Thus, a comprehensive study with large animal numbers to investigate these associations in the commercial setting is still guaranteed.

ACKNOWLEDGEMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection. We also appreciate the donation of blank CIDR by Zoetis Animal Health (Kalamazoo, MI). This project was partially supported by the National Institute of Food and Agriculture (Washington, DC) Hatch project (GEO00916).

DECLARATION OF INTEREST

The authors are associated with the University of Georgia or Zhejiang University and declare no conflicts of interests.

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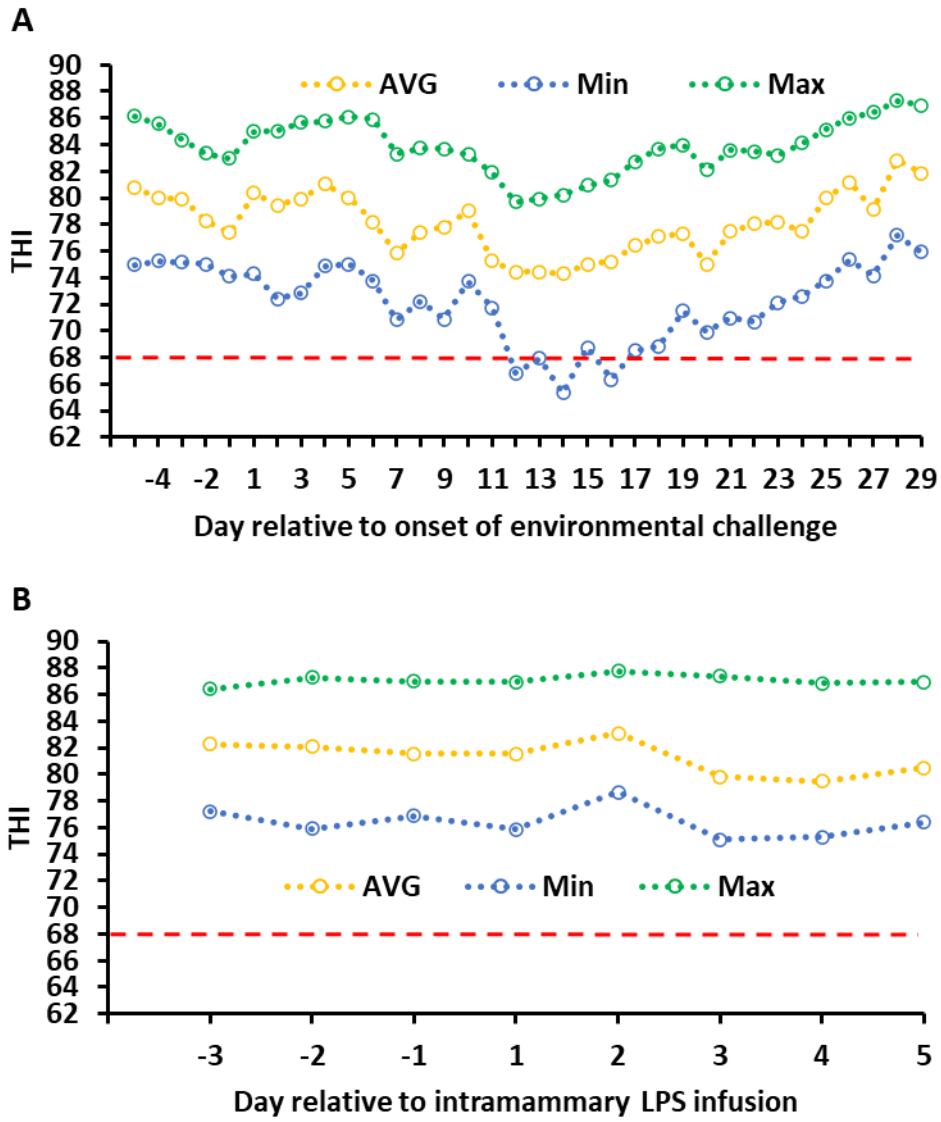

Figure 7.1: Daily average (AVG, yellow dotted line), minimum (Min, blue dotted line), and maximum (Max, green dotted line) temperature-humidity index (THI) relative to onset of environmental challenge (A) and during an intramammary lipopolysaccharide (LPS) infusion (B). Dashed red line: critical THI threshold (68).

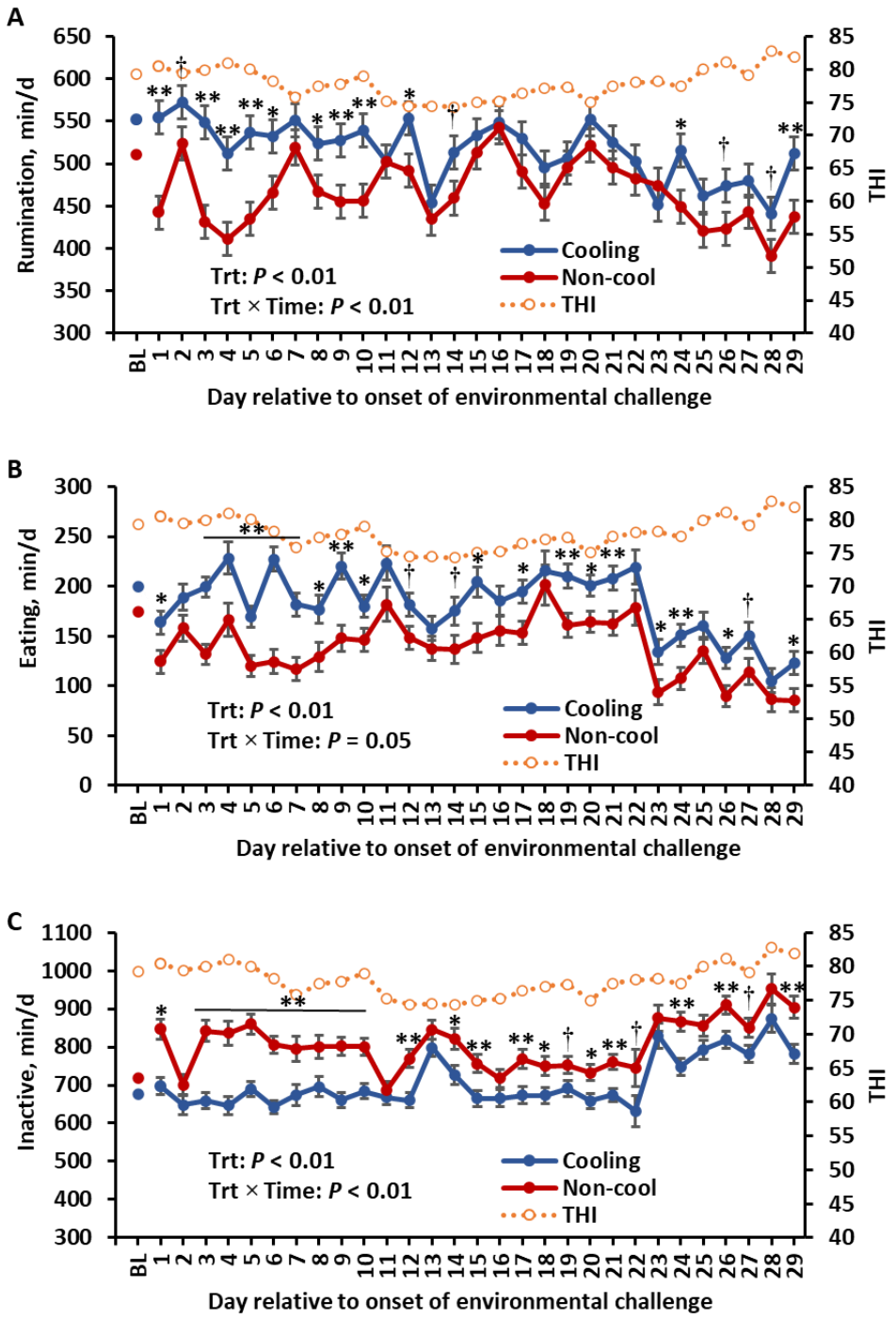

Figure 7.2: Rumination time (min/d, A), eating time (min/d, B), and inactive time (min/d, C) of cows exposed to evaporative cooling (blue line) or not (red line) relative to onset of environmental challenge. Error bars represent SEM. $^{**}P \leq 0.01$, $^{*}0.01 < P \leq 0.05$, †$0.05 < P \leq 0.10$. Daily average temperature-humidity index (THI): yellow dotted line. Data collected at baseline (BL) were used as covariate. TRT represents treatment.

