EFFECTS OF HEAT STRESS AND DIETARY ZINC SOURCE ON PERFORMANCE AND PERMEABILITY OF MAMMARY EPITHELIUM OF LACTATING DAIRY COWS

by

XISHA WENG

(Under the Direction of Sha Tao)

ABSTRACT

Heat stress may negatively alter the barrier integrity of mammary epithelium, whereas organic source of dietary zinc has been shown to enhance epithelial integrity. The objective of the first study was to evaluate the effect of environmental heat stress and dietary zinc source (zinc-MET complex vs. zinc hydroxychloride) on lactating Holstein cows' performance and permeability of mammary epithelium. Heat stress impaired cow's performance and increased gene expression of tight junction proteins in mammary tissue. Replacing a portion of zinc hydroxychloride with zinc-MET complex had no impact on cow performance but improved mammary epithelial integrity.

The objective of the second study was to examine the effect of repeated mammary tissue collections during lactation on cow performance and udder health of lactating dairy cows. Lactating cows recovered rapidly from mammary tissue collections and no long term impacts of repeated mammary biopsy procedures on DMI, milk yield and composition, or udder health were observed.

INDEX WORDS: Heat stress, Zinc, Mammary epithelial junction, Lactating cow, Mammary biopsy, Lactation performance

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XISHA WENG

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XISHA WENG

Major Professor:	Sha Tao
Committee:	John K. Bernard
	Stephen C. Nickerson

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my beloved parents and brother. Thank you all for your love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Environmental heat stress is a great concern in the dairy industry, including the southeastern area of the U.S. Heat stress has negative impacts on milk production, dry matter intake (DMI), health, and reproduction in mature cows. In addition, calves born to heat-stressed dry cows have altered metabolism, impaired immune function, and reduced future performance. Therefore, heat stress brings great economic losses in the dairy industry. Although cooling methods such as shade, fans, sprinklers, and nutritional strategies have been widely adopted in the dairy farm today, understanding the mechanisms of how heat stress affects milk production in depth is required in developing more effective methods to cope with this issue.

It had been widely accepted that the reduced DMI during heat stress is the major reason for the reduced milk production, since cows under restricted nutrition cannot synthesize more milk during the hot season. However, when comparing heat-stressed cows with pair-fed counterparts under thermo-neutrality, it was found that the decreased DMI explains approximately half of the reduction in milk production (Rhoads et al., 2009; Wheelock et al., 2010). Thus, postabsorptive metabolic changes or physiological adaptations may occur due to heat stress, which in turn reduce milk synthesis and secretion. Mammary epithelial cells are the milk-secreting cells, and their function is maintained during lactation with an impermeable mammary epithelial junctional complex (Stelwagen and Singh, 2014). An impaired mammary

epithelial junctional structure is related to impaired mammary function, which may explain the reduced milk production (Stelwagen and Singh, 2014) caused by heat stress. Thus, one of our objectives of first study was to evaluate the effect of heat stress on cow performance and mammary epithelial integrity.

Dietary zinc is one of the essential micronutrients in dairy animals, and is a critical element in cow metabolism, performance, as well as the immune system (Vallee and Falchuk, 1993). Recently, researches have also suggested a potential role of dietary zinc in improving epithelial integrity in both in vivo and in vitro conditions (Finamore et al., 2008; Wang et al., 2013; Sanz Fernandez et al., 2014; Pearce et al., 2015). Moreover, different sources of zinc (organic form vs. inorganic form) may perform differently due to the higher bioavailability of the organic form of dietary zinc. Thus, the other objective of our first study was to evaluate the effects of different dietary zinc sources (zinc hydroxychloride vs. zinc-MET complex) on cow performance as well as on mammary epithelial integrity.

Mammary tissue collection is useful to assess physiological changes in animal research, such as evaluating protein expression and localization, or gene expression profiling of the mammary gland. However, the risks of disease, such as mastitis, and potential impairment of performance related to mammary tissue collection are of great concern in dairy research as well as animal welfare communities. Studies indicated that the procedure of mammary tissue collection, using a biopsy needle (Lima et al., 2016) or a drill operating instrument (Farr et al., 1996), only affected milk yield and composition transiently and had no apparent impact on udder health. The long term impact of repeated mammary tissue collection on cow performance and udder health is unclear. Thus, the objective of our second study was to evaluate the effect of

repeated mammary tissue collection using the drill operating instrument on milk production, milk composition, as well as on udder health in an extended period.

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CHAPTER 2

LITERATURE REVIEW

Thermoregulation of Dairy Cows

As homothermic animals, dairy cows are able to maintain a constant body temperature under a temperate environment to coordinate normal physical, biochemical, and physiological processes with their body metabolism (Kadzere et al., 2002). However, climate change, including changes in the environmental temperature, humidity, rainfall, air movement, and radiation, threatens homothermic animals by negatively affecting the balance between heat accumulation and dissipation which results in heat stress (West, 2003).

External forces from the environment, such as heat stress, may cause adverse consequences such as discomfort or even death of animals (West, 2003; Das et al., 2016). In the Southeastern U.S., including Georgia, the extended high ambient temperature combined with high humidity and rainfall, makes heat stress a major issue for the dairy industry (West, 2003). The temperature-humidity index (THI) is a general term to indicate the level of heat stress and estimates cooling requirements for dairy cows (Zimbelman et al., 2009). Although it was generally accepted that a THI above 72 adversely affected high producing dairy cow (Armstrong, 1994), Zimbelman et al. (2009) suggested that a THI above 68 significantly reduced milk production in modern high producing dairy cows, and heat abatement should be implemented before reaching a THI of 68 to prevent adverse effects of heat stress.

The total heat load of a cow includes the heat production associated with internal body metabolism and the accumulated heat from external environment (West, 2003). Cows utilize several routes of heat exchange to dissipate accumulated heat to maintain body temperature, including conduction, convection, radiation, and evaporation. Conductive heat transfer is mediated by direct contact with a material, whereas convection occurs by transferring heat to the surrounding air (Kennedy, 1999). Cows are able to dissipate heat by conduction and convection through cutaneous blood flow to the peripheral environment (Choshniak et al., 1982).

Radiation is also of importance for dairy cows. Cows usually have a net heat gain through radiation during the day and a net heat loss to the cooler surroundings at night (Kennedy, 1999), and cooling methods such as shades is often implemented to reduce solar radiation during the day. However, all non-evaporative heat exchange methods (conduction, convection, and radiation) are temperature dependent and limited by high peripheral temperatures. Their effectiveness for heat dissipation is reduced when the ambient temperature increases. Thus, under a high temperature environment, cows rely on evaporation of water through panting and sweating to dissipate the extra heat (West, 2003). Although temperature independent, evaporative cooling is hindered by high relative humidity (West, 2003). Thus, evaporative cooling systems using sprinklers and fans are utilized by modern dairy farms in high relative humidity areas, such as southeastern U.S., to dissipate heat.

Under heat stress, when heat gain exceeds heat loss through conduction, convection, radiation, or evaporation to the environment, cow's body temperature rises. Physiological adaptations such as reduced milk synthesis or digestion may occur in lactating dairy cows to minimize the internal heat production (Vermunt, 2011). Cows are also able to adapt to heat stress by modifying water metabolism. Under heat stress, cows are more likely to be dehydrated

due to additional evaporative water loss (Silanikove, 1992). It was suggested that heat stress is associated with increased water intake as well as increased water losses via the respiratory tract and the skin (Beede and Collier, 1986; West, 1999). As reviewed by West (1999), cows adapted to thermo neutral condition (21.1°C) increased water intake by 110% after exposed to 32.2°C for 2 weeks, and increased water losses from the respiratory tract and skin surface by 55% and 177%, respectively. The rumen, which serves as a reservoir, is able to compensate for the majority (55-60%) of total water loss during dehydration (Silanikove, 1992). It is suggested that the expansion of rumen volume occurs under thermal stress to increase water content in the rumen and to cope with the water turnover (Silanikove, 1992).

Heat Stress Effects on Lactating Dairy Cows

Heat Stress on Milk Production

Numerous studies have shown that heat stress dramatically reduces milk production in dairy animals. Lactating dairy cows have lower feed intake, decreased milk production, and reduced metabolic rate when the ambient temperature exceeds 25℃ , in order to minimize the internal heat production and to maintain normal body temperature (Beede and Collier, 1986). Zimbelman et al. (2009) reported a daily loss of 2.2 kg/d in milk production in high producing (i.e. daily milk production greater than 35 kg) lactating Holstein cows when the average THI equaled 68. West (2003) found that one unit increase in daily average THI of two days ago resulted in the decrease in milk yield by 0.88 kg/d in Holstein dairy cows. Bouraoui et al. (2002) found a negative correlation between daily average THI and milk yield ($r = -0.76$) or feed intake ($r = -0.24$), as milk yield decreased by 21% and DMI by 9.6% when the THI increased from 68 to 78 in lactating Holstein-Friesian cows. Shwartz et al. (2009) and Wheelock et al. (2010)

observed an approximate 30 to 40% reduction in milk yield of mid-lactation dairy cows when exposed to environmental heat stress relative to thermo neutral conditions. However, studies comparing heat-stressed dairy cows with pair-fed thermal neutral animals found that the reduction in DMI only explains about half of the reduction in milk production (Rhoads et al., 2009; Wheelock et al., 2010). Thus, metabolic or physiological adaptations in dairy animals may occur during heat stress to cope with the excess heat load.

Dairy cows in different stages of lactation have different responses to heat stress in milk production. As reviewed by Bernabucci et al. (2010), mid-lactating dairy cows had the greatest decline in milk yield due to heat stress compared with their early and late lactating counterparts. This may be explained by different nutritional-metabolic conditions of dairy cows during different stages of lactation. Due to negative energy balance, early lactating cows mobilize body tissues to support milk synthesis, thereby producing less metabolic heat per kg of milk produced compared with mid-lactating cows (Bernabucci et al., 2010). Moreover, high producing dairy cows are more sensitive to heat stress compared with low producing or non-lactating dairy cows because of the increased body heat production from metabolic process of milk synthesis (West, 2003).

Effect of Heat Stress on Milk Composition

Environmental heat stress negatively affects milk composition, which alters the quality of milk as well as milk-derived products. Bernabucci et al. (2010) reported a decline in milk protein content when THI exceeded 72, but the impact of THI on milk fat content was inconsistent. However, it was suggested that milk fat content decreases in lactating cows exposed to severe heat stress when air temperature reached or exceeded 31°C (Bernabucci and Calamari, 1998). In the summer season, lactating Holsteins cows had lower milk percentages of fat, protein, total

solids, and solids-not-fat compared with spring or winter seasons (Bernabucci et al., 2015). Similar results were found in heat-stressed Friesian cows, that increasing THI values (from less than 72 to more than 78) were associated with significant decreases in percentages of milk fat, protein, lactose, total solids, and solids-not-fat (Zeinhom et al., 2016). In addition, colostrum of Holstein heifers exposed to heat stress during the prepartum period had lower concentrations of immunoglobulin G and A, total protein, casein, lactalbumin, fat, and lactose compared with cows under a thermal comfort environment (Nardone et al., 1997). During the summer, a higher milk content of IgG and serum albumin was observed for mid-lactation Holstein cows compared with those in spring and winter seasons, which indicates a leakage of mammary epithelium when exposed to environmental heat stress (Bernabucci et al., 2015).

Effect of Heat Stress on Cow Health and Reproduction

As reviewed by Kadzere et al. (2002), the incidence of health problems in livestock, including dairy cows, increases during summer, because of the higher survivability of vectors and other disease-causing agents under warm and humid environments. Moreover, environmental heat stress increases the incidence of reproductive diseases in dairy cows, such as retained placenta and metritis, which may be explained by the influence of environmental heat stress on physiological functions of high producing cows (reviewed by Kadzere et al., 2002).

It is suggested that high ambient temperature is associated with low breeding efficiency in Holstein cattle (Cavestany et al., 1985). Gwazdauskas et al. (1981) reported a dramatically reduced conception rates and increased embryonic losses in heat-stressed cattle during the summer season. The mechanisms of impaired reproductive performance by heat stress are multifactorial; for example, heat stress alters hormonal profiles associated with reproduction, impairs embryo development, and increases embryo mortality (reviewed by Bernabucci et al.,

2010). In addition to declined female reproduction efficiency, bulls have lower semen concentrations and decreased semen quality under elevated environmental temperature, which further contribute to the compromised reproductive efficiency of dairy cattle by heat stress (reviewed by Bernabucci et al., 2010). Due to the extensive genetic selection for higher milk production, dairy cattle experience reduced thermoregulatory ability (Berman et al., 1985), which magnifies the heat stress effect on reduced fertility during the summer season (al-Katanani et al., 1999).

Heat Stress Effects on the Mammary Gland

Traditionally, reduced milk yield under thermal stress was considered as a result from reduced DMI. However, more recent studies indicate that up to 50% of reduced milk yield under heat stress is independent of reduced DMI (Rhoads et al., 2009; Wheelock et al., 2010). Baumgard and Rhoads (2013) suggested that heat stress initiated postabsorptive changes in carbohydrate, lipid, and protein metabolism independent of reduced DMI in dairy cows, which seem to be adaptive mechanisms in order to maintain euthermia. A consequence of the changes in postabsorptive mechanisms is the reduced ability of heat-stressed animals to employ glucose-sparing mechanisms to prioritize the synthesis of products (milk in this case) via homeorhesis (Baumgard and Rhoads, 2013). The postabsorptive metabolic adaptations are shown to be an indirect effect of heat stress independent of reduced feed intake and whole-animal energy balance (Baumgard and Rhoads, 2013).

Mammary Epithelial Cells

The persistency of lactation is determined by the number and activity of mammary secreting cells, also known as mammary epithelial cells (Capuco et al., 2003). The increased

milk yield from calving to peak lactation is a result of the increased secretory activity per mammary epithelial cell, rather than the increasing number of mammary epithelial cells (Capuco et al., 2003). After peak lactation, the decrease in milk yield is mainly due to the increased mammary apoptosis (Capuco et al., 2003). Thus, proper regulation of the number and function of mammary epithelial cells is of importance in maintaining milk synthesis. Thermal stress may directly impact mammary gland function via influencing mammary epithelial cells to impair milk synthesis in dairy cows. In an in vitro study, Collier et al. (2006) reported that the thermotolerance of bovine mammary epithelial cells (BMEC) appeared to be lost after 8 h of thermal stress exposure, which was associated with increased expression of genes in apoptotic pathways, indicating that the BMEC underwent programmed cell death. Similar results have been reported in another in vitro study, in which exposure to heat stress immediately reduced cell viability of buffalo mammary epithelial cells mainly due to the activation of apoptotic pathway (Kapila et al., 2016). In an in vivo study, cows which were cooled during the dry period (approximately 46 d before calving) had higher mammary epithelial cell proliferation rate than non-cooled heat-stressed cows before calving, suggesting that heat stress during the dry period inhibits mammary growth and subsequent milk production (Tao et al., 2011). Thus, it is indicative that heat stress affects mammary gland function by mammary apoptosis and cell proliferation, which impairs lactation persistency as well as milk production.

Mammary Gland Epithelial Barrier

In addition to viability, activity, and number of mammary epithelial cells, the proper three-dimensional structure of the alveolar epithelium is important in maintaining the normal function of the mammary gland. In all vertebrates, junctional complex formed by adjacent

epithelial or endothelial cells plays essential roles in cell-cell interaction, maintaining an intact epithelial cell layer, and a functional paracellular pathway (Turner, 2009; Stelwagen and Singh, 2014). The cell-cell junctional complex is composed of tight junction, subjacent adherens junction, and desmosome (Itoh and Bissell, 2003; Turner, 2009). Tight junctions, as the most apical components of the cell-cell junctional complex, play the main role in separating apical and basolateral domains, and act as barriers regulating paracellular movement of ions and molecules through the epithelium (Itoh and Bissell, 2003). Furthermore, the adherens junctions are required for the assembly and polarization of tight junctions, facilitating the closure of the paracellular barrier (Turner, 2009).

