Response of lactating dairy cows fed different supplemental zinc sources with and without evaporative cooling to intramammary lipopolysaccharide infusion: intake, milk yield and composition, and hematologic profile¹

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ABSTRACT: The objective of this study was to determine the effect of dietary supplemental Zn source and evaporative cooling on intake, milk yield and composition, and the rate of leukocyte migration into the mammary gland following intramammary lipopolysaccharide (LPS) infusion. Multiparous Holstein cows ($n = 72$) were assigned to one of four treatments with a 2×2 factorial arrangement including two sources of supplemental Zn: 75 mg/kg Zn hydroxychloride or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (ZMC) each with or without evaporative cooling. The cooling system was implemented by the use of fans and misters over the freestall and feeding areas. On day 34 of the experiment, cows ($n = 16$; days in milk = 263 ± 63 d) received an infusion of 10 µg of LPS, or a saline control, in the left or right rear quarters. Individual milk samples from both quarters were collected at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion and analyzed for composition and bovine serum albumin. Rectal temperature and respiration rate were assessed and blood samples were collected at the same time points (with an additional sample at 3 h) for analyses

of lactose and cortisol. Complete blood counts were performed on samples collected within the first 24 h post infusion. Intramammary LPS infusion reduced ($P < 0.01$) milk yield, DMI and feed efficiency regardless of dietary or cooling treatments. Non-cooled cows tended ($P = 0.09$) to have greater feed efficiency (=milk yield/DMI) at 1 d after infusion than those subjected to cooling. Intramammary LPS infusion dramatically increased ($P < 0.01$) milk somatic cell count (SCC) but treatments had no apparent impact on milk SCC. Compared with cooled cows, non-cooled cows had greater ($P < 0.05$) plasma lactose concentrations, but lower ($P < 0.03$) blood concentrations of neutrophils and lymphocytes at 3 h post infusion. This suggests a greater leukocyte migration into the mammary gland of heat-stressed cows. In conclusion, noncooled cows tended to maintain greater feed efficiency and appeared to have greater leukocyte migration into the mammary gland immediately after intramammary LPS infusion compared with cooled cows. Dietary supplemental Zn source had no impact on measures assessed after intramammary LPS infusion.

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

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INTRODUCTION

Udder health is related to the productivity of animals and the economic return of dairy producers. A large portion of the economic loss due to impaired udder health (i.e., clinical mastitis)

is attributed to decreased milk yield (Rollin et al., 2015). Therefore, it is important to understand how environment and nutritional factors influence udder health and alter cow responses to mammary infection. Milk somatic cell count (SCC) and incidences of clinical mastitis increase during summer (Hogan et al., 1989; Kadzere et al., 2002; Olde Riekerink et al., 2007), which can be attributed to both environmental and animal factors (Kadzere et al., 2002; Hogan and Smith, 2012). During summer, the hot and humid environment supports greater growth and survival of pathogens in the environment of the cow compared with cold and dry weather (Kadzere et al., 2002), increasing the chance of mammary infection. Further, immune cell function is compromised with heat stress. Peripheral blood mononuclear cells exposed to heat stress *in vitro*, or *in vivo*, have a lower mitogen-induced proliferative capacity (Kamwanja et al., 1994; Lacetera et al., 2006; do Amaral et al., 2010), suggesting impaired cellular immunity. Neutrophils cultured under elevated ambient temperature or isolated from heat-stressed cows have reduced phagocytosis and killing ability by oxidative burst (do Amaral et al., 2011; Lecchi et al., 2016). Heat-stressed lactating cows also have a reduced leukocyte migration into the mammary gland in response to an intramammary chemotactic challenge of oyster glycogen (Elvinger et al., 1992). However, the impact of heat stress on lactating cows' responses, such as milk yield, intake and leukocyte migration into the mammary gland, to intramammary infection or to mammary inflammation is seldom studied.

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

inorganic or AA chelated Zn will affect the response of cows to mammary infection, or induced inflammation, particularly during heat stress.