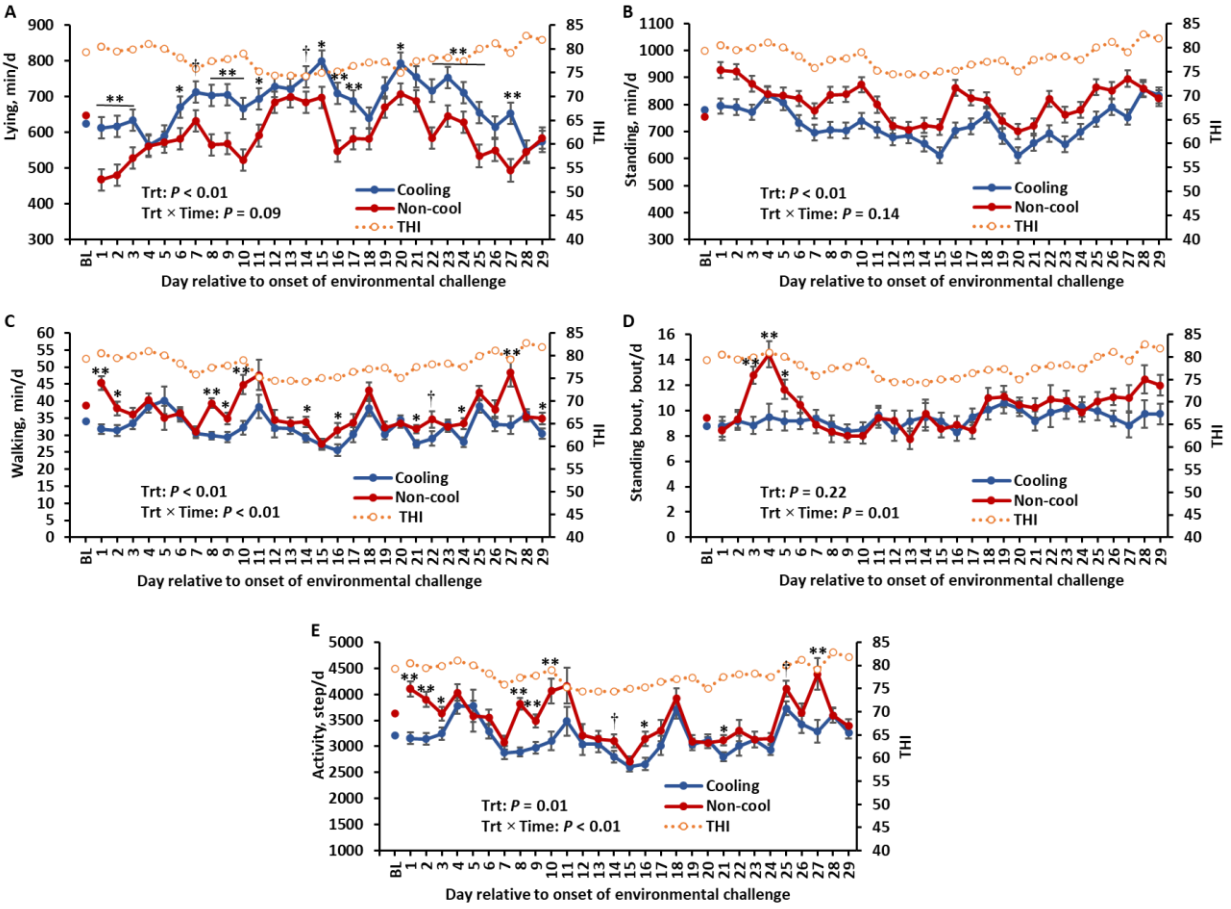

Figure 7.3: Lying time (min/d: A), standing time (min/d: B), walking time (min/d: C), standing bout (bout/day: D), and walking activity (step/day: E) of cows exposed to evaporative cooling (blue line) or not (red line) relative to onset of environmental challenge. Error bars represent SEM. $^{**}P \leq 0.01$, $^{*}0.01 < P \leq 0.05$, †$0.05 < P \leq 0.10$. Daily average temperature-humidity index (THI): yellow dotted line. Data collected at baseline (BL) were used as covariate. TRT represents treatment.

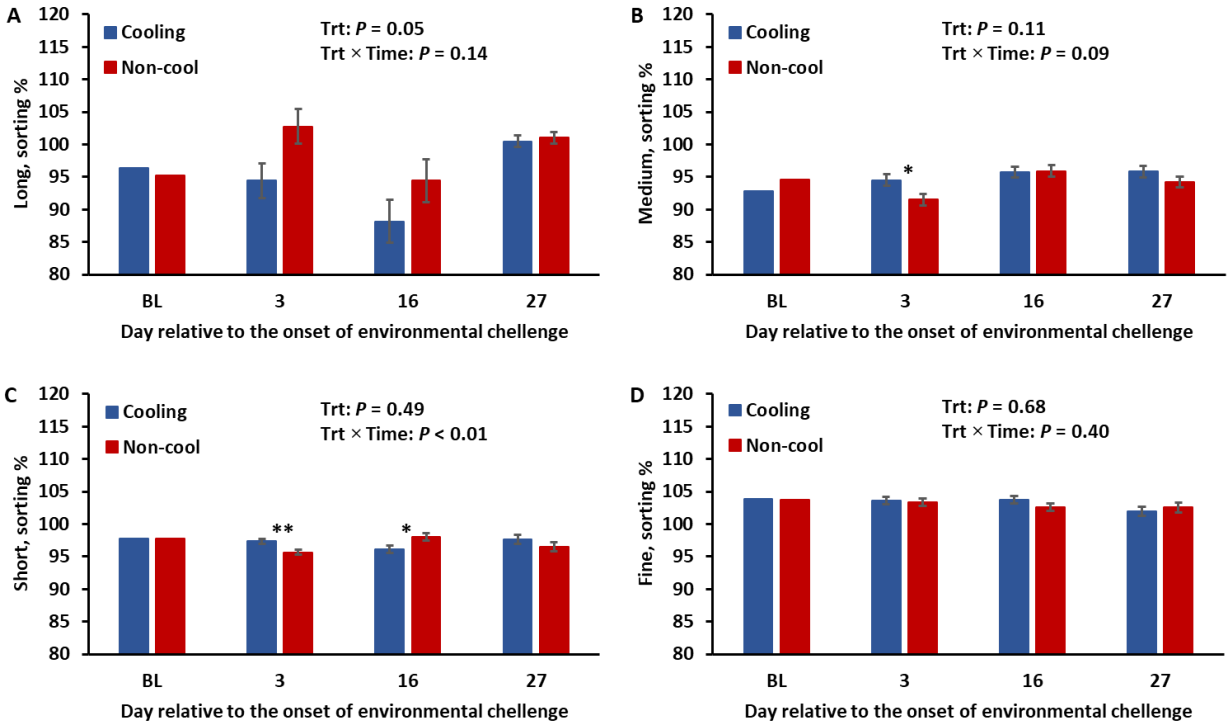

Figure 7.4: Sorting activity of long particle size (A), medium particle size (B), short particle size (C), and fine particle size (D) of cows exposed to evaporative cooling (blue bar) or not (red bar). Data collected at baseline (BL) were used as covariate. Error bars represent SEM. $^{**}P \leq 0.01$, $^{*}0.01 < P \leq 0.05$. TRT represents treatment.

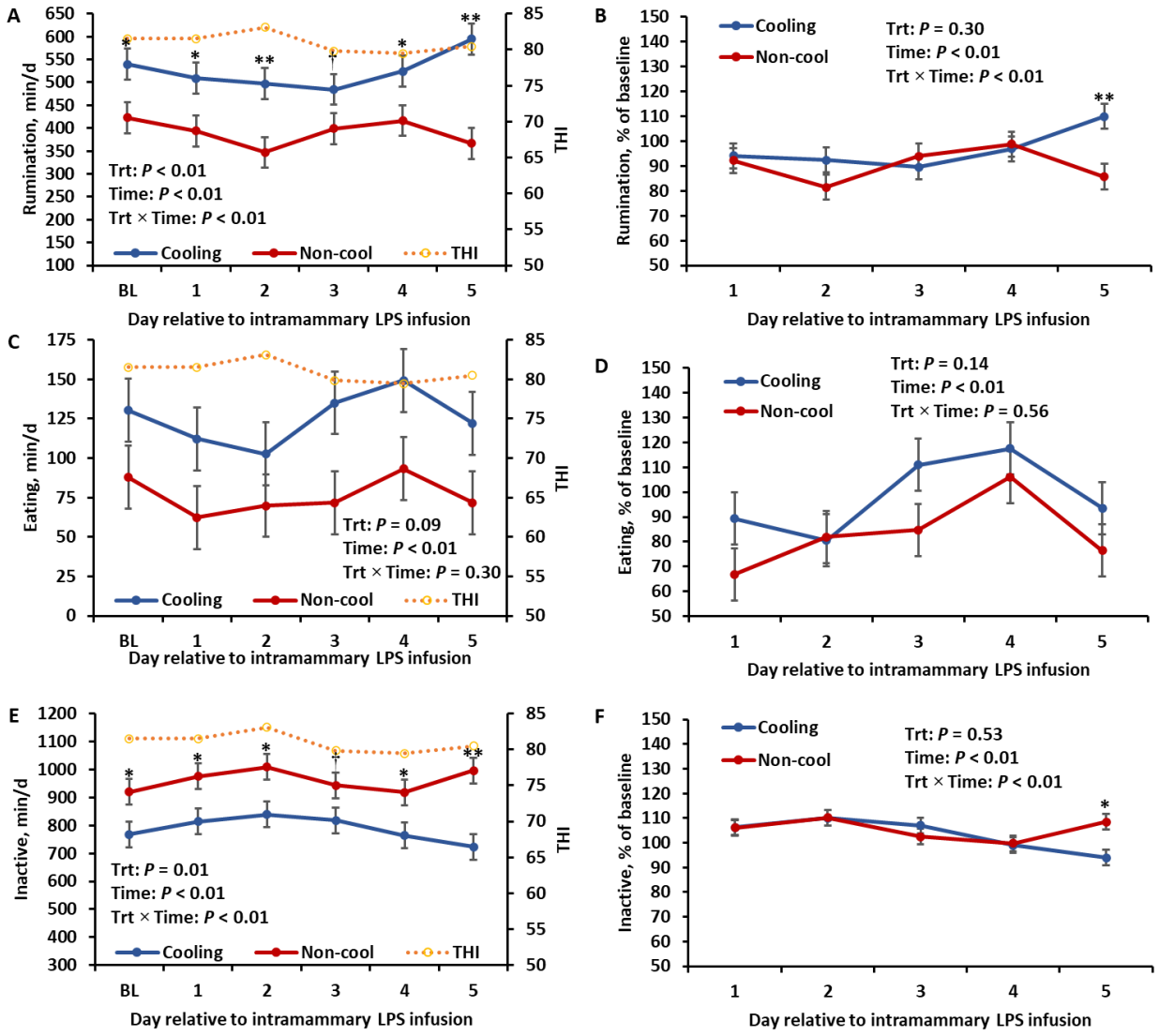

Figure 7.5: Rumination time (min/day: A; percentage relative to baseline data: B), eating time (min/day: C; percentage relative to baseline data: D), and inactive time (min/day: E; percentage relative to baseline data: F) of cows exposed to evaporative cooling (blue line) or not (red line) during an intramammary LPS infusion. Error bars represent SEM. $^{**}P \leq 0.01$, $^{*}0.01 < P \leq 0.05$, †$0.05 < P \leq 0.10$. Daily average temperature-humidity index (THI): yellow dotted line. TRT represents treatment. BL represents averaged data collected at baseline.