Proteins of Mammary Epithelial Barrier

Certain proteins are required for the formation of cell-cell junctional complex. Tight junctions are formed by two major transmembrane spanning structural proteins, occludin and claudin, which are linked intracellularly to the actin cytoskeleton via scaffolding proteins, the zonula occludens (ZO) proteins (Stelwagen and Singh, 2014). Occludin is the first identified transmembrane tight junction protein, and serves in epithelial barrier regulation (Itoh and Bissell, 2003). The claudins are a family of transmembrane proteins consisting of more than 25 members (Stelwagen and Singh, 2014), which play important roles in regulating paracellular permeability and cell polarity in the epithelium or endothelium (Lal-Nag and Morin, 2009). However, the claudin family is variably expressed in different cell types and not all members appear to be expressed in the mammary gland (Turner, 2009; Stelwagen and Singh, 2014). In the murine lactating mammary gland, claudin 3 appears to be important in maintaining a normal milk-blood barrier, whereas claudin 4 is not detectable in the alveolar epithelial cells (Kobayashi et al., 2013). In addition, markedly increased protein expression of claudin 1 and 3 and decreased

protein expression of claudin 2 and 16 were observed after milk accumulation for 20 h in murine mammary glands (Markov et al., 2012). Another important protein family in assembling tight junctions are peripheral membrane proteins ZO proteins, which consist of multiple protein interaction domains for claudins, occludin, and actin (Turner, 2009). The adherens junctions are composed of a family of transmembrane proteins called cadherins, which strongly interact with molecules on adjacent cells (Turner, 2009). E-cadherin is found as a cytoplasmic tail of the epithelial cadherin and contributes to the regulation of local actin assembly as well as tight junction assembly (Turner, 2009).

Functions of the Mammary Epithelial Barrier

The cell-cell junctional complex of mammary epithelial cells is crucial in maintaining mammary function, i.e. milk synthesis. As reviewed by Stelwagen and Singh (2014), the formation of intact mammary epithelial barrier at the onset of lactation is important for the initiation of milk synthesis, and a highly impermeable mammary epithelium is critical to maintain lactation. Further, the loss of tight junction integrity is associated with reduced milk yield, impaired mammary function, and increased paracellular transport of blood components into milk and vice versa (Stelwagen and Singh, 2014).

Physiological or pathophysiological stimuli disrupt epithelial integrity. In goats, intramammary administration of the calcium chelator EGTA disrupted mammary epithelial integrity and reduced milk production (Neville and Peaker, 1981; Stelwagen et al., 1995). However, the mammary epithelium resealed after 2 d of treatment when the milk yield returned to the pre-treatment level (Neville and Peaker, 1981; Stelwagen et al., 1995), suggesting a transit disruptive effect of EGTA on the mammary epithelium. Additionally, in goat mammary glands, after 18 h to 21 h of milk accumulation, mammary epithelial barrier was disrupted, which was

associated with dercreased mammary blood flow and milk synthesis even before the amount of accumulated milk reached the maximal capacity in the mammary gland (Stelwagen et al., 1994b). Other stimuli such as mammary gland inflammation (including mastitis) also cause a leaky mammary epithelial barrier (Stelwagen and Singh, 2014). Thus, understanding the structure and function of the mammary epithelial barrier as well as factors affecting the integrity of the epithelial barrier is important to maximize the productivity of dairy cattle.

Parameters in Evaluating Mammary Epithelial Barrier

To examine the integrity of the epithelial barrier, gene and protein expression of essential proteins involved in tight junctions as well as adherens junctions can be evaluated. Additionally, the changes in blood concentrations of milk components or vice versa are better parameters to assess the integrity of the paracellular pathway. Ions and molecules with different sizes or charges are transported across the epithelial junctional complex via two main pathways to control the transepithelial potential difference (Turner, 2009; Dokladny et al., 2016). One is the leak pathway, which allows the paracellular transportation of non-charged large solutes, such as proteins and bacterial lipopolysaccharides (Turner, 2009; Dokladny et al., 2016). The maximal particle size for the leak pathway has not been precisely defined, although it is well accepted that particles as large as bacteria cannot pass through the epithelial barrier through the leak pathway. The other route, also known as the pore pathway, is mediated through small pores, which allow select charged solutes with a radius of less than 4 Å (0.4 nm) to be transported. The size of the pores are determined by the claudin proteins associated with the tight junction complex of the epithelium or endothelium (Turner, 2009).

To study mammary epithelial integrity, indicators such as milk concentrations of blood components or vice versa are required. In addition, the secretory function of a mammary

epithelial cell requires the existence and maintenance of a small transepithelial potential difference (about 30 to 35 mV) between the basolateral and the apical side of the cell (reviewed by Stelwagen and Singh, 2014). The transepithelial potential difference is instrumentally maintained by the tight junction, and the decrease of transepithelial potential difference indicates a disrupted epithelial tight junction (reviewed by Stelwagen and Singh, 2014). In goats, milk accumulation for 36 h caused a temporarily increased permeability of the mammary epithelium indicated by elevated plasma lactose concentration and decreased transepithelial potential difference of the mammary epithelial barrier (Stelwagen et al., 1994b). Reducing milking frequency from twice daily to once a day of lactating Friesian cows increased plasma lactose concentrations by 4- to 5-fold and milk concentrations of bovine serum albumin (BSA) by 42 to 55%, suggesting mammary epithelial leakage (Stelwagen et al., 1994a). In lactating caprines, intramammary administration of EGTA caused a rapid (< 1 h) increase in blood plasma concentration of lactose indicating disrupted mammary tight junction integrity (Stelwagen et al., 1995). In lactating dairy cows, milk accumulation for one day transiently increased plasma lactose and alpha-lactalbumin levels, which suggested an interrupted mammary epithelial integrity by milk accumulation (Stelwagen et al., 1997). Indeed, lactose is solely synthesized in the mammary epithelial cells of lactating animals and secreted at the apical end of mammary epithelial cells (Nguyen and Neville, 1998). It is a non-charged disaccharide with a small radius, which passes through the epithelial junctional complex if the epithelium is “leaky” via the leak pathway (Turner, 2009; Dokladny et al., 2016). Thus, the concentration of lactose in circulating blood provides a reliable measurement of the leakage rate from the lumen of the mammary gland into the blood stream (Nguyen and Neville, 1998; Shennan and Peaker, 2000). Moreover, as indicated by Stelwagen et al. (1997), the plasma concentration of alpha-lactalbumin is highly

correlated (r = 0.82) with plasma lactose level and is a reliable indicator to assess the integrity of mammary epithelium.

Bovine serum albumin (BSA) is the most abundant protein in circulating blood with a negative charge (Böhme and Scheler, 2007). It can be transported into the mammary gland through a transcytosis pathway, similar to immunoglobulin, under normal physiological conditions (Stelwagen et al., 1994a; Monks and Neville, 2004). However, the increased influx of BSA from blood to milk indicates a disrupted mammary epithelial barrier (Stelwagen et al., 1994a). Other solutes such as sodium (Na), potassium (K) and chloride (Cl) are commonly used to measure the permeability of the mammary epithelial paracellular barrier. The increased Na and Cl concentrations in milk coupled with the decreased milk K concentrations indicate a leaky tight junction (Shennan and Peaker, 2000; Stelwagen et al., 1995). However, the change of Na and K gradients may simply be due to the transportation of these ions across transcellular pathways through the Na-K pump rather than paracellular pathways (Shennan and Peaker, 2000). Thus, care must be exercised when evaluating mammary epithelial integrity using the alteration of these ion solutes in milk (Shennan and Peaker, 2000).

In addition, the transepithelial electrical potential is a preferred indicator of the tightness or leakiness of an epithelium (Shennan and Peaker, 2000). However, relative to an in vitro model using a cultured membrane monolayer, it is technically difficult to directly measure the transepithelial electrical potential under in vivo conditions (Shennan and Peaker, 2000).

Heat Stress Effects on Epithelial Integrity

Studies examining the effect of heat stress on mammary gland epithelial integrity in lactating dairy cows are limited. However, research in other species suggested that heat stress

negatively affects epithelial integrity. For example, , increasing the ambient temperature by 1.3°C rapidly disrupted the epithelial integrity of the Madin-Darby canine kidney epithelial (MDCK) cell monolayer in vitro as indicated by the increased transepithelial electrical conductance of the cell monolayer; this impaired epithelial barrier was completely reversed when cells were cultured under thermo neutral conditions (Moseley et al., 1994). Similarly, heat exposure to porcine renal epithelial cells (LLC-PK_1) (Ikari et al., 2005), human colon adenocarcinoma cells (Caco-2) (Dokladny et al., 2006a), and MDCK cells (Dokladny et al., 2006b) showed decreased transepithelial resistance, which indicated impaired epithelial integrity due to heat stress. In rodents, increasing the environmental temperature from 37 to 41.5 – 42°C for over 90 min increased the permeability of both small intestinal and colonic sacs to 4,000-Da fluorescein isothiocyanate-labeled dextran (FITC-dextran) (Lambert et al., 2002). FITC-dextran is a large fluorescent molecule and widely used as a probe to assess epithelial integrity. Its movement across the epithelium reflects the GI permeability in both in vivo and in vitro models (Lambert et al., 2002). Similarly, heat exposure (42°C for 90 min) caused marked elevations in the permeability to 4,000-Da FITC-dextran within intestinal segments near the duodenum and moderate elevations in the jejunum and ileum in the mouse (Novosad et al., 2013). In monkeys, heat stress caused a rapid increase in plasma LPS, an indicator of a compromised reticuloendothelial system by the leakage of endotoxin from the gut lumen into the portal circulation (Gathiram et al., 1987). In swine, decreased ileum transepithelial electrical resistance and increased FITC-dextran, indicators of increased epithelial permeability, were observed under short term heat stress (37℃ for 2 h) (Pearce et al., 2014). In addition, heat exposure (35℃) for 1, 3, or 7 d in crossbred gilts caused increased plasma endotoxin levels and decreased jejunum transepithelial electrical resistance, suggesting decreased intestinal integrity (Pearce et al., 2013).

Similarly, decreased ileal and colonic transepithelial electrical resistance were observed in swine when exposed to heat stress for either 1 or 7 d, which further demonstrate the detrimental effects of heat stress on gut integrity (Sanz Fernandez et al., 2014).

Unfortunately, whether heat stress has a similar impact on the mammary epithelial barrier remains unclear. However, due to the similar junctional structure of all epithelium and endothelium among different tissues and vertebrates (Turner, 2009; Stelwagen and Singh, 2014), it is logical to speculate that heat stress impairs mammary epithelial junctional complex and increases the permeability of mammary epithelial barrier in lactating dairy cows.

Nutritional Strategies for Heat Stressed Lactating Dairy Cows

Concentrate-to-Forage Ratio

A common nutritional strategy in commercial dairies during summer is to increase the energy and nutrient densities of the diet, to compensate for the remarkably reduced DMI under heat stress conditions (Beede and Collier, 1986; West, 1999). The heat increment of feedstuff consumed by animals under heat stress conditions is a consideration. The metabolism and fermentation of fiber produce more heat compared with concentrates (Baumgard et al., 2014). Thus, increasing the concentrate-to-forage ratio to a suitable extent is suggested to reduce metabolic heat production and overall heat load in dairy cattle (Beede and Collier, 1986; West, 1999). Indeed, increasing the digestible energy density in the diet during heat stress is an effective management strategy for improving productivity of ruminants (Beede and Collier, 1986). However, adequate fiber is required to maintain optimal ruminal pH and cow health by promoting cud chewing and rumination (West, 1999).

Dietary fat

Higher concentrations of dietary fat offer similar benefits to reduce basal heat increment. As reviewed by West (1999), supplementation of dietary fat between 3 to 5% in the ration does not have a toxic effect on ruminal microflora. Feeding additional dietary fat improves the utilization of metabolizable energy (ME) for lactation and the efficient conversion of dietary fat to body fat, which is especially beneficial during heat stress due to the lower heat increment (West, 1999). However, the results of feeding dietary fat on cow thermo-physiology and performance are inconsistent among studies. O'Kelly (1987) reported that heat-stressed steers fed 9.2% dietary fat had lower body temperature compared with animals fed 2.5 % dietary fat, indicating less heat increment of a high fat diet. In heat-stressed lactating dairy cows, feeding 10% soybean oil improved fat-corrected-milk production and altered milk fatty acid profile, but had negligible effects on physiological measurements such as body temperature and respiration rate (Moody et al. 1971).

Dietary protein

Additional dietary protein is thought to be required during heat stress due to the reduced DMI (West, 1999). However, the effects of increasing dietary protein content on thermal stressed dairy cows are inconsistent and more research is still warranted. As reviewed by Baumgard et al. (2014), the addition of dietary crude protein was unable to improve cow performance during heat stress, and diets containing highly degradable protein appear to be deleterious to cows during heat stress. It was suggested that both rumen motility and rate of passage decline during heat stress, which allows for a longer residence time of feed and more extensive protein degradation to ammonia (Linn, 1997). The excessive ammonia in the body requires additional energy cost as

well as heat production because it needs to be metabolized to urea and excreted in the urine and milk (Linn, 1997).

Water

Water intake is essential for milk production and thermal homeostasis (Baumgard et al., 2014). The excessive water loss through panting and sweating under thermal stress increases the possibility of dehydration (Silanikove, 1992). Water availability and tank hygiene are more important to help cows remain thermobalance and productivity during environmental heat stress (Baumgard et al., 2014).

Minerals

Serum and urinary K concentrations decline under thermal stress, which may be related to depressed aldosterone secretion (Beede and Collier, 1986) as well as increased excretion of K in sweat, since cows utilize K as the primary osmotic regulator of water secretion from sweat (Baumgard et al., 2014). Thus, dietary K requirements are increased during thermal stress (Beede and Collier, 1986; Baumgard et al., 2014). In addition, in order to minimize urinary K losses, Na excretion increases during heat stress, which stresses the requirement of increasing dietary Na supplementation (Beede and Collier, 1986).

Dietary Zinc Supplements in Dairy Cows

Dietary zinc (Zn) is one of the essential trace minerals and a component of many metalloenzymes and metalloproteins (NRC, 2001). The catalytic, coactive, or structural role of Zn in numerous enzymes is fundamental in regulating many physiological processes, including metabolism, growth, and immune function (Vallee and Falchuk, 1993). For example, the coactive role of Zn in superoxide dismutase (Kapila et al., 2016) facilitates the transformation of

free radical superoxide to hydrogen peroxide (Gressley, 2009). Dietary Zn is required for the production of protective keratins in the hoof and teat, thus plays an essential role in maintaining structural integrity and health of the hoof and udder (Gressley, 2009).

Organic Zinc vs. Inorganic Zinc

However, different chemical structure of dietary Zn supplements, such as organic and inorganic Zn sources, may function differently in animals. Traditionally, trace minerals including zinc, copper, and manganese have been fed as inorganic salts, such as zinc sulfate, cupric sulfate, or magnesium sulfate. Those trace minerals are associated with sulfate in a dry form but dissociate when hydrated in the rumen (Gressley, 2009). However, the absorption across the rumen epithelium is minimal and the majority of trace minerals are absorbed in the small intestine (Wright et al., 2008). Further, the dissociated trace minerals in reticulo-rumen, omasum, and abomasum can form insoluble or indigestible compounds with other feed components such as plant polyphenols and sugars, which prevent efficient mineral absorption in the small intestine (Gressley, 2009). The "organic" forms of trace minerals bound to an organic molecule, such as amino acid or protein, have been widely used in the dairy cow ration and extensively studied. There are varieties of organic forms of dietary Zn such as Zn methionine complex, Zn amino acid complex, or Zn proteinate.

Previous studies indicate that organic forms of trace minerals have higher bioavailability than inorganic forms due to reduced dissociation in the reticulo-rumen, omasum, and abomasum. The complex of organic molecules and trace minerals remain intact in the upper gastro-intestinal tract and do not form insoluble and indigestible compounds, thus, yielding higher availability of trace minerals for absorption in the small intestine (Gressley, 2009). As reported by Spears (2003), in Zn-deficient lambs, feeding Zn from Zn methionine complex and Zn oxide had similar

apparent absorption of Zn, but lambs fed Zn oxide had higher urinary excretion of Zn, indicating lower Zn retention. In another study, calves supplemented with 300 mg of zinc/kg of diet from a combination of Zn lysine and Zn methionine complexes had higher liver and plasma Zn concentration than calves fed Zn oxide (Kincaid et al., 1997). Thus, feeding organic form of trace mineral is beneficial to support production due to the higher bioavailability compared with inorganic trace minerals.