The hypothesis of this study was that heat stress impairs leukocyte migration into the mammary gland and exaggerates the reduction of milk yield after intramammary lipopolysaccharide (LPS) infusion into lactating quarters of dairy cows, and that replacement of portion of the inorganic source of supplemental Zn with ZMC in the lactating cow diet will improve immune cell migration into the mammary gland and alleviate the reduction in milk yield after intramammary LPS infusion. The objective was to determine the effects of supplemental inorganic or AA chelated Zn in the lactating cow diet and evaporative cooling on milk yield and composition, DMI, feed efficiency, hematologic profile, blood lactose, and milk bovine serum albumin concentrations in the lactating dairy cow to an intramammary LPS infusion during the summer.

MATERIALS AND METHODS

Animals and Experimental Design

The animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia before initiation of the experiment. This study was conducted from April to September, 2015 at the Dairy Research Center, located on the University of Georgia-Tifton campus.

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

<85%. Cows were milked three times each day at 0800, 1600, and 2400 h, and were fed a total mixed ration (TMR) once daily at 1300 h. The TMR was supplemented with either IOZ or ZMC. Each Zn supplement was mixed with TMR before feeding. The composition and nutrient content of experimental diets were reported by Weng et al. (2018). Milk yield of individual cows was recorded at each milking (Alpro, DeLaval, Kansas City, MO), and the daily feed intake was measured using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH). Barn air temperature and relative humidity were monitored by Hobo Pro Series Temp probes (Onset Computer Corporation, Pocasset, MA) every 15 min throughout the experiment and temperature-humidity index (THI) was calculated based on $THI = (1.8 \times T + 32) - ((0.55 - 0.0055 \times RH) \times (1.8 \times T - 26))$, where T = air temperature (°C), RH = relative humidity (%) (Dikmen and Hansen, 2009). The THI during the entire experiment averaged 77.7. Compared with cows receiving cooling, cows that were deprived of cooling had increased ($P < 0.01$) respiration rates (64.8 vs. 73.9 breath/min) and vaginal temperature (39.03 vs. 39.94 °C), suggesting increased heat load. Supplemental Zn source did not affect vaginal temperature or respiration rates ($P > 0.25$). Noncooled cows had lower ($P < 0.01$) milk yield (24.5 vs. 34.1 kg/d), DMI (21.6 vs. 26.7 kg/d), and feed efficiency (=milk yield/DMI; 1.21 vs. 1.34) compared with cooled cows. Supplemental Zn source did not influence these parameters ($P > 0.60$). Neither supplemental Zn source nor cooling impacted milk somatic cell score or concentrations of protein and fat ($P > 0.10$), but noncooled cows had higher ($P < 0.01$) concentrations of milk urea nitrogen (MUN) relative to cooled cows.

Intramammary infusion of LPS or saline was performed on day 34 of the experiment. Three days before infusion, individual milk samples from the left and right rear quarters of all cows were aseptically collected to measure SCC and cultured to detect common mastitis-causing pathogens. Twenty cows ($n = 5$ per treatment) with culture negative milk samples and SCC below 200,000 cells/mL from both rear quarters were included in the infusion study. Because there are no studies reported in the literature examining the impact of sources of supplemental Zn in the diet of lactating cows or heat stress on cow responses to intramammary LPS infusion, power analysis was performed based on data reported by Zarrin et al. (2014). Zarrin et al. (2014) studied the impact of induced hyperketonemia on mammary immune responses to intramammary

LPS infusion of lactating dairy cows. The milk $\log_{10}$ (SCC) data collected at 3 h after intramammary LPS infusion of hyperketonemic and control cows (5.10 vs. 5.65, SD = 0.24, respectively) were used to calculate the anticipated number of cows needed to provide sufficient statistical power. A sample size of five cows per treatment was calculated using a level of significance of 0.05 and 80% power to detect differences if present.