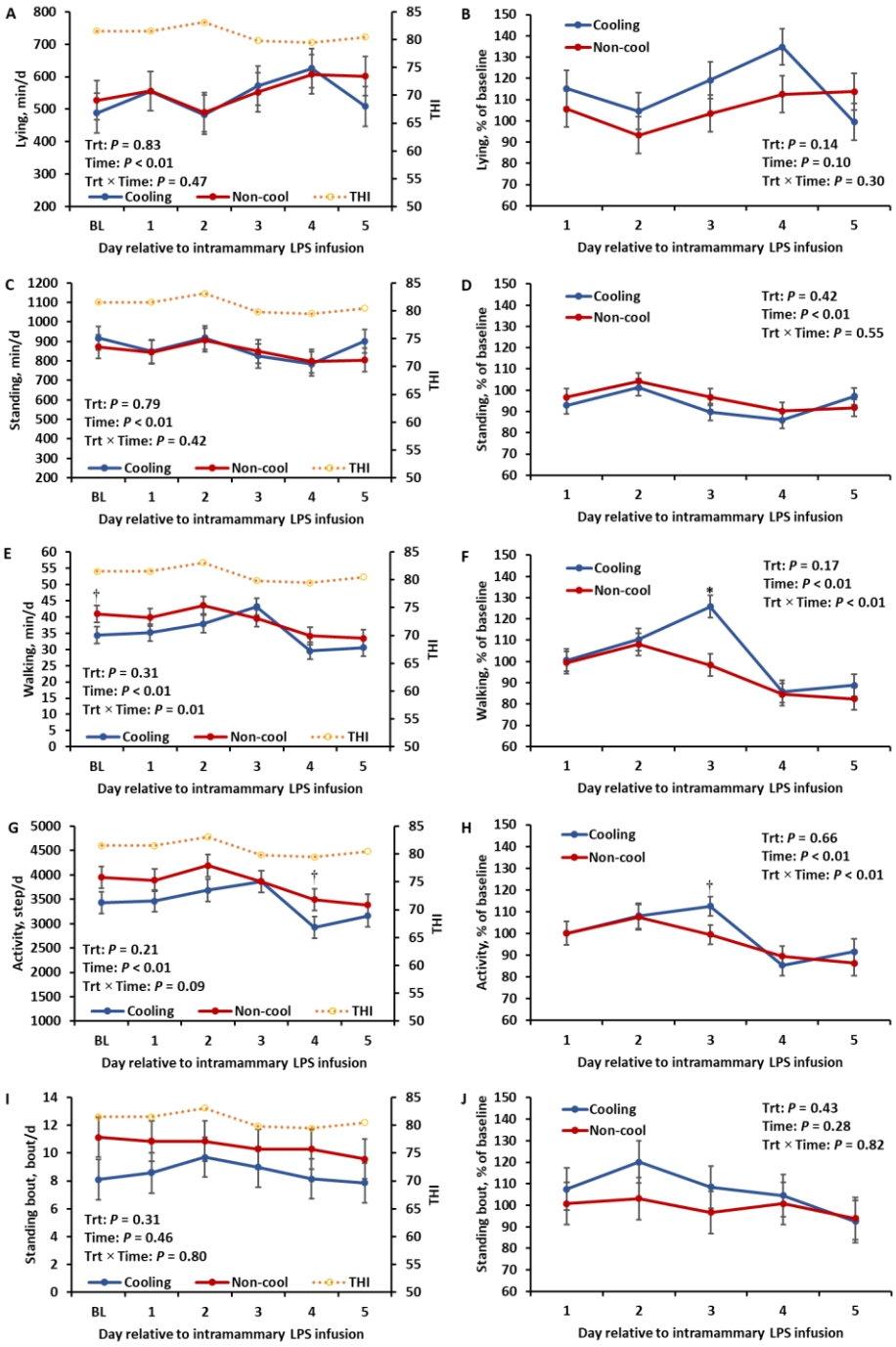

Figure 7.6: Lying time (min/day: A; percentage relative to baseline data: B), standing time (min/day: C; percentage relative to baseline data: D), walking time (min/day: E; percentage relative to baseline data: F), activity (step/day: G; percentage relative to baseline data: H), and standing bout (bout/day: I; percentage relative to baseline data: J) of cows exposed to evaporative cooling (blue line) or not (red line) during an intramammary LPS infusion. Error bars represent SEM. $^{**}P \leq 0.01$, $^{*}0.01 < P \leq 0.05$, †$0.05 < P \leq 0.10$. Daily average temperature-humidity index (THI): yellow dotted line. TRT represents treatment. BL represents averaged data collected at baseline.

CHAPTER 8

ASSESSING THE ASSOCIATION BETWEEN DAILY BODY TEMPERATURE AND BEHAVIORAL RESPONSES OF MULTIPAROUS LACTATING COWS ON FARMS WITH DIFFERENT COOLING SETTINGS AND MILKING ROUTINES[6]

[6] Marins, T.N., J. Gao, C. G. Savegnago, Y-C. Chen, A. A. C. Alves, and S. Tao. To be submitted to *Journal of Dairy Science*.

ABSTRACT

The assessment of animal behavior serves as a valuable approach to identify illness and animal responses to environmental stimuli. Understanding the link between behavioral response and body temperature is critical to enhance animal welfare and performance during heat stress. Our aim is to assess associations between behavior and body temperature of multiparous dairy cows under different types of management during summer. Mid to late lactating Holstein dairy cows from 3 commercial farms were randomly enrolled. Cows in all farms were housed under freestall barns with sand-bedding and fed twice daily. Cows were milked twice daily with a conventional milking system on Farm A (n = 27; parity = 2.2 ± 0.5 [means ± SD]; DIM = 166 ± 44 d; milk yield [MY] = 36.2 ± 7.0 kg/d) and cooled with fans and misters over the feedline. On farm B, cows were milked three times daily in a conventional milking parlor (n = 55; parity = 2.7 ± 0.7; DIM = 221 ± 40 d; MY = 39.0 ± 8.9 kg/d) and cooled with fans over the freestalls and feedline and sprinklers over the feedline. On farm C, cows were milked using automatic milking systems (n = 43; parity = 2.3 ± 0.6; DIM = 210 ± 31 d; MY = 37.5 ± 10.8 kg/d) and cooled with fans only over the freestalls and feedline. Daily resting, walking, and ruminating behaviors were recorded using automated sensor devices (NEDAP). Vaginal temperature (VT) was measured every 5 min by using a thermometer for 5-7 d. Behavioral data were summed by 24-hour periods and the daily average (AvgVT), minimum (MinVT), and maximum (MaxVT) of VT were calculated. A multilevel linear mixed model was developed to investigate the association between behavioral measures and VT variables. On farm A and C, the greater VT of the cow was associated with lower lying time and greater standing time, however, no association was detected on farm B. These data suggest that the effectiveness of the cooling facility affects the associations between lying/standing behavior and the cow's body temperature. No strong associations were observed

between the cow's VT and walking and ruminating behavior partly because cows were under chronic heat stress condition and consistent management. Therefore, this study reinforces the significance of conducting farm-level assessments on cow's physiological and behaviors during summer and highlights the importance of adoption and maintenance of an effective cooling system to mitigate the effect of heat stress on behavioral responses.

Key words: behavior, vaginal temperature, heat stress

INTRODUCTION

It is widely known that heat stress impairs dairy production causing a tremendous negative impact on dairy industry (St-Pierre et al., 2003; Key and Sneeringer, 2014). Compared with other regions in the U.S., the environmental conditions are extremely challenging in the Southeast, like Georgia, due to the longer, hotter, and humid summers (West, 2003; Bohmanova et al., 2007; Key and Sneeringer, 2014). As climate change progresses, heat stress will continue to pose challenges to livestock production (Bernabucci, 2019; Thornton et al., 2021; Cheng et al., 2022) and the adoption of cost-effective heat abatement strategies is becoming more critical to maintain animal productivity, achieve the optimal welfare of dairy cows, and ensure the producer's economic returns (Polsky and von Keyserlingk, 2017; Gunn et al., 2019).

The magnitude of heat stress experienced by dairy cows is influenced by production systems, the surrounding microenvironment, and management practices (Dikmen and Hansen, 2009; Shephard and Maloney, 2023; Leandro et al., 2024). The adopted cooling settings can be different depending on farm infrastructure, investment capacity, and management practices (Toledo et al., 2022; Espinoza-Sandoval and Calsamiglia, 2023; Grinter et al., 2023). Thus,

seeking improvements in heat abatement facilities to promote better comfort for dairy cows is continuously needed in different management systems.

The use of temperature-humidity index (THI) has been widely used for assessing the environmental conditions to which cows are exposed to and for evaluating the intensity of heat stress (Bohmanova et al., 2007; Zimbelman et al., 2009). However, animal-related measurements using phenotypic indicators can be more effective to identify and assess the severity of heat stress in individual dairy cows or groups (Becker and Stone, 2020; Hoffmann et al., 2020). For instance, in dairy cattle, the change in body temperature is used in daily management for the detection, diagnosis, and prediction of diseases, reproductive status, and heat stress events (Suthar et al., 2011; Koltes et al., 2018; Lee et al., 2024). The surrounding thermal environment alters cow body temperature, and the disrupted thermo-balance leads to increased heat load carried by a cow and consequently the increment of body temperature (Dikmen and Hansen, 2009; Wheelock et al., 2010). Additionally, measuring animal behaviors such as rumination and resting behaviors is a valuable tool for predicting or detecting illness, then offering insights into how animals immediately respond to environmental changes. These may improve management strategies, farm performance, and animal welfare (Tucker et al., 2004; Weary et al., 2009; von Keyserlingk and Weary, 2017; Eckelkamp and Bewley, 2020).

In dairy cattle, exposure to heat stress is associated with behavioral shifts to reduce heat load (Nordlund et al., 2019; Toledo et al., 2022). The increased body temperature has been reported to correlate with reduced lying time (Cook et al., 2007; Herbut and Angrecka, 2018; Tullo et al., 2019) and rumination time (Soriani et al., 2013; Abeni and Galli, 2017; Moretti et al., 2017). On-farm assessments with facility-specific data empowers farmers to identify areas for improvement and animal-based measures, such as body temperature and behavior, are essential on guiding target

management decisions to optimize animal welfare, mitigate heat stress, and consequently enhance farm productivity (Whay et al., 2003; Sumner et al., 2018; Reuscher et al., 2024). The use of technology for behavioral measurements offers valuable insights into the dynamics between animals and their environment within modern farming systems, offering key information on how cows interact and engage to various stimuli (Linstädt et al., 2024). However, it is known that cow's physiological and behavioral responses may be affected by intrinsic and extrinsic factors (e.g., parity, reproductive status, milk yield, feeding management, type of housing system, climate, type of heat abatement, etc.) (Stone et al., 2017; Beauchemin, 2018; Tucker et al., 2021). Thus, a comprehensive understanding of the relationship between body temperature and cow behavior under heat stress in different types of facility and heat abatement settings in individual farms becomes essential to achieve better decisions on cow management to improve animal production and welfare.

We hypothesized that the association between daily body temperature and behavioral responses varies with different facility, cooling setting and management in different commercial dairy farms. Thus, the objective was to evaluate the association between body temperature and behavioral responses of multiparous lactating cows in commercial dairy farms with different milking management and cooling systems.

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MATERIALS AND METHODS

All procedures and animal handling were approved by the University of Georgia Institutional Animal Care and Use Committee (AUP#: A2021 03-030-Y1-A0) before the initiation of the study. This study was conducted during a summer (June to August) in 3 different commercial dairy farms in Georgia, U.S. Data used herein were retrieved from heat audits aiming to assess the

effectiveness of cooling systems in different areas of dairy operations and to provide feedback and recommendations to dairy producers for improvement on heat abatement management during summer.

Farms and Data Collection

Upon the approval of farm owners, 3 farms were enrolled in this project. In each farm, mid-to-late lactating Holstein dairy cows were randomly selected based on the availability of animals, excluding cows in their first lactation and with clinical diseases within two months prior to the data collection period. In all farms, cows were housed in freestall barns with sand-bedding and fed twice daily using farm-specific rations formulated by their own nutritionists. The adopted milking system and the heat abatement strategies differed between farms. The specific management information of 3 farms is summarized in Table 8.1.