Role of Dietary Zinc in Cow Performance

Previous research has suggested that replacing a portion of inorganic Zn with organic Zn may be beneficial to cow performance and udder health. Nayeri et al. (2014) studied the ratio of Zn sulfate to Zn amino acid complex in pre- and postpartum Holstein cows fed diets containing 75 mg of supplemented Zn / kg, and found that replacing 66.6 mg of Zn sulfate/kg of DM in the close-up diet and 40 mg of Zn sulfate/kg of DM in the lactating cow diet with Zn amino acid complex increased milk yield and feed efficiency in multiparous and primiparous cows. Cope et al. (2009) found that lactating cows fed high level (600 mg/day) of organically chelated Zn had lower milk somatic cell counts (SCC) and higher milk yields compared with those fed a low level (396 mg/day) of organically chelated Zn or a high level (600 mg/day) of Zn oxide. In a meta-analysis including 12 trials, Kellogg et al. (2004) reported that feeding Zn methionine complex to lactating cows increased milk yield, energy-corrected milk, and fat corrected milk, and reduced SCC compared with inorganic Zn, such as Zn sulfate or Zn oxide. The beneficial effect of organic Zn shown in those studies may be due to the higher bioavailability of organic form of Zn compared with inorganic forms (Spears, 2003). However, contrary results have also been reported in other studies. Sobhanirad et al. (2010) reported that feeding additional dietary Zn (basal diet plus 500 mg Zn / kg of DM) tended to increase milk yield and reduce SCC, but no

differences were found between two dietary Zn treatments (Zn sulfate vs. Zn methionine complex). However, the duration of dietary Zn supplementation, the parity of cows, the mineral levels in basal diet or other factors may all count for the disparities among studies.

Overall, previous studies reported positive effects of supplementing dietary zinc on improving cow performance, such as increasing milk yield, under temperate climate condition. How dietary zinc supplementation affects cow performance under heat stress condition is still unclear.

Effect of Dietary Zinc on Epithelial Integrity

Supplementing dietary Zn has recently been reported to regulate epithelial barriers in a number of epithelial cell types and animal tissues. In an in vivo study, Zn supplementation improved tight junction barrier function in porcine LLC-PK_1 renal cell layers indicated by increased cytosolic pool of claudin-2 and claudin-4 (Wang et al., 2014). In a human intestinal Caco-2 cell model, Zn deficiency decreased transepithelial electrical resistance, resulting in an increase in neutrophil transepithelial migration through the cell layer (Finamore et al., 2008). This disrupted epithelial permeability was associated with altered structures of tight junctions and adherens junctions with delocalization of ZO-1, occludin, β-catenin, and E-cadherin (Finamore et al., 2008). Zinc oxide supplementation has also been reported to protect the human intestinal Caco-2 cell barrier from *Escherichia coli* exposure by preventing the increase in tight junction permeability and regulating cytokine gene expression, suggesting a protective role of Zn on intestine under *E. coli* infection (Roselli et al., 2003). In heat-stressed growing pigs, increasing dietary zinc concentration from 120 ppm to 220 ppm by supplementing zinc amino acid complex improved small intestine integrity (Sanz Fernandez et al., 2014). Interestingly, Pearce et al. (2015) found that feeding additional Zn amino acid complex to heat-stressed

growing pigs tended to mitigate the heat stress induced disruption of ileum integrity compared with those fed with same level of Zn sulfate, suggesting the advantage of Zn amino acid complex on maintaining epithelial integrity over Zn sulfate. Therefore, zinc plays potential roles in regulating compositions of epithelial barrier junctional complex and maintaining proper epithelial barrier function. However, precise mechanisms of the effects of amount and source of Zn on epithelial barriers, including mammary epithelial barriers, are still unclear. Future studies, either in vivo or in vitro, are warranted to investigate the potential function of supplementing Zn on maintaining epithelial barrier integrity.

Common Methods for Evaluating Molecular Function in Cows

Other than the alteration in performance, such as milk yield and DMI, animals' responses to certain stimuli, including environmental heat stress, can be detected on the molecular level, such as cellular structure and gene and protein expression. To assess mammary morphology and function, methods such as histological analysis, PCR, quantitative PCR, western blotting, as well as immunofluorescent staining are commonly used. However, a large amount of tissue is required to perform these analyses,

Mammary tissue collection

Traditionally, mammary gland tissues in the bovine are collected by sacrificing the animals. Enormous amount of tissue can be extracted by this approach; however, it is economically expensive. In recent years, mammary biopsy collection has become a more popular method for histological and biochemical evaluation of mammary tissue samples. However, there are concerns that the mammary biopsy collection procedure may cause hemorrhaging and mammary infection, and adversely affect milk yield due to the vascularity of lactating mammary

gland (Farr et al., 1996). Thus, researches have been focused on improving and developing a mammary biopsy collection procedure which causes less adverse effects on dairy production animals.

Several approaches for mammary tissue collection in lactating cows have been studied. Mammary tissues can be sampled using esophageal biopsy forceps directly through the teat canal, which yieldy large samples (~ 4 × 12mm) but only tissue from the cisternal region of the udder can be obtained (Farr et al., 1996). Farr et al. (1996) described a drill-operating instrument connected with a rotating cannula as the tissue collecting tool, which obtains a large tissue fragment (~ 0.75 to 1 g). This method yields a large quantity of tissue that is sufficient for most of routine research analysis with only one attempt, however, it may cause substantial bleeding. Lima et al. (2016) used a biopsy needle that was inserted into the mammary gland through a ~ 1.5 cm skin incision. This approach reduced external bleeding but obtained only a small amount of mammary tissue of ~ 6 × 3mm, and the procedure requires multiple attempts until an appropriate amount of tissue is obtained. A vacuum pump-assisted approach using an outer cannula needle (12 mm or 20 mm) with a trocar tip for mammary tissue collection has been evaluated, which minimizes external bleeding but yields a smaller amount of tissue (79.1 and 153.9 mg for 12 or 20 mm aperture needle, respectively) (VanKlompenberg et al., 2012). This method also requires several attempts to acquire enough tissue for histological or biochemical analyses (VanKlompenberg et al., 2012). de Lima et al. (2016) developed a similar instrument to the one described by VanKlompenberg et al. (2012) with modification, which has a trocar with cannula and a 30-mL syringe to create the vacuum environment instead of a vacuum pump console, and obtained 200 to 700 mg of mammary tissue from the lactating Holstein cow.

Using the drill-operating instrument on a lactating dairy cow, intramammary bleeding was cleared from milk by 6.5 d and the incision was healed without infection within 6 d, and no clinical mastitis was observed throughout the remainder of the lactation (Farr et al., 1996). Moreover, milk yield and composition were affected only transiently from the quarters subject to biopsy collection using this tissue collection procedure (Farr et al., 1996). Lima et al. (2016) reported that the biopsy needle method can be conducted and repeated on same lactating cow without apparent negative effects on milk yield and composition in a short term. Milk yield decreased after biopsy collection but returned to pre-biopsy collection level 1 day after each mammary tissue collection (Lima et al., 2016). Milk composition and SCC were affected only transiently in the subsequent days after tissue collection (Lima et al., 2016). However, within 60 d from the first mammary tissue collection, 12% of biopsied quarters were diagnosed and treated for clinical mastitis, among which 90% of the quarters recovered (Lima et al., 2016).

Each mammary tissue collection procedure has advantages or disadvantages, such as the amount of tissue obtained from one attempt of mammary tissue collection or length of wound healing. The method using drill-operating instrument is suitable for research but requires experienced technician and good management. The biopsy needle method is more commonly used to avoid possible substantial hemorrhage but multiple attempts may be required. Regardless of the methods adopted, the effect of mammary tissue collection on udder health and cow performance in a short or long term is of interest for researchers.

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CHAPTER 3

EFFECTS OF HEAT STRESS AND DIETARY ZINC SOURCE ON MAMMARY EPITHELIAL INTEGRITY OF LACTATING DAIRY COWS[1]

[1] Weng, X., A. P. A. Monteiro, J. Guo, C. Li, J. K. Bernard, D. T. Tomlinson, J. M. DeFrain, S. E. Wohlgemuth, S. Tao. To be submitted to *Journal of Dairy Science.*

Abstract

Dietary Zn and heat stress alter gut integrity in monogastrics. However, the effect of Zn on mammary epithelial integrity in heat-stressed lactating dairy cows has not been studied. Multiparous lactating Holstein cows (n = 72) were randomly assigned to 1 of 4 treatments with a 2×2 factorial arrangement to study the effects of environment and Zn source on performance and mammary epithelial integrity. Treatments included two environments: cooled (CL) or not cooled (NC) and two Zn sources: 75 ppm supplemental Zn as Zn hydroxychloride (IOZ), or 35 ppm Zn hydroxychloride + 40 ppm Zn-MET complex (ZMC). The experiment was divided into baseline (B) and environmental challenge (EC) phases, 84 d each. During B, all cows were cooled (temperature-humidity index [THI] = 72.5) while NC cows were not cooled during EC (THI = 77.7). Mammary biopsies were collected d 7 and 56 relative to the onset of EC to analyze gene expression of claudin 1, 4, and 8, zonula occludens (ZO) 1, 2, and 3, occludin and E-cadherin and protein expression of occludin and E-cadherin. Deprivation of cooling increased respiration rate (64.8 vs. 73.9 breath/min), vaginal temperature (39.03 vs. 39.94 ℃), and decreased DMI (26.7 vs. 21.6 kg/d). Energy-corrected milk yield decreased for NC cows relative to CL (24.5 vs. 34.1 kg/d). An interaction between environment and Zn source occurred for milk fat content as CL cows fed ZMC had lower milk fat percentage than other groups. Relative to CL, NC cows had lower concentrations of lactose (4.69 vs. 4.56%) and solids-not-fat (8.46 vs. 8.32%) but higher concentration of milk urea nitrogen (9.07 vs. 11.02 mg/mL). Compared with IOZ, the plasma lactose concentration of cows fed ZMC was lower and tended to be lower during B and EC phases, respectively. Relative to CL cows, plasma lactose concentration tended to increase at 3, 5, and 41 d after the onset of EC in NC cows. Treatments had no effect on milk BSA concentration. Cows fed ZMC tended to have higher gene expression of E-cadherin relative to

IOZ. Compared with CL, NC cows had increased gene expression of occludin and E-cadherin and tended to increase claudin 1, ZO-1, and -2 gene expression in the mammary gland. Protein expression of occludin and E-cadherin were unchanged. In conclusion, removing active cooling impairs lactation performance, affects gene expression of proteins involved in mammary epithelial barrier integrity, and feeding a portion of dietary zinc as ZMC improves the integrity of mammary epithelium.

Key words: heat stress, zinc, mammary epithelial junction

Introduction

Heat stress (**HS**) is well known to negatively impact the performance of lactating dairy cows. Studies (Shwartz et al., 2009; Wheelock et al., 2010) have shown that environmental HS reduced milk yield by approximately 30 to 40% in mid-lactation dairy cows. While the decreased DMI explains approximately half of the reduction in milk yield (Rhoads et al., 2009; Wheelock et al., 2010), physiological and cellular mechanisms related to the reduced milk synthesis caused by HS in addition to the decreased DMI are still unclear. Milk yield of the lactating dairy cow is determined by the secretory capacity of mammary gland including both the number and activity of mammary secreting cells (Capuco et al., 2003). Thus, HS may affect the cow's milk production by impairing mammary gland function. In an in vitro study (Collier et al., 2006), mammary epithelial cells underwent increased apoptosis when incubated under high ambient temperature compared with thermal neutral conditions, providing evidence that HS directly influences mammary cell abundance.

The intercellular junction complex is a barrier to regulate the movement of molecules and ions through paracellular pathway in both endothelium and epithelium, including mammary

epithelium (Nguyen and Neville, 1998). During lactation, the mammary epithelium forms a highly impermeable barrier to separate milk and interstitial fluid to maintain optimal milk synthesis and secretion (Stelwagen and Singh, 2014). In contrast, the increased mammary epithelial permeability caused by extended milking frequency or mastitis negatively impacts normal mammary function and health of lactating dairy cows (Nguyen and Neville, 1998; Stelwagen and Singh, 2014). The effect of environmental HS on epithelial integrity has been the subject of many studies in species other than cow. For example, under in vitro condition, HS increased the permeability of canine and porcine kidney epithelial cell junctions and human colon and kidney epithelial junctions (Dokladny et al., 2016). Similarly, in vivo, exposure to HS impaired gut integrity in swine (Pearce et al., 2013, 2014; Sanz Fernandez et al., 2014), rodents and primates (Dokladny et al., 2016). However, the impact of HS on permeability of mammary epithelium in lactating dairy cows has never been studied.

In addition to environmental factors, dietary Zn, an essential micronutrient, is important to maintain critical body functions, such as immunity and metabolism, and is critical for animal growth, health, and optimal performance (NRC, 2001). Dietary Zn also improves epithelial integrity and maintains proper tissue function. Sanz Fernandez et al. (2014) reported that increasing dietary Zn concentration from 120 to 220 ppm with supplemental Zn-AA complex improved the integrity of small intestine of growing pigs under HS conditions. However, the role of Zn in maintaining bovine mammary epithelial integrity is still unknown. The source of dietary Zn also influences epithelial integrity. Pearce et al. (2015) reported that replacing a portion of $ZnSO_4$ with Zn-AA complex mitigated the HS-induced reduction in ileum integrity in pigs. In heat-stressed steers, replacing a portion of $ZnSO_4$ with Zn-AA complex decreased duodenum villi width and increased both jejunum villi height and the ratio of villi height to crypt depth,

indicating an improved intestinal barrier function during HS (Abuajamieh et al., 2016). Whether Zn-AA complex has a similarly positive effect over inorganic sources on maintaining the integrity of mammary epithelium in the bovine is unclear.

We hypothesized that environmental HS impairs mammary function through increasing the permeability of the mammary epithelium of lactating dairy cows, and that replacing a portion of dietary inorganic Zn with Zn-AA complex improves the integrity of the mammary epithelium, especially under HS conditions. The objective was to evaluate the effect of environmental HS and supplementation of different Zn sources (Zn-MET complex vs. Zn hydroxychloride) on lactating Holstein cows' performance and permeability of the mammary epithelium.

Materials and Methods

Animals, Experimental Design

The study was conducted at the Dairy Research Center of the University of Georgia-Tifton campus from April to September, 2015. All procedures and animal handling were approved by the Institutional Animal Care and Use Committee of the University of Georgia prior to initiation of the trial.

Seventy-two multiparous lactating Holstein cows were blocked by parity (2.9 ± 0.3) and DIM (99.7 ± 13.4 d), and then assigned randomly to 1 of 4 treatments within each block with a 2 × 2 factorial arrangement: 1. diet supplemented with 75 ppm Zn as Zn hydroxychloride (**IOZ,** Micronutrients, Indianapolis, IN) with cooling (**CL**), (**IOZCL**, n = 18), 2. **IOZ** without cooling (**NC**), (**IOZNC**, n=18), 3. diet supplemented with 35 ppm Zn as Zn hydroxychloride and 40 ppm as Zn-MET complex (**ZMC**, Zinpro Corporation, Eden Prairie, MN) with cooling, (**ZMCCL**,

n=18), 4. **ZMC** without cooling, (**ZMCNC**, n=18). During the course of the experiment, seven animals were excluded from the trial: one cow from IOZCL due to a leg problem, three cows from IOZNC due to toxic mastitis and indigestion, one cow from ZMCCL due to indigestion, and two cows from ZMCNC due to ketosis and indigestion. Cows were housed in adjacent CL and NC pens in the same barn according to their environmental treatments. All animals were fed the same diet except for different dietary Zn sources (Table 3.1). The dose of supplemental Zn was selected based on Nayeri et al. (2014) who reported that replacing 40 mg/kg $ZnSO_4$ with Zn-AA complex in the diet (75 ppm total supplemental Zn) increased milk yield and reduced SCC in multiparous lactating cows. The experiment was divided into a baseline and a subsequent environmental challenge phase, 84 d each. During the baseline phase, all cows were cooled while during the environmental challenge phase cooling was deprived from NC cows. The cooling system included misters attached to the front face of fans over feed bunks and free stalls. Fans ran continuously when the environmental temperature was $\geq 20\ ^{\circ}C$ and misters were activated whenever ambient relative humidity (**RH**) was less than 85%. The onset of environmental challenge phase was considered as d 1 of the experiment.