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

Intramammary Infusion of LPS or Saline, and Data and Sample Collection

During and after the intramammary infusion, all cows remained in their respective pens. Two hours after morning milking, 10 μ g of *Escherichia coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO; Greco et al., 2015) dissolved in 5 mL pyrogen-free saline solution was infused into the left rear quarter and 5 mL pyrogen-free saline solution was infused into the right rear quarter. Teats from infused quarters were massaged thoroughly to move the infused solution into the gland cistern. Daily milk yield and DMI were collected from 7 d before until 7 d after intramammary infusion. Rectal temperature and respiration rate were assessed, and individual quarter milk samples (50 mL) collected at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion. Rectal temperatures were measured using a GLA M700 digital thermometer (GLA Agricultural Electronics, San Luis Obispo, CA) and respiratory rates were assessed by counting flank movements for 1 min. Before milk sample collection, teats were dipped with an iodine-based

solution (0.1%), cleaned using paper towels and alcohol pads, and the first five stream of milk was discarded. One aliquot of the milk sample was stored with bronopol-B-14 as a preservative for analyses of milk components (fat, protein, MUN, and SCC; Dairy One Cooperative, Ithaca, NY) using a Foss 4000 instrument (Foss North America, Eden Prairie, MN). A second aliquot was stored without a preservative at -20°C for milk BSA analysis. Blood was collected following the same schedule as milk samples with an additional sample collected at 3 h after infusion. All blood samples were collected from coccygeal vessels into vacutainer tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately placed on ice and then centrifuged at $2,619 \times g$ for 30 min at 4°C to obtain plasma. Additional blood samples were collected at 0, 3, 6, 12, and 24 h relative to infusion into K3 EDTA vacutainer tubes (Becton Dickinson) to assess hematologic profile at the University of Georgia Tifton Veterinary Diagnostic Laboratory (Tifton, GA) using a hematology analyzer (ADVIA 2120i Hematology System, Siemens, Tarrytown, NY).

Sample Analyses

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

Statistical Analyses

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

RESULTS

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

Supplemental Zn source and its interaction with cooling or time had no ($P \geq 0.13$) effect on milk yield, DMI, feed efficiency, rectal temperature, and respiration rate of cows following intramammary LPS infusion (Table 1). Regardless of the supplemental Zn source, cooled cows produced more ($P \leq 0.05$) milk from -7 to -1 d and at 2, 3, 4, 5, and 7 d relative to intramammary LPS infusion, but tended ($P \leq 0.08$) to have greater milk yield at 1 and 6 d after LPS infusion compared with noncooled cows (cooling $\times$ time interaction: $P < 0.01$, Figure 2A). Noncooled cows consumed less ($P < 0.01$) DM, and had greater ($P < 0.01$) rectal temperature and respiration rate relative to cooled cows (Table 1 and Figures 1 and 2B). Interestingly, noncooled cows tended ($P = 0.09$) to have higher feed efficiency at 1 d after intramammary LPS infusion compared with cooled cows (cooling $\times$ time interaction: $P = 0.01$, Figure 2C).

Table 1. Milk yield, DMI, feed efficiency (milk yield/DMI), rectal temperature, and respiration rate of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either cooling (CL) or not (NC), in response to intramammary lipopolysaccharide (LPS) infusion

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

The daily milk yield and DMI were measured from -7 to 7 d relative to intramammary infusion, and rectal temperature and respiration rate were measured at -12 , -4 , 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion.

¹Z = Zn source effect; C = cooling effect; T = time effect.