During the heat audit, the working conditions of the heat abatement facility (e.g., fans or water-based system) were recorded, and producers were informed and recommended to take actions.

Farm A

Farm A housed 130 lactating Holstein cows who were milked 2×/d in a double-8 herringbone milking parlor. Individual milk yield was recorded at each milking (Alpro, DeLaval, Kansas City, MO). Cows were housed in different pens under the same free-stall barn and had ad libitum access to water. Feed was delivered as TMR 2×/d. The cooling system included fans over the feedline and holding area. Misters were placed on the front face of each fan over the feedline. Fans run continuously when the air temperature exceeds 18 °C, and mistered were turned on when air temperature exceeds 18 °C with a 1 min on and 4 min off cycle. A total of 27 lactating cows (parity = 2.2 ± 0.5 [means ± SD] (Range: 2-4); DIM = 166 ± 44 d (Range: 101-248); MY = 36.2

± 7.0 kg/d (Range: 22.1 - 48.2) were selected from two pens. Twelve cows were selected from pen 1 (parity = 2.1 ± 0.3; DIM = 167 ± 41 d; milk yield [MY) = 34.0 ± 7.8 kg/d) and 15 cows were selected from pen 2 (parity = 2.3 ± 0.6; DIM = 165 ± 48 d; MY = 38.0 ± 6.0 kg/d). Both pens had same size and were at similar distance to the milking parlor.

Farm B

Farm B housed approximately 950 lactating Holstein cows. Cows were milked 3×/d in a double-20 parallel parlor. The milking parlor was not equipped with milk meters and the monthly DHIA test-day milk yield performed two days after the data collection period was used to assess the MY of individual cow. Cows were housed in four adjacent sand-bedding free-stalls barns with ad libitum access to water. The TMR was delivered 2×/d. The cooling system included fans over the bedding area, feedline and holding area. The sprinklers were placed over the feedline and in the holding area. Fans run continuously when the air temperature exceeds 17 °C, and sprinklers were turned on when the air temperature exceeds 17 °C with a 1 min on and 5 min off cycle. A total of 55 lactating cows (parity = 2.7 ± 0.7 (Range: 2-4); DIM = 221 ± 40 d (Range: 137-290 d); MY = 39.0 ± 8.9 kg/d (Range: 23.0 - 55.9 kg/d)) were selected from 3 pens. Twenty eight cows were selected from pen 1 (parity = 2.8 ± 0.7; DIM = 193 ± 28 d; MY = 42.7 ± 7.7 kg/d), 13 cows were selected from pen 2 (parity = 2.7 ± 0.6; DIM = 254 ± 31 d; MY = 34.5 ± 6.6 kg/d), and 14 cows were selected from pen 3 (parity = 2.6 ± 0.6; DIM = 245 ± 26 d; MY = 35.8 ± 10.2 kg/d). Pen 2 was adjacent with Pen 3 under the same barn. Pen 1 was in the adjacent barn.

Farm C

Farm C housed approximately 250 lactating Holstein cows with a guided automatic milking system (AMS). Individual milk yield was recorded at each milking by the integrated AMS milk meters. Cows were housed in two pens under the same freestall barn. All cows had ad libitum

access to water and received the same pellet in AMS. Cows were fed the same partially mixed ration (PMR) 2×/d. Both pens had the same size and cows had access to 2 units of DeLaval VMS V300 robotic milkers (DeLaval, Tumba, Sweden) in each pen. The cooling system includes fans placed over the feed line and bedding area. No fans were placed within the AMS waiting area. No sprinklers or misters were equipped. A total of 43 lactating cows (parity = 2.3 ± 0.6 (Range: 2-4); DIM = 210 ± 31 d (Range: 100-261 d); MY = 37.5 ± 10.8 kg/d (Range: 17.8 - 62.6 kg/d)) were selected. In pen 1, 21 cows were selected (parity = 2.3 ± 0.7; DIM = 210 ± 38 d; MY = 37.6 ± 10.3 kg/d) and 22 cows were selected from pen 2 (parity = 2.3 ± 0.5; DIM = 211 ± 24 d; MY = 37.4 ± 11.5 kg/d).

Data collection followed the same protocol for all farms. The data collection period lasted 7 d. Two days before the onset of data collection, all equipment was installed, sensors were placed on cows, and cooling facility was evaluated. In addition, a survey with the farm manager was conducted to collect information related to management and facility operation. Following the data collection period, all equipment and sensors were removed. Herd and individual performance data were retrieved from PCDart (Dairy Records Management Systems, Raleigh, NC) of farm A and B, and from DELPRO™ FarmManager system (DeLaval International AB, Tumba, Sweden) of Farm C.

The air temperature and relative humidity of the barn were monitored every 10 min throughout the study using two Hobo Pro Series Temp probes (Onset Computer Corp., Pocasset, MA) in each pen, then the averages were calculated. The THI was calculated based on THI = (1.8 × T + 32) – ([0.55 – 0.0055 × RH] × [1.8 × T – 26]), where T = air temperature (°C) and RH = relative humidity (%) (NRC, 1971).

The vaginal temperature was measured using an iButton (Mouser Electronics, Mansfield, TX) attached to a blank (progesterone-free) intravaginal implant (CIDR - Zoetis Animal Health) every 5 min, as described by Dikmen et al. (2014) and Kaufman et al. (2018). The accuracy of iButton loggers were tested using a 37.5°C water bath with several increments of temperature at varying intervals to reach 40°C, ensuring gradual changes. Only the units with similar values compared among them were used in the study, as described by Burdick et al. (2012) and Kaufman et al. (2018).

All cows were fitted with automated sensor devices (NEDAP SmartTag Neck and SmartTag Leg sensors, NEDAP Livestock Management, Groenlo, the Netherlands). SmartTag Leg was attached to the right front leg to record lying, standing, and walking time, and activity (step count) every 15 minutes (Van Erp-Van der Kooij et al., 2016; Nielsen et al., 2018). SmartTag Neck measured ruminating time every minute (Borchers et al., 2021). SmartTags have a G-sensor, which measures accelerated movements in a particular direction, based on a three-dimensional (x-, y-, and z-axes) accelerometer. Data was transferred via an Ethernet connection from a local receiver station to NEDAP servers, and then datasets were generated.

Further, locomotion score (LS) was individually assessed for each cow on the first day and last day of the study. The 5-point locomotion scoring procedure suggested by Sprecher et al. (1997) was implemented, which is based on characteristics of gait and posture both during locomotion as well as when the animal is standing. A cow was considered as lame when LS equaled to or exceeded 3. Only cows with LS 1 or 2 were introduced in the study. Additionally, clinical mastitis was diagnosed as “abnormal milk” in line with National Mastitis Council (Smith et al., 2001) by any physical change in the milk, such as clots and flakes during the foremilking at each milking. During the study period, the cows were constantly monitored for any abnormal signal that could

represent an illness. In addition, all cows were pregnant on Farm B, while there were both pregnant and non-pregnant cows on Farm A and Farm C. However, during the data collection period, no cows had visual signals of a heat event and did not receive heat alert from the automated sensor devices.

Data processing and statistical analysis

The behavioral data collected from NEDAP servers were visually checked for errors and then summed by day (minute/day or step/day) using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). If the sum of lying, standing, and walking on a specific day was lower than 1440 min/day, data were removed from analysis. The vaginal temperature (VT) measurements were used to calculate the daily average (AvgVT), minimum (MinVT), and maximum (MaxVT). The daily Avg, Max, Min, and Cv of barn THI were calculated. The percentages of time when barn THI $\geq$ 68 (PCT68), $\geq$72 (PCT72), and $\geq$78 (PCT78) within a day were calculated as well. The lactation stage was categorized into: mid lactation with 100 < DIM $\leq$ 200 and late lactation with DIM $\geq$ 201. The production level (MYC) of the cow was categorized into: low where 18 $\leq$ MY $\leq$ 36 kg/d and high where MY > 36 kg/d.

On Farm A, two cows lost the intravaginal device, two cows had lameness (score 3 and 4) during the data collection period, and one cow had malfunctioned leg sensor tag. On Farm B, one cow had lameness (score 3), and two cows had clinical mastitis. On Farm C, two cows had clinical mastitis, two cows had lameness (score 3 and 4), one cow lost her intravaginal device, and one cow had malfunctioned neck tag. The data from these cows were removed from analysis. Therefore, data from 27 cows on farm A, 55 cows on farm B, and 43 cows on farm C were included in the final analysis. On the last day of the data collection period, the freestalls were rebedded with sand on farm A. On farm B, pregnancy diagnosis was performed on the second last day of the data

collection period followed by cow regrouping on the last day of the data collection period. Data collected during these days were removed from final analysis.

Data preprocessing and statistical modeling were performed in RStudio (version 2025.05.0; RStudio Team, 2025) using R 4. 4. 2. (R Core Team, 2024). The normality of behavioral variables was assessed using histogram plots and Quantile-Quantile plots of the calculated residues and confirmed to be normally distributed.

Pearson correlation analyses between the response variable (lying time, standing time, walking time, activity, or ruminating time) and the explanatory variable (AvgVT, MinVT, and MaxVT) were conducted to explore the relationship between two variables. Additionally, a multilevel linear mixed model (LMM) was developed to investigate the association between the response variable and the explanatory variable. Initially, a base model was constructed using stepwise selection to identify the optimal combination of fixed effects (Farm [combined analysis only], MYC, and the stage of lactation) using Akaike Information Criterion (AIC) as the criterion. Random effects (Cow and Day_Farm_Pen) were included to properly account for the nested data structure and avoid pseudoreplication. Subsequently, individual explanatory variable (AvgVT, MinVT, and MaxVT) was added to the base model to assess its contribution to the model's explanatory power, and the models were compared using likelihood ratio tests to assess the statistical significance of the inclusion of the explanatory variable. The marginal coefficient of determination (R^2m) was calculated for the base model and full model (baseline model + VT variable) to quantify the proportion of variance explained by the fixed effects in the mixed model, then ΔR^2 values (R^2m of full model - R^2m of base model) were reported to indicate the change in explained variance.

Initially, a combined analysis using data collected from 3 farms was performed. The best fitted base models only included Farm as a significant fixed effect for lying, standing, walking, and activity. The best fitted base model for ruminating time included Farm and MYC as fixed effects. These suggest the behavioral patterns of cows were farm specific. Therefore, data collected from 3 farms were analyzed separately. All statistical tests were performed at a significance level of $\alpha = 0.05$, and then an association was declared when $P \leq 0.05$.