Sample Collection and Analyses

Environment and body temperature

The air temperature and RH in the barn were monitored by Hobo Pro Series Temp probes (Onset Computer Corporation, Pocasset, MA) every 15 min throughout the experiment and 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 ($^{\circ}C$) (Dikmen and Hansen, 2009). Respiration rate was counted (1500 h) for all cows once each week during the baseline phase and then three times each week during the environmental challenge phase. Vaginal temperature was

monitored every 5 min for 4 continuous d using an ibotton (Mouser Electronics, Mansfield, TX) attached to a blank CIDR every 2 wk during the baseline phase and then each week during the environmental challenge phase in a subset of cows (n=16/treatment).

Feed intake, sample collections, BW and BCS

Cows were fed once daily (1300 h) and the daily feed intake was measured using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH) throughout the experiment. The individual dietary ingredients and TMR were sampled three times each week (Mon-Wed-Fri). The DM content of samples was measured by drying at 55 °C for 48 h in a forced air oven. Rations were adjusted as needed based on the changes in ingredient DM content when necessary. Samples of TMR were composited every 2 wk and grounded to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) for nutrient analysis (Table 3.2) by Dairyland Laboratories (Arcadia, WI). The drinking water was sampled at 2 and 5 mo after the onset of experiment at multiple water troughs from the pens for mineral and hardness analyses (Table 3.3) by Dairyland Laboratories (Arcadia, WI). The sampling procedures provided by Dairyland Laboratories were followed. Every 2 wk, BW was recorded and BCS assigned (Wildman et al., 1982) throughout the entire experiment.

Milk and blood samples

Cows were milked three times (0800, 1600, and 2400 h) each day and milk yield recorded at each milking (Alpro, DeLaval, Kansas City, MO). Milk samples were collected from 3 consecutive milkings each week with bronopol-B-14 as a preservative for analyses of milk components (fat, protein, lactose, SNF, MUN, and SCC, Dairy One Cooperative, Ithaca, NY) using a Foss 4000 instrument (Foss North America, Eden Prairie, MN). The somatic cell score (SCS) was calculated based on the equation: $SCS = \log_{10}(SCC/12.5)/\log_{10}2$, for analysis.

Additional milk samples were collected without a preservative from 3 consecutive milkings at d -88, -74, -46, -18, -3, 1, 3, 5, 18, 25, 39, 53, 67 relative to the onset on EC phase and stored at -20°C for milk BSA analysis. Milk BSA concentration was analyzed using colorimetric method with bromocresol green dye (Sigma-Aldrich, St. Louis, MO) as described by Lieske et al. (2005) and the inter- and intra-assays CV were 5.2% and 2.9%. Briefly, milk samples were pooled by day proportional to milk yield at each milking and centrifuged at 3000 × g for 15 min at 4 °C to obtain skim milk. Master mix made from bromocresol green dye, succinic acid (Thermo Fisher Scientific, Waltham, MA), and Brij™-35 (30%, Thermo Fisher Scientific, Waltham, MA) was used in the colorimetric assay. A skim milk sample (200 µL) was fully mixed with 900 µL master mix and centrifuged at 1900 × g for 10 min at 25 °C. Supernatant (150 µL) was transferred to a 96 well plate to measure the absorbance at 640 nm. The BSA concentrations in skim milk were then calculated based on a standard curve. The BSA concentrations in the whole milk were calculated based on following formula: BSA (in whole milk, mg/g) = (BSA in skim milk/ $d_{20°C}$)/(100 - Fat) × 100; where $d_{20°C}$ = skim milk density of each pooled sample at 20 °C (g/mL) = 100/(F/0.93+SNF/1.608+Water%) (Chandan, 2006); Fat = fat concentration in whole milk from the results of milk component analysis; F = Fat concentration in skim milk considered as 0.2% (Pereira, 2014); SNF = Solids-not-fat concentration in skim milk from the milk component analysis; Water% = water concentration in skim milk subtracted from skim milk fat and SNF concentrations.

Blood samples were collected from coccygeal vessels into sodium-heparinized vacutainers (Becton Dickinson, Franklin Lakes, NJ) before feeding (~1230 h) on d -86, -73, -45, -17, -3, 1, 3, 5, 12, 26, 41, 54, 68 relative to the onset of EC phase from a subset of the animals (n=6, 5, 5, 7, for IOZCL, IOZNC, ZMCCL, ZMCNC, respectively). Samples were immediately

placed in ice and then centrifuged at 2,619 × g for 30 min at 4 °C to collect plasma. Colorimetric methods were used to measure plasma lactose concentration using a commercially available kit (BioVision, Milpitas, CA) and the inter- and intra-assays CV were 3.5% and 1.9%, respectively.

Mammary biopsy collections

Mammary biopsies were collected at enrollment as baseline samples, d 7 and 56 relative to the onset of environmental challenge from a subset of animals (n=8/treatment). After the first biopsy collection, one animal from ZMCNC was excluded from the trial due to ketosis related indigestion. One quarter of another animal from ZMCNC spontaneously dried off and this animal was excluded from tissue collection as well. The first and third biopsies were collected from the left rear quarter, and the right rear quarter was subjected to the second biopsy collection. The procedure for biopsy collection followed the method reported by Farr et al. (1996). Briefly, cows were sedated by intravenous injection of xylazine hydrochloride (20 µg/kg of BW, Phoenix Pharmaceuticals, St. Joseph, MO). The region for biopsy collection in the midpoint of the quarter was shaved, and then sanitized three times by scrubbing with iodine followed by rinsing with 70% ethanol. Local anesthesia was achieved with subcutaneous injection of 3 mL lidocaine hydrochloride (Animal Rx Pharmacy, Atlanta, GA) administered in a line block above the biopsy collection site. A 3-4 cm incision was made through skin and connective tissue, avoiding any large subcutaneous blood vessels. A core of mammary tissue (~0.75 g) was obtained using a rotating stainless steel cannula with a retractable blade connected with a cordless drill (Farr et al., 1996). Incisions were then closed using 18-mm stainless steel Michel wound-clips (GerMedUSA, Garden City Park, NY) and sprayed with an aerosol bandage (Neogen Corporation, Lexington, KY) to prevent infection. Tissue was rinsed with PBS,

trimmed of fat, and separated and stored in liquid nitrogen and RNAlater (Qiagen, Valencia, CA) for western blotting and qRT-PCR analyses, respectively.

RNA extraction, cDNA synthesis and qRT-PCR

Mammary gland tissue was ground in liquid nitrogen and the total RNA was extracted using TRI-reagent solution (Sigma-Aldrich, St. Louis, MO) and PureLink^{TM} RNA Mini Kit (Invitrogen, Carlsbad, CA) with an on-column DNase (PureLink^{TM} DNase Set, Invitrogen) treatment following the manufacturer's instructions. A second DNase treatment was performed on the purified RNA extract using Turbo DNA-free kit (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA).

Relative quantitative RT-PCR was used to examine the gene expression of claudin 1, 4, and 8, zonula occludens (**ZO**)-1, -2, and -3, occludin, and E-cadherin in mammary biopsies. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) and are shown in Table 3.4. The qRT-PCR was carried out using the SYBR green supermix (BioRad, Hercules, CA) in a LightCycler 480 instrument (Roche, Florence, SC) following the manufacturer's instructions. The cDNA from 50 ng of purified RNA was used for each reaction under the following condition: 95°C for 5 min for initial denaturation and enzyme activation, 40 cycles of 95°C for 15 seconds and 57°C for 30 seconds for amplification. The *GAPDH* was used as a housekeeping gene. The $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle, was used to calculate the relative gene expression with data of samples collected at enrollment (baseline sample) as calibrator samples.

Protein extraction and western blotting analysis

Tissue samples were homogenized in 1× PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) for 1 min on an ice slurry using a Polytron PT 2100 (Kinematica, Littau-Lucerne, Switzerland). The homogenate was kept on an ice slurry for 1 h and then centrifuged at 3,500 × *g* for 10 min at 4°C. The supernatant was collected and the protein concentration determined using the Bradford protein assay (BioRad). Supernatants were then stored at -80°C for future western blotting analysis.

Samples were thawed and diluted in Laemmi Buffer (BioRad) containing 5% of β-mercaptoethanol (Sigma-Aldrich) with a dilution of 11 parts of sample plus 4 parts of buffer, and heated for 10 min at 95°C. Diluted samples containing 30 or 15 μg protein were loaded on an any kD TGX precast gel (BioRad) for occludin and E-cadherin protein expression analysis, respectively, with β-actin as a loading control. A same cross sample was loaded in each gel for data normalization to minimize gel to gel variations. After electrophoretic separation, proteins were transferred to a PVDF membrane (Millipore, Bedford, MA) using a semidry blotter (BioRad) at 20V for 1 h. Transferred proteins were subsequently stained with Ponceau S (Sigma-Aldrich) to evaluate the quality of protein transfer. The membrane was then blocked for 40 minutes at room temperature in TBS-t (1 × Tris-buffered saline [150 mM NaCl, 20 mM TrisBase, pH = 7.4] with 0.05% Tween-20 [Fisher Scientific, Hampton, NH]) containing 5% non-fat dry milk (BioRad) for Occludin and β-actin detection, and 5% BSA (Millipore) for E-cadherin detection. The blocking step was followed by an overnight incubation at 4°C with primary antibody solution. Mouse anti-occludin (Invitrogen, 1:500, dilued in 5% non-fat dry milk), rabbit anti-E-cadherin (Cell Signaling, Danvers, MA, 1:1000, diluted in 5% BSA) and mouse anti-β-actin (Sigma-Aldrich, 1: 32,000, dilued in 5% non-fat dry milk) were used as primary antibodies. After primary antibody incubation, membranes were washed in TBS-t buffer,

and incubated for 1 h at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, 1:16,000, diluted in 5% non-fat dry milk) for occludin; anti-rabbit horseradish peroxidase conjugated secondary antibody (Cell Signaling, 1:2,000, diluted in 5% non-fat dry milk) for E-cadherin; and anti-mouse alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich, 1:60,000, diluted in 5% non-fat dry milk) for β-actin. The chemiluminescent substrate (Duolux, Vector Laboratories, Builingame, CA) for horseradish peroxidase or alkaline phosphatase, respectively, was applied according to the manufacturer's recommendation, and the chemiluminescent signal was visualized using a cooled charge-coupled device camera and acquisition software (G-BOX imaging system, Syngene, Frederick, MD). The chemiluminescence intensity was analyzed and quantified by densitometry using the manufacturer's analysis software (GeneTools, Syngene). Band intensity was normalized by the cross sample run in the same gel. After normalization, the ratio of the band intensity of each protein to the accordingly loading control (β-actin) were calculated and used for data analysis, with the baseline sample as a covariate.

Data analysis

PROC UNIVARIATE of SAS 9.4 (SAS Institute, Cary, NC) was used to calculate the mean ± standard deviation of THI. Parity and DIM were analyzed by PROC GLM of SAS 9.4 and the least squares means ± standard error of the mean are reported. Repeated measures data (DMI, vaginal temperature, respiration rate, milk yield and composition, plasma lactose concentration, and milk BSA concentration) were analyzed by PROC MIXED procedure of SAS 9.4. The SAS models included block, cooling, Zn source, time, and their respective interactions, with cow nested within cooling and Zn source as a random variable. For milk yield analysis, the average milk yield of cows before the onset of the experiment was included in the model as a

covariate. For analyses of plasma lactose and milk BSA concentrations, the data obtained at -86 d were included in the model as covariates. The gene and protein expression data were analyzed by PROC MIXED procedure of SAS 9.4. The SAS models included block, cooling, Zn source, time, and their respective interactions, with cow nested within cooling and Zn source as a random variable. The protein expression data obtained from mammary biopsies collected at enrollment were included in the SAS models as covariates. 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

Thermal Environment, Vaginal Temperature and Respiration Rate

The average THI in CL and NC pens were 72.7 (SD = 5.3) and 72.3 (SD = 5.3), respectively, during the baseline phase, and 77.6 (SD = 3.8) and 77.8 (SD = 3.8), respectively, during environmental challenge phase, suggesting that all animals were exposed to similar environmental HS throughout the study. During the baseline phase, all cows had similar ($P \geq 0.47$) vaginal temperature and respiration rates (Table 3.5). During the environmental challenge phase, regardless of the Zn source, CL cows had lower ($P < 0.01$) vaginal temperatures (39.03 vs. 39.94 °C, SEM = 0.05 °C, respectively) and respiration rates (64.8 vs. 73.9 breaths/min, SEM = 1.3 breaths/min, respectively) compared with NC, indicating that cooling was effective for reducing cows' heat load (Table 3.6). There were no ($P \geq 0.20$) differences observed for Zn or Zn by cooling interactions on vaginal temperature or respiration rates (Table 3.6).

Milk Yield and Composition

During both baseline and environmental challenge phases, dietary Zn source had no ($P \geq 0.12$) effect on milk yield, SCS, and concentration or yield of milk lactose, protein, SNF, and MUN (Table 3.5, 3.6). However, relative to IOZ, cows fed ZMC had a lower ($P = 0.05$) milk fat percentage resulting in lower ($P = 0.03$) fat yield during the baseline phase (Table 3.5). During the environmental challenge phase, regardless of the Zn source, NC cows had lower ($P \leq 0.04$) milk yield, FCM, ECM and concentration and yield of milk lactose and SNF, but higher concentration of MUN compared with CL cows (Table 3.6). Milk SCS and concentration of protein were not affected ($P \geq 0.80$) by cooling treatment (Table 3.6). However, due to the lower milk yield, NC cows had lower ($P < 0.01$) milk protein yield relative to CL. There was a Zn by cooling interaction ($P = 0.04$) for milk fat percentage because milk fat concentration of ZMCCL cows was lower ($P = 0.01$) than IOZCL and tended ($P = 0.06$) to be lower than ZMCNC (Table 3.6).

DMI, BW, BCS, and Feed Efficiency

During the baseline phase, there were no ($P \geq 0.13$) Zn effects on DMI, BW, BCS, or feed efficiency (Table 3.5). Similarly, during the environmental challenge phase, no differences ($P \geq 0.54$) were observed between cows fed IOZ and ZMC for DMI, BW, BCS, and feed efficiency (Table 3.6). Regardless of Zn treatment, NC cows had lower ($P < 0.01$) DMI (21.6 vs. 26.7 kg/d, SEM =0.5 kg/d, respectively), BW (664.0 vs. 711.4 kg, SEM =4.2 kg, respectively), BCS (2.74 vs. 2.89, SEM =0.03, respectively), and feed efficiency (1.16 vs. 1.28, SEM =0.03, respectively) compared with CL cows (Table 3.6).

Milk BSA and Plasma Lactose

Relative to IOZ, feeding ZMC decreased ($P = 0.03$) and tended ($P = 0.11$) to decrease plasma lactose during the baseline and environmental challenge phases, respectively (Figure

3.1a). No cooling effect or Zn by cooling interaction ($P \geq 0.36$) was observed during the environmental challenge phase, but there was a tendency for cooling by time interaction ($P = 0.09$) such that, relative to CL cows, plasma lactose concentration tended to be higher ($P < 0.10$) in NC cows at d 3, 5, and 41 (Figure 3.1b). There was no treatment effect or interaction ($P \geq 0.29$) for milk BSA content throughout the study (Table 3.5, 3.6).