Figure 1. Temperature-humidity index (solid line, no symbols) and rectal temperature of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]) in response to intramammary lipopolysaccharide (LPS) infusion. ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

Milk Composition

Regardless of environmental and dietary treatments, intramammary LPS infusion increased concentrations of milk fat, protein, MUN, and $\ln(\text{SCC})$ in LPS infused quarters (time effect: $P < 0.01$, Table 2 and Figure 3). In saline infused quarters, the percentages of milk fat, MUN, and $\ln(\text{SCC})$ increased but milk protein concentration decreased after saline infusion (time effect: $P < 0.01$, Table 2 and Figure 4). There were no effects of supplemental Zn source or its interaction with cooling or time on milk composition in either LPS or saline infused quarters (Table 2). LPS infused quarters of noncooled cows had greater ($P = 0.05$) milk fat concentration at 24 h and tended ($P = 0.10$) to have greater milk fat concentration at 6 h after infusion compared with cooled cows (cooling $\times$ time: $P = 0.07$, Figure 3A). A cooling $\times$ time interaction ($P = 0.01$) was observed for milk $\ln(\text{SCC})$ in LPS infused quarters (Figure 3C). Compared with cooled cows, the $\ln(\text{SCC})$ of LPS infused quarters of non-cooled cows was greater ($P < 0.01$) at -12 h, lower ($P = 0.02$) at 96 h, and tended ($P = 0.10$) to be lower at 120 h relative to infusion (Figure 3C). Relative to cooled cows, milk collected from LPS infused quarters of noncooled cows had lower ($P \leq 0.10$) protein concentrations before infusion (cooling $\times$ time interaction: $P < 0.01$, Figure 3B), and greater ($P \leq 0.06$) MUN concentrations at 12, 48, 96, 120, 144, and 168 h following infusion (cooling $\times$ time interaction: $P = 0.06$, Figure 3D). Similarly, MUN concentrations of saline infused quarters of non-cooled cows were greater ($P < 0.01$) at 168 h and tended ($P \leq 0.08$) to be greater at 72, 120, and 144 h following infusion (cooling $\times$ time: $P = 0.02$, Figure 4).

Figure 2. Milk yield (A), DMI (B) and feed efficiency ([milk yield/DMI], C) of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]) in response to an intramammary lipopolysaccharide (LPS) infusion. ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

Hematologic Profile

Regardless of supplemental Zn source or cooling, intramammary LPS infusion resulted in temporary reductions of blood concentrations of plasma protein, white blood cells, eosinophils, monocytes, lymphocytes, and neutrophils (time effect: $P \leq 0.01$) (Table 3). Supplemental Zn source and its interactions with cooling or time had no ($P \geq 0.17$) impact on hematologic profiles (Table 3). Relative to cooled cows, noncooled cows had lower ($P = 0.02$) blood monocyte concentrations (Table 3 and Figure 5D). Cooling by time interactions ($P \leq 0.03$) were observed for blood concentrations of plasma protein, white blood cells, lymphocytes, and neutrophils after infusion.

Table 2. Milk composition of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either cooling (CL) or not (NC), in response to intramammary lipopolysaccharide (LPS) or saline infusion. Milk samples were collected from LPS and saline infused quarters at -12, -4, 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h relative to infusion

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

¹Z = Zn source effect; C = cooling effect; T = time effect.

Superscripts: a vs. b: $P < 0.10$.

Figure 3. Milk concentrations of fat (A), protein (B), ln(somatic cell count [SCC]) (C), and urea nitrogen (D) in lipopolysaccharide (LPS) infused quarter of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]). ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

Compared with cooled cows, noncooled cows had lower ($P \leq 0.03$) blood concentrations of white blood cells, lymphocytes, neutrophils, and plasma protein at 3 h after intramammary LPS infusion, and tended ($P = 0.07$) to have lower concentration of blood lymphocytes at 6 h after intramammary LPS infusion (Table 3 and Figure 5A–C).

Plasma Lactose and Cortisol and Milk BSA

Intramammary LPS infusion increased plasma lactose concentrations (time effect: $P < 0.01$, Fig. 6A). Consistently, milk BSA concentrations were increased (time effect: $P < 0.01$) in LPS infused quarters, but not in saline infused quarters (Figure 6B). There was no

effect of supplemental Zn source, and its interaction with cooling or time ($P \geq 0.72$) on plasma lactose concentrations. Supplemental Zn source or cooling had no ($P \geq 0.28$) impact on milk BSA concentrations in LPS infused quarters following infusion. Compared with cooled cows, the plasma lactose concentrations of noncooled cows was greater ($P < 0.05$) at 0 and 3 h and tended ($P = 0.09$) to be greater at -4 h relative to intramammary LPS infusion (cooling $\times$ time: $P < 0.01$, Figure 6A).