RESULTS

General observations of cooling system

During the heat audits, on Farm A, fans were not provided in the bedding area. Over the feedline, we observed several broken fans. In addition, the wind speed provided by individual fans were examined using a hand-held anemometer (Kestrel 3000; Nielsen-Kellerman Co., Boothwyn, PA) for a 1 min-period. Some fans over the feedline (9 out of 10) and holding area (2 out of 6) failed to maintain a consistent air speed equals to or above 3 m/s (Brouk, et al., 2003) at 1.5 meters above the floor at a distance equivalent to two fan spacings (pole to pole) due to the incorrect angle of fan position and the debris accumulated on the fans that reduced the efficiency of airflow. Several misters placed on the front face of each fan (3 out of 10) over the feedline were clogged causing reduced water emission. Further, there was a water pressure issue on farm A, especially during the hottest time of the day when the water consumption was maximized due to drinking and mistering. These all compromised the efficiency of misters for watering cows. These findings indicate that the cooling system was not efficient, and cows were not sufficiently cooling on farm A.

On Farm B, only few fans did not keep a consistent air speed above 3 m/s at 1.5 meters and 1 m/s at 0.5 meters (Reuscher et al., 2024) above the floor at a distance equivalent to two fan spacings over the bedding area (8 out of 30), over the feedline (3 out of 15) and the holding area (2 out of 6). We observed that 3 sprinklers over the feedline (out of 75) and 2 in the holding area (out of 12) were not functioning properly due to clogging, compromising the efficiency of watering cows at certain areas of the feedline and in the holding area. Although some minor issues were found, the heat abatement system on farm B was considered more intense and efficient for cooling cows compared with the other 2 farms.

Farm C adopted fans as the only cooling strategy. No fans were placed within the AMS waiting area. All fans placed over the feed line and bedding area had consistent air speed above 3 m/s at 1.5 meters above the floor at a distance equivalent to two fan spacings. The lack of watering system was concerning especially when air temperature exceeds the cow's surface temperature, and the conductive and convective cooling was minimized. The lack of effective evaporative cooling is a major weakness of the heat abatement management on farm C compared to the other 2 farms.

Environment

The THI varied during the data collection period at each farm (Table 8.2). Farm A had the lowest average THI (73.2 ± 4.7 [mean ± SD], ranged from 70.6 to 76.4), followed by farm B (75.4 ± 3.9 ranged from 73.3 to 77.7) and farm C (77.4 ± 3.7 ranged from 75.8 to 78.4). During the data collection period, cows on farm C were always exposed to THI ≥ 68 while cows experienced 98% of the time with THI ≥ 68 on farm B and 84% of the time on farm A. Further, cows on farm C experienced 44.0% of the time with THI ≥ 78 while 27.6% on Farm B, and 14.7% on Farm A.

Vaginal temperature

The descriptive data of daily vaginal temperature for each farm is shown in Figure 8.1. On farm A, the daily AvgVT ranged from 39.6 ± 0.5°C (Day 2) to 39.9 ± 0.6°C (Day 6), MaxVT ranged from 40.3 ± 0.6°C (Day 1) to 40.6 ± 0.07°C (Day 6), and MinVT ranged from 39.0 ± 0.47°C (Day 2) to 39.2 ± 0.50°C (Day 6). On farm B, the daily AvgVT ranged from 39.2 ± 0.6°C (Day 1) to 39.4 ± 0.5°C (Day 5), MaxVT ranged from 39.9 ± 0.5°C (Day 2) to 40.3 ± 0.6°C (Day 5), and MinVT ranged from 38.5 ± 0.6°C (Day 1) to 38.6 ± 0.5°C (Day 5). On farm C, the daily AvgVT ranged from 39.7 ± 0.5°C (Day 1) to 40.0 ± 0.6°C (Day 5), MaxVT ranged from 40.3 ± 0.6°C (Day 1) to 40.8 ± 0.7°C (Day 5), and MinVT ranged from 39.0 ± 0.5°C (Day 1) to 39.3 ± 0.6°C (Day 5).

Behavioral response

Daily lying and standing time, walking time and activity, and ruminating time for each farm across the data collection periods are presented in Table 8.3, 8.4, and 8.5, respectively. On farm A, the lying time ranged from 731.2 to 847.0 min/d, standing time ranged from 564.3 to 680.2 min/d, walking time ranged from 28.7 to 34.0 min/d, activity ranged from 2737.2 to 3196.6 steps/d, and ruminating time ranged from 483.5 to 529.7 min/d. On farm B, the daily lying time ranged from 705.2 to 800.4 min/d, standing time ranged from 578.3 to 672.8 min/d, walking time ranged from 61.1 to 62.1 min/d, activity ranged from 4768.2 to 4922.6 steps/d, ruminating time ranged from 489.7 to 521.1 min/d. On farm C, cows spent 650.5 to 741.1 min/d lying down, 674.6 to 761.5 min/d standing up, 24.2 to 28.0 min/d walking with 2499.6 to 2769.2 step/d, and 426.4 to 465.2 min/d ruminating.

The combined analyses including data from all three farms is provided in Table 8.6. The separate mixed model analyses for farm A, farm B, and farm C are shown in Table 8.7, 8.8, and 8.9, respectively. In the combined analysis, all behavioral responses revealed weak to moderate correlations ($P < 0.01$) with VT variables. The lying time was negatively correlated with all VT variables, while standing time was positively correlated with VT variables. Both walking time and activity of cows were negatively correlated with AvgVT, MinVT, and MaxVT. The ruminating time was positively correlated with MaxVT. In the mixed model analyses, farm was the only significant fixed effect to be included in the base models for lying, standing, walking, and activity. The base model for ruminating time included farm and MYC. The explanatory improvement of mixed model was observed when AvgVT and MaxVT were included in the model with MaxVT having the greatest ΔR^2 for both lying (0.06) and standing time (0.05). The inclusion of VT variables did not improve ($P > 0.1$) the fit of base models of daily walking time and activity. The inclusion of AvgVT ($\Delta R^2 = 0.03$) and MaxVT ($\Delta R^2 = 0.02$) improved (LRT $P = 0.01$) the R^2 of base model for daily ruminating time.

On farm A, lying time exhibited negative ($P < 0.01$) correlations while the daily standing time had positive correlations ($P < 0.01$) with all VT variables. Walking time, activity and ruminating time of cows were positively ($P \leq 0.04$) correlated with AvgVT, MinVT, and MaxVT. In the mixed model analyses, the stages of lactation were the only significant fixed effect to be included in the base models for all behavioral variables. For lying and standing time, additions of AvgVT, MinVT and MaxVT improved the fit of the base model, with MaxVT having the greatest improvements ($\Delta R^2 = 0.09$ and 0.10 for lying and standing time, respectively). Adding VT variables into the base models of walking time and activity did not ($P \geq 0.33$) improve the R^2. For

daily ruminating time, the addition of AvgVT had a significant improvement ($\Delta R^2 = 0.08$, LRT $P = 0.02$) on the R^2 of the model.

On farm B, the daily walking time and activity had positive correlations ($P < 0.01$) with AvgVT, MinVT, and MaxVT. The ruminating time had a negative correlation with MinVT ($P = 0.01$). In the mixed model analyses, the base models for lying time, standing time and ruminating time included the MYC, while the base models for walking time and activity included the stages of lactation. Interestingly, the addition of VT variables did not improve the fits of the models for lying time, standing time, walking time, and activity. For daily ruminating time, adding MaxVT ($\Delta R^2 = 0.04$, LRT $P < 0.01$) improve the fit of the model.

On farm C, lying time exhibited negative correlation with MaxVT ($P < 0.01$), while the standing time had positive correlations with MaxVT ($P < 0.01$). The walking time had a negative correlation with MinVT ($P = 0.03$), while the activity had no correlations with VT variables ($P > 0.05$). The daily ruminating time had ($P < 0.01$) positive correlations with AvgVT, MinVT, and MaxVT. In the mixed model analyses, the base models for lying time, standing time and activity included the stages of lactation, while the base models for walking time and ruminating time included the MYC. The addition of AvgVT ($\Delta R^2 = 0.14$) and MaxVT ($\Delta R^2 = 0.15$) into the base model of lying time or standing time resulted in significant (LRT $P < 0.01$) improvements on the R^2 of the model. However, no significant improvement of the model for walking time (LRT $P > 0.05$) and activity (LRT $P > 0.05$). For the daily ruminating time, adding MinVT improved the fit of the model ($\Delta R^2 = 0.04$, LRT $P = 0.03$).

DISCUSSION

This study was performed during mid-summer; therefore, THI did not show extreme fluctuation across days at each farm. During the heat audit conducted on farm A, cows were exposed to lower AvgTHI compared with cows on farm B and farm C, but cows on all farms experienced intensive and consistent heat stress. Zimbelman et al. (2009) reported that the reduced lactation performance of high-producing dairy cows was observed when daily average THI exceeds 68. However, the THI threshold for heat stress could be lower depending on geographic locations and the animal traits examined, such as milk component yields (Campos et al., 2022; Moore et al., 2024) and reproductive traits (Gernand et al., 2019). When using behavioral responses to determine the THI threshold, Heinicke et al. (2018) reported that high-yielding Holstein-Friesian dairy cows in the moderate climatic zone of Germany had significantly decreased lying time when THI exceeds 67. In addition, Allen et al. (2015) reported fewer cows standing when THI was below 68 compared with cows exposed to higher THI. Hut et al. (2022) evaluated the behaviors of lactating dairy cows from commercial farms with conventional milking parlor and AMS and with inadequate cooling facility (equipped with few fans but without water systems) in a temperate climate and reported that lying time started to decrease when THI reaches 56 with a more significant reduction when THI exceeds 72. More recently, Lemal et al. (2024) proposed THI thresholds of 66 for the reduced rumination time of Holstein cows in the Walloon Region of Belgium with a temperate maritime climate. A clear THI threshold for behavioral changes by heat stress has not been identified, especially in subtropical areas with elevated temperature and humidity such as the Southeastern U.S. In this present study, because the daily AvgTHI was consistently greater than 68, the behavioral responses of cows may have already been altered by heat stress. Thus, the data presented herein do not reflect the cow responses to immediate

heat stress exposure but represent the associations between behaviors and body temperature of chronically heat-stressed cows under different management conditions.

The increase in body temperature not only occurs during heat stress conditions but also results from feverish responses following infectious diseases (Lee et al., 2016) and estrus (Dolecheck et al., 2015). In this present study, cows were free of diseases or heat events, therefore, variations in body temperature among cows can be attributed to individual animal factors (Buhr et al., 2010), including but not limited to stages of lactation and production levels, and differences in farm management practices, such as milking routines and the effectiveness of heat abatement systems (Gaughan et al., 2023; Harvatine, 2023). Montes et al. (2023) indicated that activities associated with walking to the milking parlor and the high temperature in the parlor are associated with increased body temperature of early-lactating dairy cows. On farm B, cows were milked 3 ×/d and the distance from free-stall barns to milking parlor is longer compared with farm A and C. Coupled with the greater intensity of heat stress (greater THI) during the data collection period, cows on farm B may experience more heat challenges. However, cows on farm B exhibited lower daily AvgVT and MinVT, further confirming that the cooling facility on farm B was effective to regulate cow body temperature. This is achieved by more efficient fans over free stalls, feedline and holding pen and sufficient soaking over the feedline.