Gene and Protein Expressions

Dietary Zn source had no effect ($P \geq 0.23$) on mRNA expression of epithelial junction related genes except that feeding ZMC tended ($P = 0.09$) to increase the gene expression of E-cadherin compared with IOZ (Table 3.7, Figure 3.2). Relative to CL, NC cows had higher ($P \leq 0.03$) gene expression of occludin and E-cadherin and tended ($P \leq 0.09$) to have higher gene expression of claudin 1, ZO-1 and -2 (Table 3.7, Figure 3.2). Cooling did not affect ($P \geq 0.13$) gene expression of claudin 4 and 8 (Table 3.7). However, there was a cooling by time interaction ($P = 0.05$) for gene expression of ZO-3 such that NC cows tended to have higher ($P = 0.09$) gene expression relative to CL on d 7 (Table 3.7, Figure 3.2). Furthermore, a cooling by time ($P = 0.05$) and a Zn by cooling by time interaction ($P < 0.01$) for gene expression of claudin 4 were observed (Table 3.7); however, the SLICE and PDIFF functions of SAS indicated time effects within each treatment rather than treatment effects at individual time points. In contrast, there were no differences ($P \geq 0.12$) among treatments or treatment by time interactions observed for protein expression of occludin and E-cadherin (Figure 3.3).

Discussion

Milk yield of lactating dairy cows starts to decrease when ambient THI exceeds 68 (Zimbelman et al., 2009). In the current study, the THI during the baseline and environmental

challenge phases averaged 72.5 and 77.7, respectively, indicating that all cows were exposed to HS during the entire experiment. However, the lower vaginal temperature and respiration rate of CL cows compare with NC during the environmental challenge phase indicates that the cooling setting was effective in reducing the heat load of cows. Dietary Zn concentrations and sources may influence animals' thermal status. In swine, increasing the dietary Zn concentrations from 120 to 320 ppm tended to increase the animal's body temperature under HS (Sanz Fernandez et al., 2014). Under non-HS conditions, replacing a portion of $ZnSO_4$ with Zn-AA complex increased the maximal rectal temperature of dairy cattle during the transition period (Nayeri et al., 2014). In contrast, diets containing 60 ppm Zn-AA complex and 60 ppm $ZnSO_4$ reduced gilts' body temperature during a short term heat challenge compared with diets supplemented with 120 ppm $ZnSO_4$ (Pearce et al., 2015). Mechanisms for the possible regulatory effects of Zn on thermal regulation are unclear. In current study, no differences were observed between IOZ and ZMC for body temperature or respiration rate, indicating that replacing a portion of Zn hydroxychloride with Zn-Met complex had no impact on cows' thermal regulation under HS.

As expected, NC cows produced 27% lower milk yield and consumed 19% less DM compared with CL, which is consistent with previous research indicating that HS compromises cow performance (Kadzere et al., 2002; West, 2003). Relative to CL, NC cows had lower feed efficiency, suggesting that the decrease in DMI cannot entirely explain the loss of milk yield by HS, consistent with previous studies (Rhoads et al., 2009; Wheelock et al., 2010).

Environmental conditions had no effect on milk protein percentage in the current study. The impact of HS on milk protein concentration is inconsistent reported in previous studies. Compared with thermo-neutrality, HS reduced the milk protein concentration (Rhoads et al., 2009; Shwartz et al., 2009, Wheelock et al., 2010). In contrast, supplemental evaporative cooling

either decreased (Tarazón-Herrera et al., 1999) or had no influence on milk protein content (Taylor et al., 1991; Chen et al., 1993; Chan et al., 1997). These discrepancies between studies may result from different experimental models or distinct basal diets or both. Relative to CL, NC cows had lower milk lactose concentrations, which is consistent with previous reports (Shwartz et al., 2009), and suggests that HS reduces mammary lactose synthesis. However, when all cows were fed the similar amount of DM, HS cows have similar milk lactose percentage compared with those under thermo-neutrality (Rhoads et al., 2009), indicating that the reduced milk lactose synthesis by HS may be feed intake dependent. In the current study, NC cows had higher MUN concentration than CL, which is similar to the results of Rhoads et al. (2009) and Shwartz et al. (2009) who reported that HS increases plasma urine nitrogen concentrations compared with thermo-neutrality. Reasons for the increased concentrations of BUN or MUN resulting from HS are unknown, but may reflect altered ruminal N metabolism and/or systemic AA metabolism (Rhoads et al., 2009).

Deprivation of cooling had no impact on milk SCS in the present experiment, consistent with previous studies (Chan et al., 1997; Tarazón-Herrera et al., 1999). It has been suggested that summer weather is correlated with elevated SCC (Hammami et al., 2013) and increased mastitis incidences (Gaughan et al., 2009) at the farm level. However, controlled studies confirmed that HS *per se* does not increase milk SCC.

To the best of our knowledge, the current study is the first experiment to compare dietary Zn hydroxychloride with Zn-MET complex on the performance of lactating dairy cows under HS. Regardless of the environmental conditions, dietary Zn source had no effect on milk yield, DMI, and feed efficiency during the entire trial, suggesting that diets supplemented with IOZ or ZMC at a concentration of 75 ppm support similar performance during lactation in summer. In

contrast, previous studies (Spears, 1996; Kellogg et al., 2004) reported that, relative to $ZnSO_4$ or Zn oxide, feeding Zn-MET complex improved milk production. The advantage of Zn-AA complex over inorganic Zn sources to improve milk yield (Kellogg et al., 2004; Rabiee et al., 2010) was attributed to the increased bioavailability (Spears, 1996). Compared with Zn oxide, feeding Zn-MET complex does not alter Zn absorption but reduces urinal Zn excretion, resulting in a higher Zn retention (Spears, 1989). On the other hand, Genther and Hansen (2015) reported that supplementing sulfate-bound trace minerals (Cu, Mn, and Zn), but not hydroxy minerals, reduced ruminal DM disappearance, suggesting that hydroxy minerals have no negative influence on rumen function compared with sulfate-bound trace minerals. However, direct comparisons between hydroxy trace mineral and mineral-AA complex on bioavability and rumen function in ruminants are still not available. Duration of the supplementation of different sources of Zn also influences the milk yield response of cows. In a meta-analysis, Rabiee et al. (2010) found that feeding Zn-AA complex starting before calving had a stronger impact on the increase in milk production than supplementation started after parturition. Using a ratio of supplemental inorganic to organic Zn similar to the current experiment, Nayeri et al. (2014) reported that cows supplemented with 35 ppm $ZnSO_4$ and 40 ppm Zn-AA complex starting one month before calving until 250 DIM had higher milk yield relative to those fed 75 ppm $ZnSO_4$. In contrast, the dietary treatments in the current study started at mid-lactation. Future studies are warranted to examine the production responses of cows supplemented with hydroxy mineral and mineral-AA complex starting during the prepartum period. Moreover, most of studies that examine cows' responses to different Zn source were conducted in temperate climates, and it is also possible that different Zn sources affect cow performance differently under HS compared with thermo-neutral condition.

Zn source had no influence on milk percentages of protein, lactose, and SNF, consistent with previous studies (Kellogg et al., 2004; Wang et al., 2013; Rabiee et al., 2010). Different from Kellogg et al. (2004) and Nayeri et al. (2014) who reported that supplementation of Zn-MET or Zn-AA complexes reduced milk SCC compared with $ZnSO_4$ or Zn oxide, in the current study, the milk SCS was not influenced by dietary Zn sources. These data may indicate that replacing 40 ppm Zn hydroxychloride with Zn-MET complex from 75 ppm supplemental Zn has no impact on milk quality during summer. However, the total Zn content of diets used in the experiment conducted by Nayeri et al. (2014) and studies reviewed by Kellogg et al. (2004) were lower than that fed in the current study. It is possible that the high dietary Zn content fed in the present study prevent us from observing any potential impact of different Zn sources on milk SCC. Interestingly, cows fed with ZMC had significantly lower milk fat concentration compared with IOZ during the baseline phase and ZMCCL cows had the lowest milk fat content among other treatment groups during the environmental challenge phase, suggesting that diets with ZMC reduce milk fat synthesis compared with IOZ, especially in cows under evaporative cooling during summer.

Reports on the effects of dietary Zn on milk fat concentration are inconsistent. As summarized by Kellogg et al. (2004) and Rabiee et al. (2010), supplementation of Zn-MET or Zn-AA complexes had no impact on milk fat percentage in lactating dairy cows. In contrast, Nayeri et al. (2014) observed that replacing 15.5 ppm $ZnSO_4$ with Zn-AA complex in lactating cow diets tended to decrease milk fat percentage compared with cows fed a control diet containing 75 ppm $ZnSO_4$; however, the diet supplemented with 40 ppm Zn-AA complex and 35 ppm $ZnSO_4$ had no impact on milk fat content relative to control. In the current study, the interaction between Zn source and environment also indicate that cows with various heat stains

may respond differently to diets containing different Zn sources regarding milk fat synthesis. However, the underlying mechanisms are still unclear.

The mammary epithelium is unique in synthesizing and secreting milk components into the alveolar lumen, and this process requires the existence and maintenance of transepithelial transport pathways for milk synthesis (Stelwagen and Singh, 2014). A well maintained mammary epithelial junctional complex is an indicator of optimal mammary function, and the loss of integrity of mammary epithelium during lactation is related to reduced milk synthesis and secretion (Stelwagen and Singh, 2014). As reviewed by Shennan and Peaker (2000), the plasma concentration of lactose is a preferable indicator to assess the tightness or permeability of mammary epithelium. In the present study, no environment and diet interaction was observed for plasma lactose concentration, indicating that HS and dietary zinc source influence the mammary epithelial barrier by different mechanisms. Environment had no overall impact on plasma lactose concentration, suggesting that cows that are under long term exposure to HS largely maintain the integrity of the mammary epithelium. However, NC cows tended to have higher plasma lactose concentrations at the beginning of the environmental challenge phase compared with CL cows, indicating that the permeability of mammary cell junction may temporarily increase when exposed to acute HS before returning to the basal level due to an adaptation to HS. Similarly, Stelwagen et al. (1997) observed a gradual decrease in leakage of milk lactose of the lactating cow with each cycle of extended milking interval, which suggests that the mammary gland is able to adapt to altered milk frequency, possibly by reorganizing mammary epithelial junctions. The reason for the transient increase in plasma lactose concentration of NC cows relative to CL cows at 41 d of the environmental challenge phase is unclear. All cows were managed and handled similarly during the entire trial.

Compared with IOZ, ZMC cows had lower plasma concentrations of lactose during both baseline and environmental challenge phases, suggesting that replacing 40 ppm Zn-hydroxychloride with Zn-MET in the diet reduces the leakage of lactose through the mammary epithelial barrier. Dietary Zn is a critical micronutrient to maintain epithelial barrier integrity. In vitro, depletion of Zn in the medium of a human intestinal Caco-2 cell culture significantly increased epithelial permeability, while repletion of Zn into the culture rescued the disrupted epithelial barrier (Finamore et al., 2008). Similarly, supplementing Zn into a porcine renal LLC-PK cell line culture reduced epithelial permeability (Wang et al., 2014), indicating a conservation of the critical role of Zn in maintaining epithelial integrity across species and cell types. In vivo, increasing dietary Zn concentration from 120 ppm to 220 ppm by supplementation Zn-AA complex attenuated the increase in ileal permeability caused by HS in growing pigs (Sanz Fernandez et al., 2014). Interestingly, replacing a portion of $ZnSO_4$ with Zn-AA complex in the swine diet also improved integrity of ileal epithelium under HS conditions (Pearce et al., 2015), suggesting improved performance of Zn-AA complex over $ZnSO_4$ in maintaining epithelial integrity, possibly due to the higher bioavailability (Spears, 2003). Nevertheless, the results obtained in the current study provide evidence that the dietary Zn source also influences the mammary epithelial integrity in lactating dairy cows, and replacing 40 ppm of Zn hydroxychloride with Zn-MET in the diet reduces the permeability of the mammary epithelium. Interestingly, milk BSA content was not affected by environmental conditions, dietary Zn source, or their interactions. Under normal physiological conditions, similar to immunoglobulin, serum albumin transports across mammary epithelial cells through a transcytosis pathway in the mouse lactating mammary gland (Monks and Neville, 2004). However, when the mammary epithelial barrier is disrupted, milk BSA content is dramatically increased due to the influx from

blood into milk through the paracelluar pathway (Stelwagen and Singh, 2014). Therefore, data in the present study indicate that the environmental and dietary treatments only altered the permeability of mammary epithelium but did not result in a disruption of the mammary epithelial barrier.

The epithelial paracellular barrier is composed of a junctional complex including tight junction, adherens junction, and desmosome (reviewed by Itoh and Bissell, 2003; Tsukita et al., 2008; Turner, 2009). The tight junction is located at the apical side of the cell-cell junction and is the rate-limiting structure regulating epithelial permeability (Turner, 2009). It is composed of transmembrane spanning structural protein families, including occludin and claudins, that are linked to the intracellular actin cytoskeleton via scaffolding proteins, the ZO family (Stelwagen and Singh, 2014). The adherens junction is adjacent to the tight junction and is critical for cell-to-cell adhesion and polarization (Itoh and Bissell, 2004; Turner, 2009). The main transmembrane protein that forms the adherens junction between epithelial cells is E-cadherin (Kundsen and Wheelock, 2005). In the current study, compared with CL, the mammary glands of NC cows had higher gene expression of occludin, claudin 1, ZO-1 and -2, and E-cadherin at both 7 and 56 d of the environmental challenge phase, suggesting an overall increase in the synthesis of protein related to the paracellular junction. The trend of the increase in gene expression of ZO-3 at 7 but not 56 d perhaps indicates that the effort to synthesize more mammary tight junction proteins is stronger when a cow is exposed to acute heat stress. Similarly, in gilts, exposure to heat stress for 7 d increases the gene expression of occludin, claudin 3, and ZO-1 in the jejunum (Pearce et al., 2013). Interestingly, although the mammary gene expressions are up-regulated in NC cows compared with CL cows, protein expressions of occludin and E-cadherin were not influenced by environments, which is consistent with the similar plasma lactose

concentration between NC and CL cows, and confirms that cows under HS are able to maintain mammary epithelial integrity. Further, these data may suggest that there is a net loss of junctional proteins due to HS and that the mammary gland of the NC cow strives to maintain the normal epithelial integrity by synthesizing more proteins relative to CL cows. Therefore, the resources (e.g., AA) and energy (e.g. ATP) in the mammary gland may shift from milk synthesis to maintain proper epithelial integrity, which may be one mechanism of the reduced milk production of HS lactating dairy cows.

Surprisingly, although cows supplemented with ZMC had reduced plasma lactose concentration compared with IOZ, no differences were observed between Zn sources for gene and protein expressions of most epithelial junction proteins examined. Similar to the results in the current study, Pearce et al. (2015) reported that replacing 60 ppm $ZnSO_4$ with Zn-AA complex in gilts' diet partially rescued the increases in intestinal permeability after 12 h exposure to HS without influencing the gene expression of tight junction proteins, compared with those fed 120 ppm $ZnSO_4$. Relative to those in the Zn-enriched medium, human intestinal Caco-2 cells cultured in a Zn-depleted medium display reduced protein expression of occludin and ZO-1, and re-organize the tight and adherens junctional proteins from epithelial junctions to cytoplasm within the cells (Finamore et al., 2008). These data may indicate that Zn affects epithelial junctions via maintaining expression and proper location of tight and adherens junctional proteins. Therefore, it is possible that replacing 40 ppm dietary Zn-hydroxychloride with Zn-MET promotes proper locations of junctional proteins between mammary epithelial cells without affecting their expressions compared with IOZ, thereby improving mammary epithelial integrity. The mechanism of the increased gene expression of E-cadherin in ZMC compared with IOZ cows is unknown. As reviewed by Oloumi et al. (2004), there are three E-box motifs located at

the 5′ promoter region of the gene of E-cadherin, which can be bound by Zn-finger proteins to regulate its expression. Zinc is the intrinsic component of Zn-finger proteins and influences their stability to regulate gene expression (reviewed by Lee and Kelleher, 2016). Thus, the different sources of dietary Zn may alter the cellular activity of Zn-finger proteins to influence gene expression. The fact that the dietary Zn source only affects mammary E-cadherin expression at the transcription level but not at the translational level may indicate a post-transcriptional regulation of E-cadherin, but, the mechanism is still unclear.