Relative to cooled cows, noncooled cows had lower plasma cortisol concentrations (cooling effect: $P = 0.03$); however, there were no ($P = 0.24$) significant interactions between cooling by time for plasma cortisol concentrations (Figure 7A). Further, there was a tendency ($P = 0.10$) for a supplemental Zn source by time interaction, such that the plasma cortisol concentration of cows supplemented with ZMC was greater ($P = 0.05$) at 3 h and tended to be greater at 0 h relative to intramammary LPS infusion compared with cows fed IOZ (Figure 7B).

Figure 4. Milk concentration of urea nitrogen in saline infused quarter of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]). ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

DISCUSSION

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

It is interesting that noncooled cows tended to have greater feed efficiency 1 d after intramammary infusion of LPS compared with cooled cows. Because there was no interaction of cooling by time for DMI, both noncooled and cooled cows had similar decreases in DMI after infusion. Thus, the greater feed efficiency of noncooled cows compared with cooled cows 1 d after infusion may be due to the lower reduction in milk yield by a local mechanism within the LPS infused mammary gland. Due to heat stress, cooled cows maintained

Table 3. Hematologic profile of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC), and exposed to either evaporative cooling (CL) or not (NC), in response to intramammary lipopolysaccharide infusion

Item	IOZ		ZMC		SEM	P-value						
	CL	NC	CL	NC		Z ²	C	Z $\times$ C	T	Z $\times$ T	C $\times$ T	Z $\times$ C $\times$ T
White blood cells, $\times 10^3$ cells/ μ L	9.79	5.86	9.46	6.35	2.05	0.17	0.60	0.59	<0.01	0.38	0.03	0.28
Lymphocyte, $\times 10^3$ cells/ μ L	5.35	2.48	4.26	2.73	1.21	0.85	0.10	0.64	<0.01	0.71	<0.01	0.37
Neutrophil, $\times 10^3$ cells/ μ L	2.69	2.33	2.98	2.98	0.32	0.17	0.60	0.59	<0.01	0.38	0.03	0.28
Monocyte, $\times 10^3$ cells/ μ L	0.37	0.22	0.30	0.21	0.04	0.36	0.02	0.57	<0.01	0.83	0.22	0.91
Eosinophil, $\times 10^3$ cells/ μ L	1.60	1.52	1.64	1.44	0.09	0.78	0.13	0.52	<0.01	0.31	0.17	0.48
Basophil, $\times 10^3$ cells/ μ L	0.09	0.06	0.08	0.08	0.03	0.92	0.65	0.56	0.43	0.58	0.41	0.63
Hematocrit, %	28.4	27.9	27.4	27.4	0.52	0.20	0.63	0.60	<0.01	0.30	0.67	0.53
Plasma protein, g/dL	8.09	7.82	7.76	7.69	0.19	0.26	0.39	0.63	0.01	0.32	0.01	0.29

Blood samples were collected at 0 h (before infusion) and at 3, 6, 12, and 24 h after infusion.

¹Z = Zn source effect; C = cooling effect; T = time effect.