Dairy cattle's behavioral responses to heat stress vary according to the duration and intensity of thermal challenges, and the cow's individual characteristics such as breed, age, and physiological state (Polsky and von Keyserlingk, 2017; Herbut et al., 2018). In this study, in the combined analysis including cows from 3 farms, farms have significant effect on the behavioral responses, which may be expected due to different milking management, cooling systems, diets

and environmental conditions. This also suggests that the associations between behaviors and VT variables are farm specific and there is a need for separate analyses by farm.

On farm A and C, in the mixed model analysis of lying or standing time, the base models only include the stage of lactation. Similarly, the stage of lactation was identified as a significant variable in explaining variations of lying time in lactating dairy cows on farms with both conventional milking system and AMS (Løvendahl and Munksgaard, 2016; Solano et al., 2016; Westin et al., 2016). Chaplin and Munksgaard (2001) reported that cows in early lactation (<100 DIM) spent less time lying than cows in late lactation (>200 DIM). Bewley et al. (2010) reported that cows at the later stage of lactation (>150 DIM) had greater lying time compared with cows in the first half of lactation (⩽150 DIM). Cows in early or mid- lactation may spend more time eating to meet the nutrient requirements for greater milk production, and consequently less time lying down compared with late lactating cows (Bewley et al., 2010; Løvendahl and Munksgaard, 2016). Additionally, high producing cows in their early and mid-lactation may reduce their lying time because of the discomfort resulting from udder engorgement (Vasseur et al., 2012; Maselyne et al., 2017). Consistently, we also observed mid-lactation cows had lower lying time but greater standing time compared with late lactation cows on farm A and C, and cows with greater milk yield had lower lying time and greater standing time than low producing cows on farm B. However, it is important to note that the marginal R^2 of the base models was very low indicating the stage of lactation or milk yield category is a significant but not a major variable that affects the cow's lying or standing behavior.

Heat stress is a major environmental factor that directly and indirectly modifies the behavior of cattle in both intensive and extensive production systems (Silanikove, 2000; Palacio et al., 2015), changing the daily behavioral budget (Cook et al., 2007). Toledo et al. (2023) reported

that early lactating multiparous Holstein cows in a sand-bedded freestall barn equipped with fans and soakers over the feed line spent 814 ± 23.9 min/d lying during winter, which is greater than the lying time (717 ± 21.1 min/d) of early lactating cows during summer. We previously reported that the deprivation of evaporative cooling (fans + mister) during summer reduced lying time and concurrently increased standing time during summer (Marins et al., 2025). On farm A and C, the addition of AvgVT or MaxVT largely improved the fit of the mixed model for lying and standing times, indicating that hotter cows have lower lying time but greater standing time. It is suggested that dairy cows during heat stress increase their standing time to expose more body surface area to cooler air for heat dissipation (Allen et al., 2015; Nordlund et al., 2019). Additionally, cows may spend more time under the fans and water system to receive cooling (Cook et al., 2007).

Interestingly, on farm B, no improvement was observed when VT variables were added into the mixed model analysis for lying or standing times, suggesting that the body temperature of cows had no apparent associations with their lying/standing behavior. During the study period, we found significant deficiencies in cooling system on farm A. For instance, there were no fans over the freestalls, fans over the feedline did not provide sufficient airflow and some misters did not function properly. On farm C, although fans provided sufficient airflow over the freestalls and feedline, the absence of water system thereby the evaporative cooling hinders the effectiveness of heat abatement especially when air temperature is high (West, 2003; Anderson et al., 2013; Macavoray et al., 2023). Conversely, the intensive and efficient cooling system on farm B was more effective in abating heat stress and regulating cow body temperature. The data in our study may suggest that the consistent and effective heat abatement system minimizes additional changes in behaviors for cooling and maintaining body temperature during summer. In other words, on farms with insufficient cooling facility, cow behavior may change as the heat load of an animal

increases to facilitate cooling. In contrast, the consistent and effective heat abatement can sufficiently reduce the heat load of a cow carries, minimizing the need for behavioral changes.

Further, regardless of the farm, the addition of VT variables had no improvement on the R^2 of mixed model analysis for walking time and activity. The increment of activity (step count) has been shown to be associated with environment (Heinicke et al., 2018; Lovarelli et al., 2024), as greater activity during summer compared to winter (Brzozowska et al., 2014). Antanaitis et al. (2023) reported greater activity (step/h) in cows exposed to THI greater than 72 compared with those exposed to THI lower than 72. In a recent controlled study, we reported that multiparous cows without evaporative cooling during summer had increased walking time and activity (steps/day) compared to those equipped with fan and misters, likely reflecting an effort to identify cooler areas in attempting to heat dissipation and increased frequency to visit water trough (Marins et al., 2025). The lack of association between walking time and activity and VT variables in this study may be explained by the consistent management and weather pattern. In the current study, the data collection periods were relatively short without significant management-related changes (e.g., pregnancy check, freestall rebedding) or extreme weather events (e.g., heat wave, thunderstorm). Thus, consistent management and schedules may minimize variations of walking activities and prevent us from observing any associations between waling time and activity and body temperature. Similarly, Lovarelli et al. (2024) reported no increased activity of cows exposed to prolonged heat stress and cooled by fans.

The mixed model analysis suggested that the stage of lactation or MYC or both is associated with daily rumination time of cows in all 3 farms. Intake is positively related to the ruminating time of a lactating dairy cow (Schirmann et al., 2012; Grant and Cotanch, 2023). Cows in the mid-lactation produce more milk and consume more feed than late lactation cows, and higher

producing cows consume more feed to support greater milk synthesis than lower producing cows. Thus, these associations between the stage of lactation or milk yield and rumination may stem from the differences in feed intake. The addition of VT variables in 3 farms slightly improved the R^2 of the mixed model for daily ruminating time (Farm A: AvgVT; Farm B: MaxVT; Farm C: MinVT), suggesting that cows with greater body temperature are associated with greater ruminating time. Climate factors are associated with the daily pattern of rumination (Müschner-Siemens et al., 2020; Song et al., 2023). There is an unfavorable association between the hot environment and the cow's rumination time (Soriani et al., 2013; Abeni and Galli, 2017), and the reduced daily rumination time has been proposed as an indicator of heat stress. Moretti et al. (2017) reported that rumination time decreases as THI increases, and this decrease was more apparent in high-producing cows. Corazzin et al. (2021) reported that late-lactating Italian Holstein Friesian had reduced daily rumination time under heat stress (THI > 72) compared with the environment with THI below 72. In a recent study, we also observed that cows without evaporative cooling had lower ruminating time compared with cows equipped with cooling (Marins et al., 2025). It is important to highlight that the data collection periods of this study were in mid-summer with a consistent heat stress condition which may lead to a less environmental impact on ruminating time. Additionally, in addition to heat stress, greater metabolic heat production due to greater intake also increases the cow body temperature (Mader and Davis, 2004; Renaudeau et al., 2012). Thus, the positive association between daily ruminating time and VT variables may also reflect the association between ruminating time and feed intakes of a cow (Stone et al., 2017; Beauchemin, 2018).

CONCLUSION

In this study, we found that the effectiveness of the cooling facility strongly affects the associations between lying/standing behavior and the cow's body temperature. In contrast, under chronic heat stress conditions and consistent management, the changes in body temperature by heat stress were not associated with daily walking activity and ruminating time. These results reinforce the importance of conducting farm-level assessments when evaluating behavioral and physiological responses of lactating dairy cows during summer. Our findings also highlight the importance of implementing effective cooling systems to reduce the adverse effects of heat stress on animal behavior.

ACKNOWLEDGEMENTS

The authors thank the participating farms for animal use and data collection. We also appreciate the donation of blank CIDR by Zoetis Animal Health (Kalamazoo, MI). This project was partially supported by the National Institute of Food and Agriculture (Washington, DC) Hatch project (GEO00916).

DECLARATION OF INTEREST

The authors are associated with the University of Georgia and declare no conflicts of interests.

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Table 8.1: Management information of the 3 participating dairy farms.

	Farm A	Farm B	Farm C
Herd lactating cows (n)	130	950	250
Average herd milk yield (kg/d)	33.4	40	30
Milking system	Conventional[1]	Conventional	AMS[2]
Milking time			
Pen 1	0300 / 1400	0700 / 1500 / 2300	-
Pen 2	0400 / 1500	0500 / 1300 / 2100	-
Pen 3	-	0600 / 1400 / 2200	-
Feeding time			
Pen 1	0500 / 1400	0800 / 1500	1000 / 1500
Pen 2	0600 / 1500	1100 / 1800	1100 / 1600
Pen 3	-	1130 / 1830	-
Feed push-up	> 4×/d	> 5×/d	> 4×/d
Pen stocking density	90 - 100%	100 - 110%	100%
Cooling system			
Parlor	Fan	Fan	-
Holding	Fan	Fan / sprinkler	-
Feed line	Fan / mister	Fan / sprinkler	Fan
Bedding	-	Fan	Fan

[1]Conventional: double-8 herringbone parlor for Farm A and double-20 parallel parlor for Farm B.
[2]AMS: Automatic milking system (DeLaval VMS V300 robotic milkers, DeLaval, Tumba, Sweden).

Table 8.2: Descriptive data of Daily THI recorded inside the pens of each farm.

Day	Mean	SD[1]	CV	Min	Max	PCT68	PCT72	PCT78
					Farm A			
Overall	73.2	4.7	6.5	61.2	82.9	84.0	65.9	14.7
1	71.3	4.4	6.2	63.2	77.5	67.4	48.3	0.0
2	70.6	5.5	7.8	61.2	77.9	64.0	46.7	0.3
3	73.5	6.2	8.5	62.9	80.5	72.1	62.4	39.3
4	72.9	2.0	2.8	69.5	77.4	99.0	64.6	0.0
5	74.6	2.4	3.2	70.8	79.2	98.6	83.5	10.9
6	76.4	3.6	4.7	70.9	82.9	98.3	85.7	36.5
					Farm B			
Overall	75.4	3.9	5.2	66.9	83.3	98.0	75.4	27.6
1	73.3	3.4	4.7	68.3	79.0	99.3	52.4	15.4
2	73.6	3.8	5.1	66.9	79.5	90.9	55.3	21.6
3	74.7	2.2	3.0	70.4	78.7	100.0	81.5	4.6
4	77.6	3.2	4.1	71.8	82.4	100.0	97.9	47.9
5	77.7	4.2	5.4	71.2	83.3	100.0	90.0	48.4
					Farm C			
Overall	77.4	3.7	4.7	70.2	84.7	100.0	94.9	44.0
1	75.8	3.7	4.9	70.2	81.1	100.0	71.9	37.9
2	77.4	2.8	3.6	73.2	82.0	100.0	100.0	49.3
3	77.3	3.9	5.0	71.6	82.7	100.0	93.1	44.4
4	78.4	3.9	4.9	72.6	83.6	100.0	100.0	52.4
5	78.1	3.7	4.7	74.0	84.7	100.0	100.0	36.1
6	77.1	3.8	4.9	71.9	82.6	100.0	99.7	43.1
7	77.7	3.3	4.2	72.4	82.5	100.0	100.0	44.8

[1]SD: standard deviation; CV: coefficient of variation (%); Min: minimum; Max: maximum; PCT68: percentage of time with THI ≥ 68; PCT72: percentage of time with THI ≥ 72; PCT78: percentage of time with THI ≥ 78.