Conclusion

Deprivation of cooling remarkably impaired the lactation performance of lactating dairy cows but NC cows largely maintained mammary epithelial integrity compared with CL cows. The protein expressions of occludin and E-cadherin were not altered by environments. However, NC cows had higher gene expression of epithelial junctional proteins, indicating HS induces epithelial junctional protein synthesis to maintain proper epithelial integrity. On the other hand, replacing 40 ppm of Zn hydroxychloride with Zn-MET complex in the diet of lactating cows had no strong impacts on cow performance but reduced plasma concentrations of lactose. These data suggest that dietary Zn-MET complex may enhance the integrity of the mammary epithelium but the cellular mechanism is still unclear.

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Table 3.1. Ingredient composition of experimental diets

Ingredient	% of DM
Corn silage	24.68
Ryegrass silage	16.45
Ryegrass hay	3.29
Brewers grains, wet	11.10
Ground corn	19.74
Molasses	4.94
Soybean hulls	1.65
Citrus pulp	6.58
Soybean meal	1.65
Amino plus[1]	2.06
Prolak[2]	3.04
Metabolys[3]	0.44
Mepron[4]	0.07
Megalac[5]	1.65
Salt	0.04
Calcium carbonate	0.49
Sodium bicarbonate	0.82
Magnesium oxide	0.28
Dynamate[6]	0.08
Rumensin[7]	0.25
OmniGen-AF[8]	0.20
Diamond V XP[9]	0.21
Trace mineral-vitamin premix[10]	0.30

[1]Ruminally protected soybean meal, Ag Processing Inc., Omaha, NE.
[2]Marine and animal RUP supplement, H. J. Baker & Bros. Inc., Westport, CT.
[3]Ruminally protected by-pass lysine, H. J. Baker & Bros. Inc., Westport, CT.
[4]Rumin protected methionine, Evonik Industries, Kennesaw, GA.
[5]Calcium salts of long-chain FA, Arm Hammer Animal Nutrition, Church & Dwight Co. Inc., Princeton, NY.
[6]Feed-grade mineral consisting of sulfate form of potassium and magnesium, Mosaic Co. Inc., Plymouth, MN.
[7]Rumensin, Elanco Animal Health, Indianapolis, IN.
[8]Immune stimulant, Phibro Animal Health, Corp., Teaneck, NJ .
[9]Microbio product containing active enzymes derived from the microorganisms *Aspergillus oryzae, Aspergillus niger, and Trichoderma viride* and selected microorganisms: *Propionibacterium freudenreichii, Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium, Lactobacillus lactis, Pediococcus cerevisiae, Bacillus subtillis, Baccillus licheniformis, and Aspergillus oryzae*. Diamond V. Inc., Cedar Rapids, IA.
[10]Mineral-vitamin premix contained (DM basis): 75.63% calcium carbonate; 5.03% Sel Plex 2000 (Alltech, Lexington KY); 4.70% IntelliBond M (Micronutrients, Indianapolis, IN); 3.90% Avalia Mn 80 (Zinpro Corp., Eden Prairie, MN); 2.42% IntelliBond Z (Micronutrients, providing approximately 35 mg/kg Zn of total DMI); 1.95% Availa Cu (Zinpro Corp.); 1.51% Copro 25 (Zinpro Corp.); 0.28% copper chloride; 0.05% Ethylenediamine dihydroiodide (EDDI); 2.74%

Vitamin E, 227,000 IU (13,869 IU/kg); 1.82% Vitamin 325,000 A (6,004,570 IU/kg), Vitamin D (600,430 IU/kg). The rest of Zn (0.007% IntelliBond Z and 0.033% of Zinpro 120 [Zinpro Corp.] relative to total DM of the ration for cows receive Zn hydroxychloride and Zn-MET complex, respectively) was hand added to the diets.

Table 3.2. Nutrient contents of the experimental diets supplemented with Zn hydroxychloride (IOZ) or Zn-MET complex (ZMC)

DM basis	IOZ (n = 12)	ZMC (n = 12)
CP, %	17.9 ± 0.8[1]	18.3 ± 1.0
SP[2], % of CP	38.8 ± 3.4	41.3 ± 2.6
NDFa[3], %	30.0 ± 1.4	30.0 ± 1.6
ADF, %	20.7 ± 1.2	20.2 ± 0.7
Lignin, %	3.0 ± 0.4	2.7 ± 0.2
Starch, %	24.3 ± 1.7	23.9 ±2.3
Sugar, %	4.4 ± 0.8	5.4 ± 0.9
EE[4], %	4.7 ± 0.3	4.7 ± 0.3
NFC, %	41.6 ± 1.6	41.1 ± 1.1
Ash, %	8.5 ± 0.9	8.8 ± 0.9
NE_L, Mcal/kg	1.64 ± 0.03	1.64 ± 0.03
Calcium, %	1.2 ± 0.3	1.3 ± 0.3
Phosphorus, %	0.5 ± 0.0	0.5 ± 0.0
Magnesium, %	0.4 ± 0.0	0.4 ± 0.0
Potassium, %	1.7 ± 0.2	1.8 ± 0.2
Sulfur, %	0.3 ± 0.0	0.3 ± 0.0
Sodium, %	0.4 ± 0.1	0.4 ± 0.1
Chloride, %	0.5 ± 0.1	0.5 ± 0.1
Manganese, mg/kg	136.8 ± 29.3	134.6 ± 31.5
Zinc, mg/kg	154.9 ± 25.6	156.4 ± 24.9
Copper, mg/kg	17.8 ± 5.8	16.9 ± 3.0
Iron, mg/kg	550.9 ± 61.8	535.6 ± 39.9
Molybdenum, mg/kg	1.8 ± 0.6	1.6 ± 0.2
Cobalt, mg/kg	1.4 ± 0.2	1.4 ± 0.2

[1]Mean ± Standard Deviation.
[2]SP = Soluble protein.
[3]NDFa = ash free NDF.
[4]EE = Ether extract.

Table 3.3. Composition, pH, and hardness of drinking water collected at 2 and 5 mo after the onset of the experiment

Drinking water	Month 2	Month 5
pH	7.76	7.87
Hardness, mg/kg	172	150
Composition, mg/kg		
Nitrate	1.30	1.55
Calcium	53.60	47.67
Magnesium	9.28	7.47
Phosphorus	0.12	< 0.01
Potassium	1.32	1.04
Copper	0.03	0.01
Iron	0.08	0.04
Zinc	0.13	0.03
Sodium	4.15	4.06
Manganese	0.03	0.01
Chloride	< 8	7
Sulfates	2.25	1.77
Total dissolved solids	220	295

Table 3.4. Names, symbols, GenBank accession numbers, primer sequences and amplicon sizes of genes examined

Gene name	Gene symbol	GenBank accession#	Primer[1]	Sequence (5' - 3')	Amplicon size, bp
Claudin 1	*CLDN1*	NM_001001854.2	F R	CGGTCAATGCCAGGTATG CTGGGTGTTGGGTAAGATG	130
Claudin 4	*CLDN4*	NM_001014391.2	F R	CCAACTGTGTGGATGATGAG CGCGGATGACGTTGTTAG	123
Claudin 8	*CLDN8*	NM_001098096.1	F R	GAGAGTGTCTGCCTTCATTG AGCAGCGAGTCGTAGATT	123
zonula occludens-1	*TJP1*	XM_015468497.1	F R	CTTTACGAGCTCCAGGCACT GGGGTCCTTCCTGTACACCT	104
zonula occludens-2	*TJP2*	NM_001102482.1	F R	GGACTCGGACAAGCCTAT GACCGGTTCTGGTCATTTC	110
zonula occludens-3	*TJP3*	NM_001045874.1	F R	GATACCCCGTTCCAGAACCT TGTAGAAGGAGTCGCCCAAG	148
Occludin	*OCLN*	NM_001082433.2	F R	CACCTGCAGCTACTGGACTCT GAGCAAAAGCCACAATAACCA	114
E-cadherin	*CDH1*	NM_001002763.1	F R	GCCAACGAGCTGATACAC CGGCATGAGAGAAGAGAATG	123
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_001034034.2	F R	GATGCTGGTGCTGAGTATG CAGAAGGTGCAGAGATGATG	112

[1]F = forward; R = reverse.

Table 3.5. Vaginal temperature, respiration rate, BW, BCS, DMI, feed efficiency, milk yield and composition of cows fed diets supplemented with Zn hydroxychloride (IOZ, n=32) or Zn-MET complex (ZMC, n=33) during the baseline phase

Parameter	IOZ	ZMC	SEM	*P*-value
VT[1], °C	39.11	39.14	0.04	0.52
RR[2], breath/min	58.1	59.6	1.8	0.47
BW, kg	675	672	6	0.53
BCS	2.75	2.73	0.04	0.29
DMI, kg/d	27.2	26.9	0.5	0.64
Milk yield, kg/d	42.2	41.7	0.8	0.64
3.5% FCM[3], kg/d	38.9	37.1	1.0	0.15
ECM[4], kg/d	37.7	35.8	0.9	0.17
FE[5] - ECM/DMI	1.48	1.42	0.05	0.13
Milk composition				
Lactose, %	4.70	4.70	0.03	0.59
Lactose, kg/d	2.00	1.95	0.05	0.41
Protein, %	2.64	2.68	0.03	0.39
Protein, kg/d	1.12	1.10	0.03	0.56
Fat, %	3.40	3.24	0.07	0.05
Fat, kg/d	1.44	1.32	0.03	0.03
SNF, %	7.71	7.76	0.05	0.63
SNF, kg/d	3.46	3.41	0.07	0.58
MUN, mg/dL	11.52	11.30	0.33	0.57
SCS[6]	2.32	2.96	0.29	0.12
BSA, mg/g	1.91	1.75	0.12	0.37

[1]Vaginal temperature.
[2]Respiration rate.
[3]3.5% FCM = (0.4324 × kg of milk yield) + (16.216 × kg of milk fat yield).
[4]ECM = (0.327 × kg of milk yield) + (12.95 × kg of milk fat yield) + (7.20 × kg of milk protein yield).
[5]Feed efficiency = ECM (kg/d)/DMI (kg/d).
[6]SCS = $\log_{10}(SCC/12.5)/\log_{10}2$.

Table 3.6. Vaginal temperature, respiration rate, BW, BCS, DMI, feed efficiency, milk yield and composition of cows fed diets with Zn hydroxychloride (IOZ) or Zn-MET complex (ZMC) exposed to either cooling (CL) or not (NC) during the environmental challenge phase

Parameter	IOZ		ZMC			*P*-value		
	CL	NC	CL	NC	SEM	Zn	CL	Zn×CL
VT[1], °C	39.06	39.88	39.00	40.00	0.07	0.64	< 0.01	0.20
RR[2], breath/min	64.4	72.4	65.3	75.5	1.8	0.28	< 0.01	0.55
BW, kg	712	666	711	662	6	0.59	< 0.01	0.78
BCS	2.88	2.76	2.91	2.73	0.04	0.97	< 0.01	0.48
DMI, kg/d	27.3[a]	21.0[b]	26.1[a]	22.1[b]	0.7	0.92	< 0.01	0.08
Milk yield, kg/d	35.5	25.9	35.3	25.8	1.1	0.91	< 0.01	0.97
3.5% FCM[3], kg/d	35.5	25.0	33.3	24.6	1.0	0.18	< 0.01	0.36
ECM[4], kg/d	35.1	24.7	33.1	24.3	1.0	0.23	< 0.01	0.40
FE[5] - ECM/DMI	1.28	1.18	1.28	1.14	0.04	0.64	< 0.01	0.52
Milk composition								
Lactose, %	4.70	4.57	4.67	4.55	0.05	0.61	< 0.01	0.94
Lactose, kg/d	1.68	1.17	1.66	1.15	0.06	0.67	< 0.01	1.00
Protein, %	2.86	2.86	2.81	2.85	0.05	0.57	0.80	0.70
Protein, kg/d	1.02	0.73	0.99	0.71	0.03	0.37	< 0.01	0.93
Fat, %	3.49[ac]	3.34[abc]	3.14[b]	3.38[c]	0.09	0.11	0.59	0.04
Fat, kg/d	1.24	0.86	1.11	0.84	0.04	0.07	< 0.01	0.12
SNF, %	8.50	8.33	8.42	8.31	0.07	0.47	0.04	0.66
SNF, kg/d	3.03	2.13	2.98	2.09	0.08	0.58	< 0.01	0.96
MUN, mg/dL	8.99	11.26	9.15	10.78	0.26	0.56	< 0.01	0.22
SCS[6]	3.47	3.16	3.26	3.76	0.39	0.62	0.93	0.29
BSA, mg/g	1.86	1.83	2.06	2.09	0.21	0.29	1.00	0.89

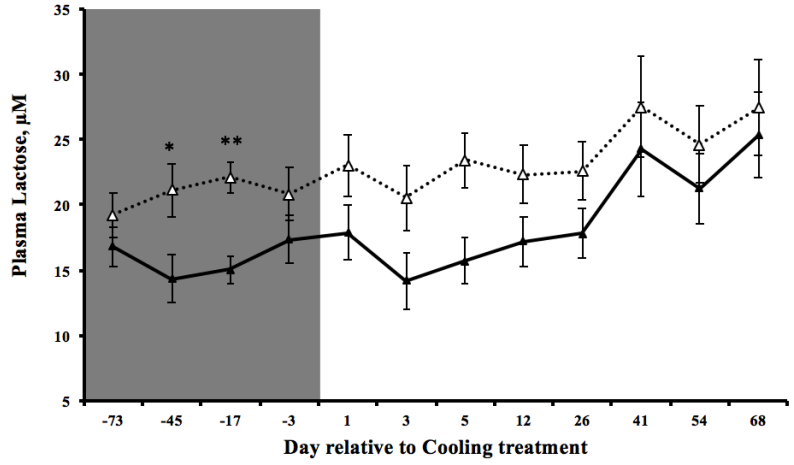
[1]Vaginal temperature.
[2]Respiration rate.
[3]3.5% FCM = (0.4324 × kg of milk yield) + (16.216 × kg of milk fat yield).
[4]ECM = (0.327 × kg of milk yield) + (12.95 × kg of milk fat yield) + (7.20 × kg of milk protein yield).
[5]Feed efficiency = ECM (kg/d)/DMI (kg/d).
[6]SCS = $\log_{10}(SCC/12.5)/\log_{10}2$.
Superscripts: a vs b: $P < 0.05$; b vs. c: $P = 0.06$.

Table 3.7. Gene expression in mammary tissue of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-MET complex (ZMC) exposed to either cooling (CL) or not (NC)

Gene	Day 7[1]				Day 56				SEM	*P*-value						
	IOZ		ZMC		IOZ		ZMC									
	CL	NC	CL	NC	CL	NC	CL	NC		Z[2]	C	Z×C	T	Z×T	C×T	Z×C×T
CLDN1	0.70	1.68	1.39	1.94	2.44	3.04	1.73	2.26	0.54	0.82	0.07	0.44	<0.01	0.14	0.99	0.54
CLDN4	1.06	1.33	2.48	1.10	1.78	1.37	1.37	2.98	0.52	0.41	0.92	0.88	0.10	0.60	0.05	<0.01
CLDN8	0.64	2.43	0.93	2.94	0.73	2.68	1.10	2.40	1.11	0.58	0.13	0.92	0.78	0.66	0.26	0.78
TJP1	0.98	1.55	0.98	3.17	1.26	1.95	1.00	4.46	0.87	0.35	0.06	0.42	<0.01	0.65	0.18	0.19
TJP2	0.87	2.00	0.74	2.59	1.41	2.88	0.85	3.59	1.09	0.91	0.09	0.83	0.04	0.42	1.00	0.56
TJP3	0.73	1.24	0.76	1.10	0.94	0.84	0.72	0.99	0.24	0.85	0.27	0.82	0.31	0.90	0.05	0.11
OCLN	0.74	1.60	1.21	2.38	1.16	1.72	1.36	1.98	0.41	0.23	0.03	0.79	0.30	0.29	0.48	0.90
CDH1	0.43	2.68	2.01	7.79	1.90	3.37	2.58	7.71	1.62	0.09	0.01	0.62	0.07	0.38	0.51	0.62

[1]Days relative to the onset of environmental challenge.
[2]Z: Zn source effect; C: cooling effect; T: time effect

a.



b.