Figure 5. The blood concentrations of white blood cells (A), lymphocytes (B), neutrophils (C), and monocytes (D) of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]) after intramammary lipopolysaccharide (LPS) infusion. ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

greater milk yield than noncooled cows before and after intramammary LPS infusion except for 1 d after infusion when cooled cows only tended to have greater milk yield than noncooled cows. This may further support that the mammary gland of noncooled cows had a lower reduction in milk yield immediately after intramammary LPS infusion relative to cooled cows. Heat shock proteins (HSP) are evolutionarily conserved cellular stress proteins constitutively expressed at low levels in all somatic cells. Their expression can be induced by stress conditions including heat stress (Kregel, 2002). In the bovine, exposure to elevated temperature in vitro resulted in increased gene expression of HSP70 by mammary epithelial cells (Collier et al., 2006). The lactating mammary gland has greater HSP27, HSP70, and HSP90 gene expression when exposed to heat stress (Orellana et al., 2017). Because HSP have cytoprotective functions as molecular chaperones and inhibit apoptosis (Kregel, 2002, Schmitt et al., 2007), the enhanced mammary HSP expression induced by heat stress in lactating mammary gland may exert a protective function for cell survival and maintenance of milk synthesis after a secondary insult, such as intramammary infusion of LPS. Because milk yield of the individual quarter was not measured, this hypothesis was only partly

tested in the current study but deserves further investigation.

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

Consistent with previous research (Aditya et al., 2017), milk fat concentrations were increased in both LPS and saline infused quarters. This is likely due to a dilution effect resulting from decreased milk

Figure 6. The plasma concentration of lactose (A) of cows exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]) in response to intramammary lipopolysaccharide (LPS) infusion. Milk concentration of bovine serum albumin (BSA) (Figure 6B) in LPS infused quarter of cows under cooling or not, and fed diets supplemented with 75 mg/kg Zn hydroxychloride (IOZ), or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (ZMC) (cooled cows fed IOZ: solid circle [●]; noncooled cows fed IOZ: open circle [○]; cooled cows fed ZMC: solid square [■]; noncooled cows fed ZMC: open square [□]). The solid triangle [▲] in B represents the milk BSA concentration in saline infused quarters. For plasma lactose concentration, effect of Zn source ($P = 0.76$), cooling ($P = 0.06$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.99$), Zn source by time interaction ($P = 0.72$), cooling by time interaction ($P < 0.01$), and cooling by Zn source by time interaction ($P = 0.52$). For milk BSA concentration in LPS infused quarter, effect of Zn source ($P = 0.94$), cooling ($P = 0.95$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.87$), Zn source by time interaction ($P = 0.57$), cooling by time interaction ($P = 0.28$), and cooling by Zn source by time interaction ($P = 0.44$). ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

yield. Compared with cooled cows, milk fat concentrations in LPS infused quarters of noncooled cows were greater at 6 and 24 h post infusion. Relative to cooled cows, noncooled cows had similar plasma β -hydroxybutyrate acid and triglyceride concentrations after intramammary LPS infusion, but lower concentrations of nonesterified fatty acids after 12 h post infusion (Marins and Tao, unpublished data, University of Georgia, Tifton). These results may suggest that cooling does not have a significant influence on milk fat synthesis after intramammary LPS infusion. Therefore, the greater milk fat concentrations at 6 and 24 h post LPS infusion may be explained by the lower reduction in milk yield in LPS infused quarters of noncooled cows compared

Figure 7. The effects of evaporative cooling (A) and dietary Zn source (B) on plasma concentrations of cortisol of lactating dairy cows in response to intramammary lipopolysaccharide (LPS) infusion. Cows were exposed to evaporative cooling (solid diamonds [◆]) or not (open diamonds [◇]), and fed diets supplemented with 75 mg/kg Zn hydroxychloride (IOZ, open triangle [Δ]), or 35 mg/kg Zn hydroxychloride + 40 mg/kg Zn-Met complex (ZMC, solid triangle [▲]). Effect of Zn source ($P = 0.18$), cooling ($P = 0.03$), time ($P < 0.01$), cooling by Zn source interaction ($P = 0.82$), Zn source by time interaction ($P = 0.10$), cooling by time interaction ($P = 0.24$), and cooling by Zn source by time interaction ($P = 0.27$). ** $P \leq 0.01$, * $P \leq 0.05$, † $P \leq 0.10$.