Table 8.3: Daily lying time and standing time (min/d) of cows on 3 farms during the data collection period.

Day	Lying				Standing			
	Mean	SD[1]	Max	Min	Mean	SD	Max	Min
				Farm A				
1	781.6	82.0	923	562	626.8	80.3	839	471
2	747.8	81.5	860	592	658.1	79.4	824	545
3	755.8	94.6	887	568	651.7	90.1	836	526
4	847.0	77.9	1001	668	564.3	76.4	736	414
5	754.2	106.4	921	511	654.7	104.9	893	481
6	731.2	89.3	911	479	680.2	88.5	931	504
				Farm B				
1	800.4	95.9	1051	527	578.3	93.3	850	332
2	764.2	93.6	998	518	614.1	91.2	837	385
3	784.3	103.5	1066	497	594.5	101.6	875	321
4	729.9	103.4	1005	515	648.1	103.3	865	384
5	705.2	97.4	943	483	672.8	96.6	892	444
				Farm C				
1	741.1	140.7	1016	411	674.6	137.9	997	400
2	695.5	123.6	940	433	719.0	120.8	968	470
3	666.9	132.2	1007	426	745.5	131.0	996	407
4	650.5	129.6	929	439	761.5	127.9	978	484
5	675.0	144.6	925	397	739.3	141.1	1000	504
6	700.0	125.3	941	422	712.7	121.4	983	475
7	672.6	126.8	927	435	740.7	124.4	974	493

[1]SD: standard deviation; Max: maximum; Min: minimum.

Table 8.4: Daily walking time (min/d) and activity (step/d) of cows on 3 farms during the data collection period.

Day	Walking				Activity			
	Mean	SD[1]	Max	Min	Mean	SD	Max	Min
				Farm A				
1	31.6	7.9	47	18	3034.4	469.5	3991	1985
2	34.0	9.2	60	18	3196.6	600.7	4697	2040
3	32.5	9.1	50	18	3174.7	623.3	4731	2101
4	28.7	6.1	41	15	2737.2	387.8	3659	1836
5	31.1	7.6	44	17	2987.3	520.3	4208	1940
6	28.7	7.1	43	15	2927.0	452.6	3724	2024
				Farm B				
1	61.3	9.4	84	47	4768.2	666.7	6547	3596
2	61.7	8.2	85	47	4850.9	603.6	6488	3968
3	61.1	8.8	83	47	4786.3	596.6	6746	3875
4	62.0	7.3	80	48	4910.1	505.5	6399	3825
5	62.1	7.9	79	46	4922.6	591.6	6679	3809
				Farm C				
1	24.2	8.8	54	11	2499.6	528.3	3993	1754
2	25.5	8.3	45	9	2633.1	530.3	4016	1523
3	27.6	6.9	45	18	2728.3	469.7	3912	1949
4	28.0	8.0	51	15	2769.2	496.1	4223	1919
5	25.6	9.2	50	11	2612.9	559.8	4097	1807
6	27.3	9.1	52	15	2710.1	567.5	4286	2003
7	26.6	7.9	49	12	2705.7	469.3	4145	1835

[1]SD: standard deviation; Max: maximum; Min: minimum.

Table 8.5: Daily ruminating time (min/d) of cows on 3 farms during the data collection period.

Day	Ruminating			
	Mean	**SD[1]**	**Max**	**Min**
		Farm A		
1	520.3	102.1	714	316
2	506.0	100.0	670	292
3	487.9	100.7	654	258
4	490.0	112.2	668	266
5	483.5	98.4	657	313
6	529.7	118.5	732	299
		Farm B		
1	489.7	90.3	706	247
2	497.9	81.8	759	291
3	521.1	90.9	752	310
4	495.1	92.1	806	283
5	512.5	90.2	770	289
		Farm C		
1	465.2	124.9	748	233
2	457.2	118.9	763	214
3	450.3	126.3	652	108
4	440.7	107.0	592	115
5	451.9	109.1	720	205
6	433.7	105.8	688	196
7	426.4	112.5	634	153

[1]SD: standard deviation; Max: maximum; Min: minimum.

Table 8.6: Associations between daily behavioral responses and vaginal temperature variables in a combined analysis including cows from 3 farms.

	Pearson		Mixed Model			
Explanatory[1]	**r**	***p*-value**	**Model**	**R^2m**	**ΔR^2**	**LRT (*p*-value)**
			Lying			
			Base (Farm)	0.08		
AvgVT	-0.18	< 0.01	Base + AvgVT	0.10	0.02	0.04
MinVT	-0.13	< 0.01	Base + MinVT	0.09	0.01	0.18
MaxVT	-0.24	< 0.01	Base + MaxVT	0.14	0.06	< 0.01
			Standing			
			Base (Farm)	0.16		
AvgVT	0.24	< 0.01	Base + AvgVT	0.17	0.02	0.03
MinVT	0.19	< 0.01	Base + MinVT	0.16	0.01	0.17
MaxVT	0.28	< 0.01	Base + MaxVT	0.21	0.05	< 0.01
			Walking			
			Base (Farm)	0.82		
AvgVT	-0.37	< 0.01	Base + AvgVT	0.82	0.00	0.93
MinVT	-0.41	< 0.01	Base + MinVT	0.82	0.00	0.93
MaxVT	-0.28	< 0.01	Base + MaxVT	0.82	0.00	0.34
			Step			
			Base (Farm)	0.78		
AvgVT	-0.35	< 0.01	Base + AvgVT	0.79	0.00	0.59
MinVT	-0.32	< 0.01	Base + MinVT	0.79	0.00	0.76
MaxVT	-0.26	< 0.01	Base + MaxVT	0.79	0.00	0.29
			Ruminating			
			Base (Farm)	0.08		
AvgVT	0.06	0.13	Base + AvgVT	0.11	0.03	0.01
MinVT	0.03	0.39	Base + MinVT	0.09	0.01	0.06
MaxVT	0.10	0.01	Base + MaxVT	0.10	0.02	0.01

[1]Explanatory variables: daily average vaginal temperature (AvgVT), minimum VT (MinVT), and maximal VT (MaxVT); r: Pearson correlation coefficient; Base: a base model that included significant fixed variables after a step-wise selection using Akaike Information Criterion; R^2m: marginal R-squared (proportion of variance explained by the fixed effects, excluding the contribution of random effects); ΔR^2: R^2m of full model (base model + VT variables) - R^2m of reduced model (base model only); LRT: likelihood ratio test; Significance declared when $P \leq 0.05$.

Table 8.7: Associations between daily behavioral responses and vaginal temperature variables on Farm A.

	Pearson		Mixed Model			
Explanatory[1]	**r**	***p*-value**	**Model**	**R^2m**	**ΔR^2**	**LRT (*p*-value)**
			Lying			
			Base (Lactation stage)	0.01		
AvgVT	-0.30	< 0.01	Base + AvgVT	0.12	0.11	0.01
MinVT	-0.25	< 0.01	Base + MinVT	0.08	0.07	0.02
MaxVT	-0.34	< 0.01	Base + MaxVT	0.10	0.09	0.01
			Standing			
			Base (Lactation stage)	0.01		
AvgVT	0.29	< 0.01	Base + AvgVT	0.13	0.11	0.01
MinVT	0.24	< 0.01	Base + MinVT	0.08	0.07	0.01
MaxVT	0.33	< 0.01	Base + MaxVT	0.11	0.10	< 0.01
			Walking			
			Base (Lactation stage)	0.05		
AvgVT	0.20	0.02	Base + AvgVT	0.05	0.00	0.76
MinVT	0.23	< 0.01	Base + MinVT	0.05	0.00	0.66
MaxVT	0.18	0.04	Base + MaxVT	0.05	0.00	0.86
			Step			
			Base (Lactation stage)	0.06		
AvgVT	0.24	< 0.01	Base + AvgVT	0.07	0.01	0.57
MinVT	0.26	< 0.01	Base + MinVT	0.07	0.01	0.49
MaxVT	0.23	< 0.01	Base + MaxVT	0.06	0.00	0.75
			Ruminating			
			Base (Lactation stage)	0.04		
AvgVT	0.26	< 0.01	Base + AvgVT	0.12	0.08	0.02
MinVT	0.28	< 0.01	Base + MinVT	0.07	0.04	0.06
MaxVT	0.20	< 0.01	Base + MaxVT	0.06	0.02	0.19

[1]Explanatory variables: daily average vaginal temperature (AvgVT), minimum VT (MinVT), and maximal VT (MaxVT); r: Pearson correlation coefficient; Base: a base model that included significant fixed variables after a step-wise selection using Akaike Information Criterion; R^2m: marginal R-squared (proportion of variance explained by the fixed effects, excluding the contribution of random effects); ΔR^2: R^2m of full model (base model + VT variables) - R^2m of reduced model (base model only); LRT: likelihood ratio test; Significance declared when $P \leq 0.05$.

Table 8.8: Associations between daily behavioral responses and vaginal temperature variables on Farm B.