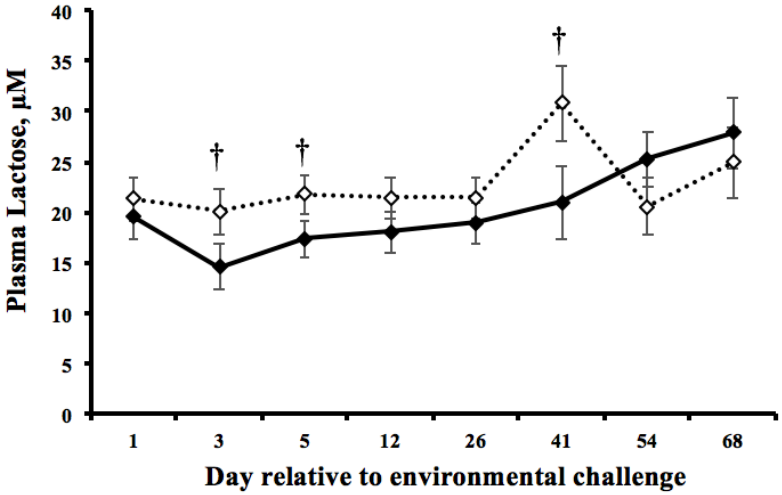


Figure 3.1a. The plasma concentrations of lactose of cows fed diets supplemented with Zn hydroxychloride (open triangles [△], n = 11) or Zn-MET (solid triangles [▲], n = 12) in the baseline (shade area) and environmental challenge phases. **Figure 3.1b.** The plasma concentrations of lactose of cows with (solid diamonds [♦], n = 11) or without cooling treatment (open diamonds [◊], n = 12) during the environmental challenge phase. In the baseline phase, effect of Zn source ($P = 0.03$), time ($P = 0.69$) and Zn source by time interaction ($P = 0.06$). In the environmental challenge phase, effect of Zn source ($P = 0.11$), cooling ($P = 0.36$), time ($P < 0.01$), Zn by cooling interaction ($P = 0.81$), Zn source by time interaction ($P = 0.52$), cooling by time interaction ($P = 0.09$), Zn source by cooling by time interaction ($P = 0.52$). $^{**}P < 0.01$, $^{*}P < 0.05$, †$P \leq 0.10$

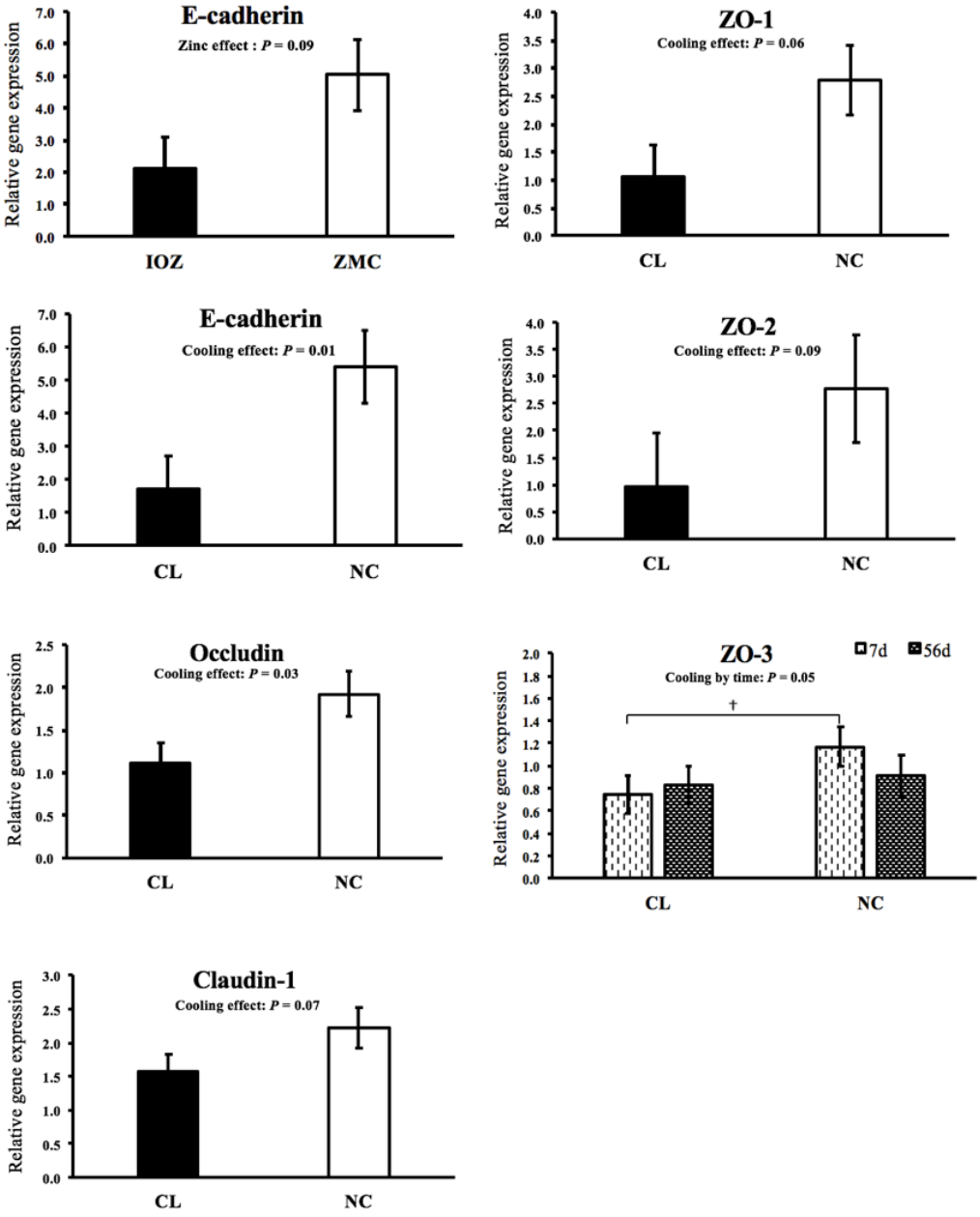


Figure 3.2. Relative gene expression of mammary tissue of cows fed diets supplemented with Zn hydroxychloride (IOZ, n = 16) or Zn-MET complex (ZMC, n = 14) exposed to either cooling (CL, n = 16) or not (NC, n = 14).

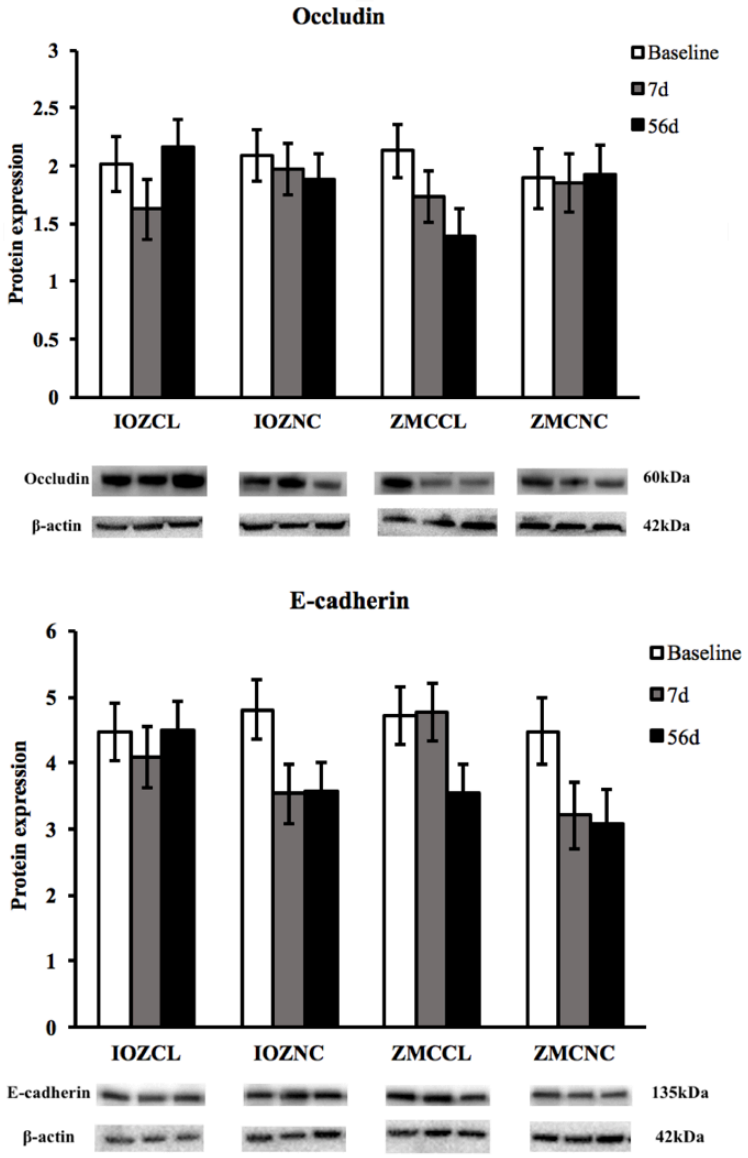


Figure 3.3. The protein expression of mammary tissue of cows fed diets supplemented with Zn hydroxychloride (IOZ, n = 16) or Zn-MET complex (ZMC, n = 14) exposed to either cooling (CL, n = 16) or not (NC, n = 14). Data are presented as least squares mean ± standard error of mean and displayed as the ratio of the band intensity of each protein to the accordingly loading control (β-actin). No treatment effects or treatment × time interaction ($P \geq 0.12$) were observed for Occludin and E-cadherin protein expression.

CHAPTER 4

REPEATED MAMMARY TISSUE COLLECTIONS DURING LACTATION DO NOT ALTER SUBSEQUENT MILK YIELD OR COMPOSITION [2]

[2] Weng, X., A. P. A. Monteiro, J. Guo, B. M. S. Ahmed, J. K. Bernard, D. J. Tomlinson, J. M. DeFrain, G. E. Dahl, S. Tao. Accepted by *Journal of Dairy Science*. Reprinted here with permission of publisher.

Abstract

Mammary biopsy collection (MB) is a valuable approach for studying mammary gland biology, but it is unclear if repeated MB impair the performance of lactating dairy cows. The objective of this trial was to examine the effect of repeated MB during lactation on udder health, dry matter intake (DMI), and lactation performance of lactating dairy cows. Sixty-four multiparous, mid-lactation Holstein cows were enrolled in a 29-wk trial and 32 cows were randomly selected for repeated MB. The MB and non-MB (NMB) cows had similar parity (2.6 ± 0.9, $P = 0.13$) and DIM (96.5 ± 56.3 d, $P = 0.13$) at enrollment. All animals were housed in the same barn and managed in the same manner. Cows were milked three times daily with milk yield recorded at each milking. Milk composition was measured weekly and DMI recorded daily. Three MB were performed per cow: 1 wk after enrollment and at 15 and 24 wk. The first and third MB were performed on the left rear quarter whereas the second MB was on the right rear quarter. The MB were performed based on procedures described by Farr et al., (1996) using a rotating stainless steel cannula with a retractable blade connected to a cordless drill with appropriate sedation and antiseptic treatment after each MB. After MB, udder health, surgical wound healing, and presence of blood in milk were visually examined at each milking. Blood was cleared from milk 3.86 ± 2.0 d after MB. During the experiment, 4 rear quarters of MB cows and 5 rear quarters from NMB cows were diagnosed and treated for clinical mastitis. No differences were observed ($P > 0.1$) in DMI, milk yield, somatic cell score, or milk concentration and yield of fat, protein, lactose, and solids-not-fat between MB and NMB. In conclusion, lactating cows recover rapidly from MB and repeated MB have no long term impacts on DMI, milk yield and composition or udder health of lactating dairy cows.

Key words: mammary biopsy, lactating cow, udder health, lactation performance

Introduction

Studies that examine morphology and the functions of the bovine mammary gland require mammary tissue collected from animals either by sacrificing the animal or performing a mammary biopsy collection (**MB**). The advantage of the sacrificial procedure is that an enormous amount of tissue can be obtained from different quarters and locations within a quarter. However, this approach is economically expensive. On the other hand, MB is relatively inexpensive and a satisfactory amount of tissue can be extracted for histological and biochemical evaluations. Two common procedures for MB have been reported frequently in bovine studies. One uses a rotating cannula connected to a slow speed electric motor by which a large tissue fragment (0.75 to 1g) can be collected (Farr et al., 1996). The other procedure uses core biopsy needles to extract small tissue fragments (20 to 25 mg) and may require several insertions to collect enough tissue for analysis (Lima et al., 2016; Soberon et al., 2010). Although a valuable approach, concerns arise as to the potential for adverse effects of MB on udder health and performance of the cow. Within 1 wk after MB using a rotating cannula, intramammary blood accumulated during MB clears from the mammary gland and mammary function (yield and composition) recovers to the pre-MB level (Farr et al., 1996). It is not uncommon to perform multiple MB on the same animal to examine the effect of time or treatment by time. It has been reported that repeated MB using a biopsy needle (Lima et al., 2016) or a trocar coupled to a syringe (de Lima et al., 2016) transiently reduced milk yield after each MB. However, in most studies, the impact of repeated MB on cow performance was examined by comparing milk yield of the same cow before and after MB without any control animals included in the analyses. Thus, the objective of this study was to extend previous studies to examine the impact of repeated MB

using a rotating stainless steel cannula on DMI, milk yield and composition, and mammary health of lactating dairy cows over a 6-month period.

Materials and Methods

Animals, Experimental Design

Data used in this study were extracted from an experiment described by Weng et al. (2016). The trial was conducted at the Dairy Research Center on the University of Georgia Tifton Campus from April to October, 2015. Treatments and animal handling were approved by the University of Georgia Institutional Animal Care and Use Committee prior to beginning the trial. Sixty-four multiparous mid-lactation Holstein cows were enrolled in the trial and randomly assigned into either MB (n=32) or non-MB group (**NMB**, n=32). Cows from both groups had similar parity (MB = 2.5 vs. NMB = 2.8, SEM = 0.2, $P = 0.13$) and DIM (MB = 85.7 vs. NMB = 107.3, SEM = 9.8, $P = 0.13$) at enrollment. At the beginning of the trial, one animal in the MB group was excluded from the experiment due to ketosis related indigestion, and two cows from the NMB group were excluded because of either lameness or refusal to eat behind a Calan gate. The data from these 3 cows were not included in the analyses. Further, one NMB cow developed toxic mastitis during the second week of the trial and was excluded from the experiment. Due to the short period on the trial for this animal (one week), the DMI and lactation performance data of this animal were not included in the analyses, but the incidence of mastitis was reported.

Cows were housed in a 4-row freestall barn for 29 wk and managed similarly during the experimental period. The MB were performed at wk 2, 15 and 24 of the trial. The first and third MB were performed on the left rear quarter, whereas the second MB was performed on the right rear quarter. Cows were milked thrice daily (0800, 1600, and 2400 h) and milk yield was

recorded at each milking (ALPRO herd management, Delaval, Tumba, Sweden) throughout the trial. Milk samples were collected weekly from 3 consecutive milkings for SCC and composition analysis (fat, protein, lactose, SNF, Dairy One, Ithaca, NY). The somatic cell score (SCS) was calculated based on the equation: $SCS = \log_{10}(SCC/12.5)/\log_{10}2$. Cows were fed ad libitum and DMI was recorded daily for individual cows using the Calan gate system (American Calan, Northwood, NH) during the first 25 wk of the trial. Milk yield and composition during the following week of each MB were not recorded because milk was discarded due to blood contamination.