with cooled cows. Similar to Aditya et al. (2017), intramammary LPS infusion increased the milk protein percentage in LPS infused quarters, which may partially result from a dilution effect due to reduced milk yield. Coliform mastitis or mammary inflammation induced by intramammary LPS infusion results in a disrupted mammary epithelial junctions accompanied with an influx of serum proteins, such as albumin, immunoglobulin, into the mammary gland (Stelwagen and Singh, 2014). Moreover, intramammary LPS infusion increases the synthesis of antibacterial proteins and peptides and acute phase proteins in the mammary gland (Lohuis et al., 1988b; Isobe et al., 2009; Gott et al., 2015). This may also contribute to the increased milk protein percentage in the inflamed gland. In contrast, milk protein concentration was decreased after saline infusion regardless of the dietary and cooling treatments, suggesting that the systemic responses induced by intramammary LPS infusion reduced milk protein synthesis in saline infused quarters.

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

Compared with cooled cows, noncooled cows had greater reductions in blood concentrations of white blood cells, neutrophils, and lymphocytes immediately after intramammary infusion of LPS. This suggests greater leukocyte migration into heat-stressed mammary gland, and trafficking to related lymph node in response to acute mammary inflammation. The greater plasma lactose concentrations at 3 h post infusion of noncooled cows further supports greater immune cell infiltration into the mammary gland compared with cooled cows. Immune cell infiltration is associated with disrupted mammary epithelial junction resulting in greater lactose leakage from the gland into the blood. As reviewed by Evans et al. (2015), hyperthermic treatment enhances lymphocyte trafficking into lymphoid organs by increasing the expression of L-selectin on leukocytes and $\alpha\beta 7$ integrin on high endothelial venules in lymph nodes, allowing the animal to mount more effective adaptive immunity. Hyperthermic treatment also augments neutrophil migration into inflamed, or infected, tissues through increased extravascular movement resulting from an enhanced inflammatory response (Evans et al., 2015). In contrast, previous studies reported that heat stress impairs neutrophil migration in response to chemoattractants in vitro and in vivo in dairy cattle (Elvinger et al., 1991, 1992). Therefore, results of the current study may suggest that the mammary gland of a noncooled cow exerts a stronger inflammatory response and subsequently releases more chemoattractants to attract immune cells in response to LPS infusion in comparison

with cooled cows. To test this hypothesis, future work needs to be conducted to examine the impact of heat stress on local inflammatory responses within the mammary gland after infection or induced inflammation. The biological significance of greater immune cell migration into the heat-stressed mammary gland immediately after intramammary LPS infusion is not understood. In dairy cattle, several lines of evidence suggest that heat stress compromises both adaptive and innate immunity. It is possible that the mammary gland of a heat-stressed cow requires more immune cells to compensate for impaired function caused by heat stress in order to cope with mammary infection.

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

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

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

CONCLUSION

In the current study, noncooled cows maintained higher body temperature, but tended to have greater feed efficiency and greater leukocyte migration into the mammary gland immediately after intramammary infusion of LPS compared with cooled cows. These results suggest that heat stress does not exaggerate the reduction in milk yield but may influence a greater recruitment of immune cells into the mammary gland during mastitis. Because heat stress impairs innate immunity in cows (do Amaral et al., 2011; Lecchi et al., 2016), it is unresolved if heat stress impacts the clearance of bacteria from the mammary gland during clinical mastitis. This question deserves further investigation. The source of supplemental Zn in dairy cow diets influences mammary health, and many studies have reported that replacing a portion of inorganic source of dietary supplemental Zn with AA chelated Zn reduces milk SCC. However, the results from the current study suggest that the source of supplemental Zn (Zn-Met vs. Zn hydroxychloride) has no impact on intake, milk yield and composition, and leukocyte infiltration into the mammary gland after intramammary LPS infusion. The

results in the current study provide valuable preliminary data to further define the impact of heat stress and nutritional factors on cow responses during intramammary infection.

Conflict of interest statement. None declared.

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