	Pearson		Mixed Model			
Explanatory[1]	**r**	***p*-value**	**Model**	**R^2m**	**ΔR^2**	**LRT (*p*-value)**
			Lying			
			Base (MYC)	0.00		
AvgVT	-0.04	0.50	Base + AvgVT	0.01	0.00	0.67
MinVT	-0.01	0.85	Base + MinVT	0.00	0.00	0.89
MaxVT	-0.11	0.07	Base + MaxVT	0.01	0.00	0.45
			Standing			
			Base (MYC)	0.00		
AvgVT	0.03	0.65	Base + AvgVT	0.01	0.00	0.62
MinVT	0.00	0.98	Base + MinVT	0.00	0.00	0.93
MaxVT	0.10	0.11	Base + MaxVT	0.01	0.00	0.50
			Walking			
			Base (Lactation stage)	0.00		
AvgVT	0.18	< 0.01	Base + AvgVT	0.02	0.00	0.59
MinVT	0.17	< 0.01	Base + MinVT	0.02	0.00	0.55
MaxVT	0.18	< 0.01	Base + MaxVT	0.02	0.01	0.24
			Step			
			Base (Lactation stage)	0.03		
AvgVT	0.19	< 0.01	Base + AvgVT	0.03	0.00	0.99
MinVT	0.20	< 0.01	Base + MinVT	0.03	0.00	0.47
MaxVT	0.16	< 0.01	Base + MaxVT	0.03	0.00	0.74
			Ruminating			
			Base (MYC)			
AvgVT	-0.09	0.12	Base + AvgVT	0.08	0.00	0.32
MinVT	-0.16	0.01	Base + MinVT	0.08	0.01	0.30
MaxVT	-0.02	0.76	Base + MaxVT	0.11	0.04	< 0.01

[1]Explanatory variables: daily average vaginal temperature (AvgVT), minimum VT (MinVT), and maximal VT (MaxVT); r: Pearson correlation coefficient; Base: a base model that included significant fixed variables after a step-wise selection using Akaike Information Criterion; R^2m: marginal R-squared (proportion of variance explained by the fixed effects, excluding the contribution of random effects); ΔR^2: R^2m of full model (base model + VT variables) - R^2m of reduced model (base model only); LRT: likelihood ratio test; Significance declared when $P \leq 0.05$.

Table 8.9: Associations between daily behavioral responses and vaginal temperature variables on Farm C.

Explanatory	Pearson		Mixed Model			
	r	*p*-value	Model	R^2m	ΔR^2	LRT (*p*-value)
			Lying			
			Base (Lactation stage)	0.01		
AvgVT	-0.06	0.31	Base + AvgVT	0.14	0.14	< 0.01
MinVT	0.02	0.71	Base + MinVT	0.02	0.01	0.32
MaxVT	-0.17	< 0.01	Base + MaxVT	0.16	0.15	< 0.01
			Standing			
			Base (Lactation stage)	0.01		
AvgVT	0.07	0.27	Base + AvgVT	0.15	0.14	< 0.01
MinVT	-0.02	0.80	Base + MinVT	0.02	0.01	0.33
MaxVT	0.17	< 0.01	Base + MaxVT	0.16	0.15	< 0.01
			Walking			
			Base (MYC)	0.02		
AvgVT	-0.09	0.18	Base + AvgVT	0.02	0.00	0.72
MinVT	-0.14	0.03	Base + MinVT	0.03	0.01	0.38
MaxVT	-0.06	0.35	Base + MaxVT	0.02	0.00	0.46
			Step			
			Base (Lactation stage)	0.01		
AvgVT	-0.06	0.37	Base + AvgVT	0.01	0.00	0.58
MinVT	-0.11	0.07	Base + MinVT	0.01	0.01	0.44
MaxVT	-0.02	0.76	Base + MaxVT	0.03	0.02	0.15
			Ruminating			
			Base (MYC)	0.05		
AvgVT	0.34	< 0.01	Base + AvgVT	0.08	0.03	0.15
MinVT	0.33	< 0.01	Base + MinVT	0.09	0.04	0.03
MaxVT	0.35	< 0.01	Base + MaxVT	0.05	0.00	0.97

[1]Explanatory variables: daily average vaginal temperature (AvgVT), minimum VT (MinVT), and maximal VT (MaxVT); r: Pearson correlation coefficient; Base: a base model that included significant fixed variables after a step-wise selection using Akaike Information Criterion; R^2m: marginal R-squared (proportion of variance explained by the fixed effects, excluding the contribution of random effects); ΔR^2: R^2m of full model (base model + VT variables) - R^2m of reduced model (base model only); LRT: likelihood ratio test; Significance declared when $P \leq 0.05$.

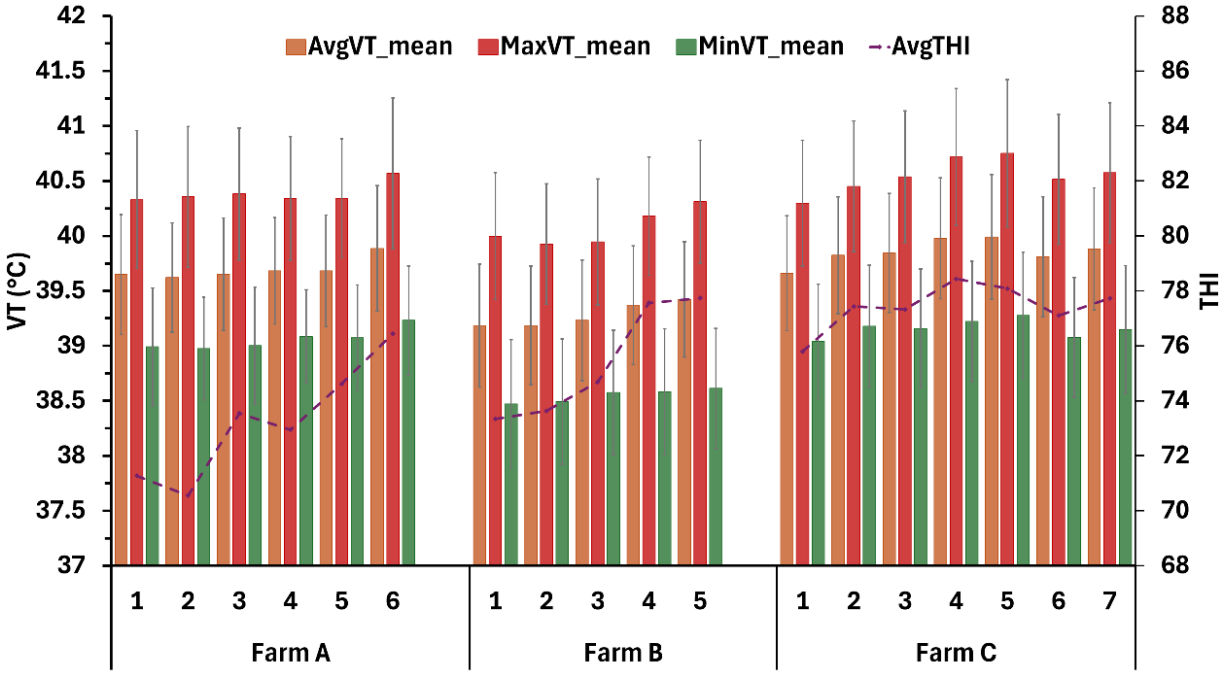

Figure 8.1: Daily average (Avg, orange bar), maximum (Max, red bar), and minimum (Min, green bar) vaginal temperature (VT) across days on Farm A (conventional milking system, 2×/d), Farm B (conventional milking system, 2×/d), and Farm C (automatic milking system). Dashed purple line: daily average temperature-humidity index (THI). Error bars represent standard deviation.

CHAPTER 9

CONCLUSIONS

Undoubtedly, heat stress compromises the health, productivity, and welfare of dairy cows, causing negative economic impact on the farm's profitability and whole dairy industry. Therefore, a comprehensive understanding of the effect of heat stress on performance, physiology, immune response, and behavior patterns allows the implementation of nutritional, environmental, and management strategies to promote productivity, health and welfare of dairy cows during summer. Despite advancements in knowledge and research efforts that have contributed to a better understanding of the impact of heat stress on dairy industry in the last decades and the initiatives that improve the technology in mitigating heat stress, additional studies are still necessary to improve the management of cows under the adverse heat stress conditions.

This dissertation combines applied and basic research to evaluate the impacts of environment, nutrition, and management on productivity and health, aiming to develop effective approaches for identifying and mitigating heat stress and consequently improving health and welfare of dairy cattle. Studies presented in this dissertation utilized various *in vitro* immune measures (e.g., lymphocyte proliferation and cytokine production) and *in vivo* challenge models (e.g., intramammary and intravenous endotoxin infusion) to elucidate the cow's metabolic, immunological and inflammatory responses under heat stress. Results from these studies have shown that deprivation of evaporative cooling influenced the performance, metabolic, immune, inflammatory, and behavioral responses of lactating dairy cows.

Further, nutritional strategies that enhance cow health during summer are poorly understood, and the presented studies have examined the effectiveness of several nutritional supplements on physiology, immune and inflammatory responses, and performance of heat-stressed dairy cows, showing potential benefits to implement nutritional supplementation as an approach to boost immune function and consequently enhance cow responses facing challenges such as heat stress and diseases.

As a summarization, presented in chapter 3-5, where we evaluated the metabolism and inflammation of cows fed two zinc sources (Zn-hydroxychloride vs. Zn-Met complex) under two environmental conditions (cooling [CL] vs. non-cooling [NC]) prior to and following an intramammary lipopolysaccharides infusion (IM-LPS) during summer, we found that non-cooling condition reduced plasma metabolite concentrations, but increased insulin concentration. Dietary Zn source had no impact on systemic and mammary metabolism. Following IM-LPS, NC induced greater leukocyte migration into the mammary gland after IM-LPS, and NC maintained lower plasma glucose concentrations but had a more rapid and prolonged increase in serum insulin concentration than CL. Further, NC influenced the systemic and mammary mineral metabolism after IM-LPS, however, dietary Zn source had no impacts.

In chapter 6, we evaluate hormonal, inflammatory and immunological responses of lactating dairy cows fed an immunomodulatory supplement (OmniGen® AF, OG) and under CL vs. NC prior to and following an intravenous LPS infusion (IV-LPS) during summer. The major results demonstrated that feeding OG enhanced cortisol release under basal conditions and after IV-LPS in CL but not NC, suggesting that heat stress inhibits the OG mediated cortisol release. Further, NC enhanced inflammatory responses of circulating lymphocytes stimulated with

mitogens ex vivo than CL. Yet, OG promoted the proliferation of immune cells isolated from cows under NC.

In chapter 7, we examined the behavioral responses of cows under CL and NC, and the study revealed that NC reduced lying, rumination, and eating time but increased walking time and activity level. In addition, cows under NC sorted more for long particles from the TMR than cows under CL condition.

In chapter 8, we evaluated associations between behaviors and body temperature of lactating dairy cows on 3 commercial dairy farms, and we demonstrated that the effectiveness of cooling facility strongly affects the associations between lying/standing behavior and body temperature but was not associated with daily walking activity and ruminating time under chronic heat stress condition and consistent management.

Additionally, the presented studies identified the cow's behavioral response as an approach to identify, manage, and mitigate the negative effects of heat stress on animals. Understanding environmental conditions and how cow responds to the environmental stimulus provide a holistic evaluation of the heat abatement system and identify areas where improvements are needed. These studies strongly suggest that implementing cost-effective heat abatement strategies are essential to maintaining productivity and maximizing economic returns in dairy operations.

The general findings from the studies present in this dissertation will not only contribute to the scientific community but also it will pave the way for innovative approaches to dairy cattle management. The results will help producers to achieve the best cow productivity and stimulate other industry professionals to identify novel products and strategic interventions to optimize cow productivity and welfare, consequently, contributing to a stronger sustainable dairy industry.