Mammary Tissue Collections

The procedure for MB was based on the method reported by Farr et al. (1996). All animals were examined with no signs for clinical mastitis (presence of visible flakes in milk during forestripping) before each MB. Cows were sedated by intravenous injection of xylazine hydrochloride (20 μg/kg of BW, AnaSed, LLOYD Laboratories, Shenandoah, IA) before each MB. The biopsied region in the midpoint of the quarter was shaved and sanitized three times by scrubbing with iodine followed by 70% ethanol. Local anesthesia was achieved with subcutaneous injection of 3 mL 2% lidocaine hydrochloride (Animal Rx Pharmacy, Atlanta, GA) above the biopsy site. A 2-3 cm incision was made through skin and connective tissue, avoiding apparent blood vessels. A core of mammary tissue was obtained using a rotating stainless steel cannula with a retractable blade connected to a cordless drill (Farr et al., 1996). Incisions were then closed using 18-mm stainless steel Michel wound-clips (GerMedUSA, Garden City Park, NY) followed by topical administration of an aerosol bandage (Neogen Corporation, Lexington, KY) to prevent infection. After MB, cows were kept in a small rubber flooring pen for 30-60 min to assure absence of any visible bleeding before returning to the freestall. After each MB, the

quarter that was subjected to MB was hand-stripped before each milking to remove intramammary blood clots. Incision healing and presence of blood in milk were visually assessed at each milking. The occurrence of clinical mastitis was monitored by examining the milk for presence of visible flakes during forestripping at each milking.

Data Analysis

The UNIVARIATE procedure of SAS 9.4 (SAS Institute, Cary, NC) was used to calculate the time elapsed after MB until blood was cleared from milk and wound clips were removed from the incision site and the means ± standard deviation are reported. The GLM procedure of SAS was used to analyze parity and DIM. The MIXED procedure of SAS 9.4 was used to analyze the repeated measures data including DMI, milk yield, and composition. The statistical model included fixed effects of treatment, time and their interaction with cow nested in the treatment as the random effect. For milk yield analysis, the average daily milk yield before onset of the experiment was included in the SAS model as a covariate. Data are reported as least squares means ± standard error of the mean. Significance and tendency were declared when $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

Results and Discussion

A core (~70 × 4 mm) of mammary tissue weighing ~ 0.75 g was obtained in the current trial. In contrast to the small tissue fragments collected using biopsy needles (Lima et al., 2016), the amount of tissue collected by rotating cannula is sufficient for most histological and biochemical studies (Annen et al., 2007; Miller et al., 2006). Only one attempt was performed during each MB and the entire procedure lasted less than 30 min. Although apparent large vessels were avoided, external bleeding was not completely eliminated in the present study. In

contrast, external hemorrhaging was minimized in MB using biopsy needles (Lima et al., 2016). In the current study, blood was cleared from milk at 3.9 ± 2.0 d (mean ± SD) after MB, consistent with previous study using rotating cannula (Farr et al., 1996) or biopsy needle (Lima et al., 2016), and wound clips were removed at 4.6 ± 1.0 d after MB (Table 4.1), indicating rapid incision healing and recovery of mammary function. Further, there was an effect of MB sequence ($P < 0.01$) on the time when blood was cleared from milk, because it took longer for mammary gland to clear the blood following the first MB compared with the subsequent MB (3.7 vs. 2.5 vs. 2.3 d [LSM] for the first, second, and third MB, respectively, SEM = 0.6 d). These data may reflect the experience of the technician to remove intramammary blood clots during the hand-stripping before milking. Throughout the entire trial, 4 (6.5%) and 5 (8.3%) incidences of clinical mastitis were identified in rear quarters of MB and NMB cows (Table 4.1), respectively, with no reoccurrence of mastitis in the same quarter of same cow, indicating MB had no influence on mammary health. A higher incidence of mastitis (12%) was reported by Lima et al. (2016), but no occurrence of mastitis was observed by Farr et al. (1996) or Annen et al. (2007) after MB. The inconsistent results of mastitis occurrence after MB in different studies may be due to different stages of lactation of experimental animals, various lengths of experiments, the application of antibiotics following MB (Annen et al., 2007; Farr et al., 1996), different housing and milking management, distinct MB methods, or a combination of all. Regardless, data from the current study indicate that a low mastitis incidence following MB can be achieved through good management even without the application of antibiotics.

No treatment effect or treatment by time interaction were observed ($P \geq 0.49$) in DMI between MB and NMB cows (25.3 vs. 25.7 kg/d, SEM = 1.2 kg/d, Figure 4.1), suggesting no apparent systemic influence of repeated MB on the cows. Further, there was no difference ($P =$

0.63) in milk yield between both groups (MB = 35.8 vs. NMB = 35.2 kg/d, SEM = 1.7 kg/d, Figure 4.2) throughout the trial, indicating that repeated MB had no long term impact on milk yield of lactating Holstein cows. One limitation of the current study was that the short-term (within one week) effect of MB on milk yield was not examined. To ensure consistent milking time and procedure, all cows were milked at the same time in the same milking parlor during the entire experiment. The milk was collected into a bucket during the following week of MB and subsequently dumped to avoid the blood contamination into the main milk line, and hence the milk yield was not recorded. Using biopsy needles, Lima et al. (2016) reported that milk yield was reduced only on the following day of MB. In contrast, using a MB procedure similar to the one used in the current experiment, Farr et al. (1996) reported that milk yield was significantly reduced after tissue collection, but recovered to the pre-MB level within 6.5 d post-MB. The short term reduction in milk yield may be due to the trauma and stress caused by MB; however, milk yield fully recovers within one week as shown in the current study. The cellular mechanisms of the rapid recovery of milk yield after MB is unknown. Lactating mammary gland experiences compensatory growth in response to various stimuli (Capuco and Akers, 1990; Knight and Peaker, 1982), therefore, it is possible that, in the current study, the mammary gland also experienced a compensatory growth due to the loss of secretory tissue. However, it is unclear if the compensatory growth occurred within the MB quarter or the non-MB quarters. Future study needs to be conducted to evaluate the milk yield response on individual quarters after MB. Moreover, in the current study, MB were performed in alternating rear quarters with extended interval (2 to 3 mo). It is not uncommon that subsequent MB are performed on the same quarter in a shorter interval, i.e, one or two wk; therefore, future research is also required to

further examine the impact of repeated MB on the same quarter with a shorter interval on milk production and mammary health at the single quarter level and the whole animal level.

Repeated MB had no long term effect on SCS and concentration or yield of milk fat, protein, lactose, and SNF ($P \geq 0.11$, Table 4.1), consistent with Farr et al. (1996). Similar to milk yield, the short-term impact of MB on milk composition was not evaluated in the current study. Previous MB research (Farr et al., 1996) in mid-lactation cows indicated an immediate but transient decrease in concentration of milk lactose due to leakage of lactose from the mammary gland into the circulatory blood system, resulting from breakage of the blood-milk barrier. In contrast, in early lactating cows, Lima et al. (2016) observed increased milk lactose concentrations but a decrease in milk protein concentrations following MB; however, data were confounded with dramatic changes of milk composition during early lactation (Linn, 1988; Tsioulpas et al., 2007). Regardless of the approaches used, milk SCC transiently increase after MB in dairy cattle as reported in previous studies (Farr et al., 1996; Lima et al., 2016), indicating a possible inflammatory response due to trauma caused by MB. Data from the current study suggested that milk SCS returned to pre-biopsy levels within 1 wk after MB. The change in milk SCS is consistent with the incidence of mastitis between MB and NMB cows, and further confirms that repeated MB have no negative long term impacts on milk quality and mammary health.

Conclusion

In conclusion, although external hemorrhage occurred during MB, blood cleared from milk within one wk. The mammary gland recovered rapidly after MB and no evidence was found

that MB impairs milk yield and composition, milk quality and mammary health. Overall, repeated MB had no impact on performance of lactating dairy cows.

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Table 4.1. Incidence of mastitis, time for blood clearance in milk and removal of wound clips and milk composition for cows subjected to repeated mammary biopsy collection (MB, n = 31) or not (NMB, n = 30)

Parameter	MB	NMB	SEM	*P*-value
Mastitis[1]	4	5	--	--
Blood clearance, d[2]	3.9	--	2.0[3]	--
Wound clips removal, d[2]	4.6	--	1.0[3]	--
Milk composition				
Fat, %	3.32	3.34	0.09	0.56
Fat, kg/d	1.23	1.24	0.08	0.93
Protein, %	2.69	2.77	0.03	0.11
Protein, kg/d	1.00	1.00	0.07	0.78
Lactose, %	4.67	4.66	0.03	0.75
Lactose, kg/d	1.75	1.72	0.16	0.60
SNF, %	8.02	8.08	0.05	0.34
SNF, kg/d	2.97	2.95	0.31	0.83
SCS[4]	2.85	3.17	0.35	0.34

[1]Number of mastitis incidences in rear quarters of MB or NMB cows during the trial.
[2]Days after MB.
[3]Standard deviation.
[4]SCC linear score (SCS) = $\log_{10}(SCC/12.5)/\log_{10}2$.

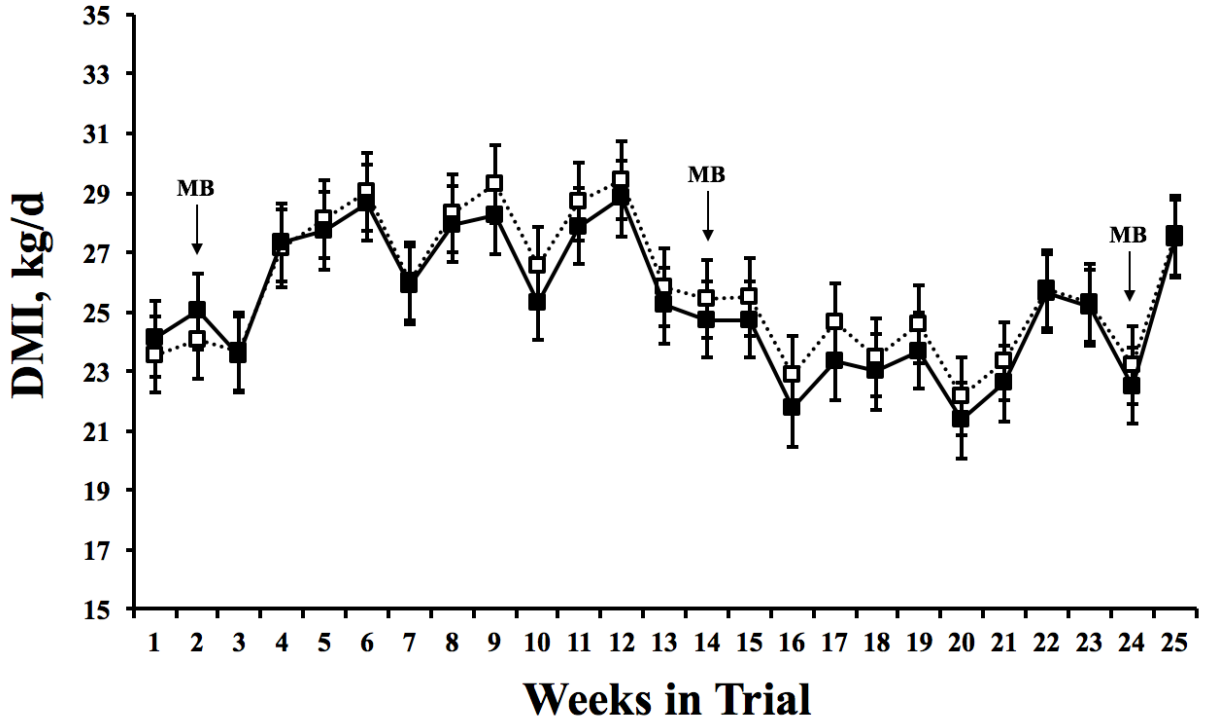

Figure 4.1. The DMI of lactating cows that were subjected to repeated mammary biopsy collection (n = 31; solid squares [■]) or not (n = 30; open squares [□]). No treatment ($P = 0.49$) or treatment by time ($P = 0.79$) effects were observed.

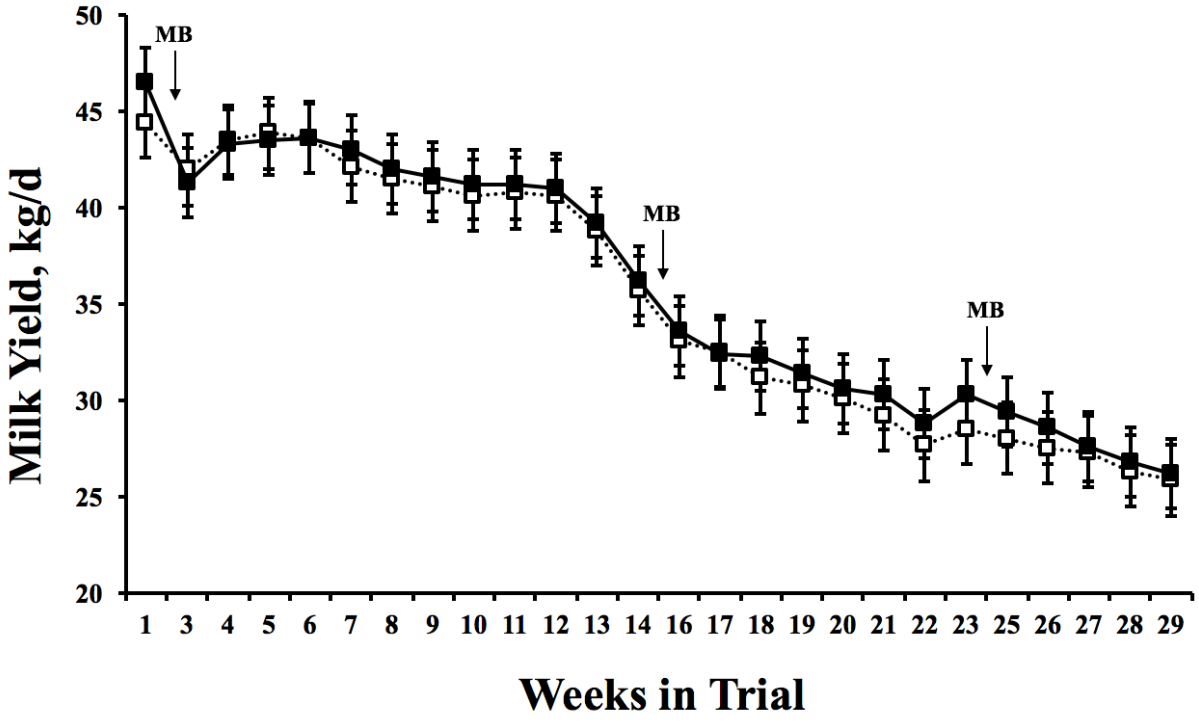

Figure 4.2. Milk yield of lactating cows that were subjected to repeated mammary biopsy collection (n = 31; solid squares [■]) or not (n = 30; open squares [□]). No treatment ($P = 0.63$) effect was observed, but there was a tendency for a treatment by time interaction ($P = 0.06$). However, the PDIFF and SLICE functions of SAS indicated time effects within each treatment rather than treatment effects at individual time points.

CHAPTER 5

CONCLUSIONS

Environmental heat stress remarkably impairs lactation performance of lactating dairy cows while dietary zinc source had no effect on lactation performance. Mammary epithelial integrity was largely maintained in heat-stressed cows, and no differences on protein expression of occludin and E-cadherin were observed. However, increased gene expression of epithelial junctional proteins was observed in mammary gland of heat-stressed cows. Results indicate that heat stress affects cellular events associated with epithelial integrity, and cows under environmental heat stress are able to maintain the mammary epithelial permeability by regulating gene expression of epithelial junctional proteins. Future study on whether protein turnover occurs under heat stress relative to thermos-neutrality is required. Replacing 40 ppm of zinc hydroxychloride with zinc-MET complex in the diet of lactating cows improved mammary epithelial integrity regardless of environmental heat stress, which may due to the higher bioavailability of organic form of zinc. Future study on evaluating the cellular mechanism of dietary zinc source on mammary epithelial barrier is required, such as re-localization of epithelial junctional proteins.

The procedure of mammary tissue collection causes external and internal hemorrhaging within a short period in lactating dairy cows. However, cows recovered rapidly and repeated mammary tissue collection had no long term effect on total milk yield, milk

composition, quality, and udder health. Nevertheless, evaluating the effect of repeated mammary tissue collections on cow performance of the single quarter is of interest to examine the possible compensatory effect from other three quarters.