Short communication: Effect of supplemental zinc source with and without evaporative cooling on systemic and mammary metabolism of lactating dairy cows during summer

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ABSTRACT

The negative effects of heat stress partly result from disturbed systemic metabolic responses and possibly altered mammary gland metabolism of lactating dairy cows. Our previous research reported that supplemental dietary Zn sources may affect milk fat synthesis of lactating cows during summer. Thus, our objective was to evaluate the systemic and mammary metabolism of cows fed 2 supplemental Zn sources under 2 environmental conditions. Multiparous lactating Holstein cows ($n = 72$; days in milk: 99.7 ± 13.4 d; parity: 2.9 ± 0.3) were randomly assigned to 4 treatments in a 2×2 factorial arrangement. Treatments included 2 different environments: cooled (CL) using fans and misters or noncooled (NC), and 2 supplemental Zn sources: 75 mg of Zn hydroxychloride/kg of DM (IOZ) or 35 mg of Zn hydroxychloride/kg of DM + 40 mg of Zn-Met complex/kg of DM (ZMC). The 168-d experiment was divided into baseline and environmental challenge phases, 84 d each. During the baseline phase, all cows were cooled and fed respective dietary treatments, and during the environmental challenge phase cows continued receiving the same diets but NC cows were deprived of cooling. Temperature-humidity index averaged 77.6 ± 3.8 and 77.8 ± 3.8 for CL and NC pens, respectively, during the environmental challenge phase. Plasma was collected before the baseline phase and at 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75, and 81 d of the environmental challenge phase for metabolites and insulin analyses. Mammary biopsies were collected before the baseline phase and at 7 and 56 d of the environmental challenge phase to measure mRNA abundance of proteins related to mammary metabolism. Compared with CL, NC reduced plasma glucose, nonesterified fatty acids,

β -hydroxybutyrate, and triglyceride concentrations, but increased insulin concentration. Cows fed ZMC had greater plasma triglyceride concentration than IOZ. Treatments had no effect on mRNA abundance of protein related to mammary fatty acid and glucose metabolism except that NC cows had greater mammary mRNA abundance of 6-phosphogluconate dehydrogenase and ATP-dependent 6-phosphofructokinase than CL cows. In conclusion, deprivation of evaporative cooling influenced the metabolism of lactating dairy cows but dietary Zn source had no apparent effect.

Key words: heat stress, zinc, metabolism

Short Communication

Heat stress influences the performance of dairy cows partly by reducing nutrient intake and disturbing metabolism. Studies conducted in environmental chambers highlighted the inhibitory effect of heat stress on adipose tissue mobilization and fatty acid utilization, and the increased glucose utilization by extramammary tissues in heat-stressed lactating dairy cows despite decreased DMI (Wheelock et al., 2010; Lamp et al., 2015). In a dry lot setting, Tarazón-Herrera et al. (1999) reported that during summer, lactating cows provided only shade had similar plasma nonesterified fatty acid (NEFA) concentrations compared with those provided shade and evaporative cooling. In a freestall barn, mid- to late-lactation cows without evaporative cooling consumed 19% less DMI compared with those with cooling (Weng et al., 2018), but how deprivation of evaporative cooling affects blood metabolites and insulin of lactating cows is unknown. In lactating goats, exposure to acute heat stress reduced mammary gland glucose uptake (Sano et al., 1985). Additionally, Weng et al. (2018) reported that deprivation of evaporative cooling during summer decreased milk lactose concentration, suggesting altered glucose metabolism of mammary gland in lactating dairy cows. To the best of our knowledge, the cellular events related to metabolism in the mammary

Received February 17, 2020.

Accepted June 10, 2020.

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gland of cows with or without evaporative cooling have not been extensively studied.

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

The animals and experimental design were described previously in detail (Weng et al., 2018). Before the initiation of the trial, experimental procedures and animal handling were approved by the Institutional Animal Care and Use Committee of the University of Georgia (AUP#: A2014 10–003-Y1-A0). The study was conducted during the summer at the Dairy Research Center of the University of Georgia–Tifton campus. Seventy-two multiparous lactating Holstein cows were blocked by DIM (99.7 ± 13.4 d) and parity (2.9 ± 0.3) and then randomly assigned to 1 of 4 treatments with a 2×2 factorial arrangement. The 2 environmental treatments were evaporative cooling (**CL**) or without evaporative cooling (**NC**), and the 2 dietary treatments were 75 mg of supplemental Zn/kg of DM supplied from an inorganic source (Zn hydroxychloride, **IOZ**; Micronutrients, Indianapolis, IN) or 35 mg of Zn hydroxychloride/kg of DM and 40 mg of Zn-Met complex/kg of DM (**ZMC**; Zinpro Corp., Eden Prairie, MN). The experiment was divided into 2 phases with an 84-d baseline phase and a subsequent 84-d environmental challenge phase. During the baseline phase, all cows were cooled and fed their respective

dietary treatments. During the environmental challenge phase, all cows remained on the same diets but NC cows were deprived of evaporative cooling. The onset of the environmental challenge phase was considered as d 1 of the experiment. The stocking density in both NC and CL pens was similar and maintained below 100%, and all cows were managed in the same manner. The evaporative cooling system included misters attached to the front face of fans mounted over feed bunks and freestalls. The fans and misters were turned off for NC cows. Cows were fed a TMR once daily (1300 h). The TMR was supplemented with either IOZ or ZMC, and each Zn supplement was mixed with TMR before feeding. Cows were milked 3 times each day (0800, 1600, and 2400 h) and yield was recorded at each milking (Alpro, DeLaval, Kansas City, MO). Ambient temperature and relative humidity in both CL and NC pens were recorded every 15 min throughout the experiment to calculate temperature-humidity index (**THI**; NRC, 1971), and vaginal temperature of a subset of cows ($n = 16/\text{treatment}$) was measured every 5 min for 4 continuous days each week during the environmental challenge phase using an iButton (Mouser Electronics, Mansfield, TX) coupled to a blank controlled internal drug-releasing device (CIDR).

Blood samples were collected from coccygeal vessels into Vacutainer tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ) before baseline phase (d -86) and at d 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75, and 81 (~ 1230 h) relative to the onset of environmental challenge phase from a subset of animals [$n = 14, 14, 14$, and 13, for IOZ-CL, IOZ-NC, ZMC-CL, and ZMC-NC, respectively, parity = 3.0 ± 1.2 , DIM = 175 ± 40 d at the onset of environmental challenge (mean $\pm$ SD)] to collect plasma. Plasma concentrations of glucose (Autokit Glucose; catalog no. 439–90901; Wako Chemicals USA Inc., Richmond, VA), NEFA [HR Series NEFA-HR(2), catalog no. 999–34691, 995–34791, 991–34891, and 993–35191; Wako Chemicals USA Inc.], BHB (Autokit 3-HB, catalog no. 439–90901; Wako Chemicals USA Inc.), triglyceride (Triglyceride M assay, catalog no. 994–02891, 990–02991, 992–02892, 998–02992; Wako Chemicals USA Inc.), and insulin (bovine insulin ELISA, catalog no. 10–1201–01; Mercodia AB, Uppsala, Sweden) were determined using commercially available kits, with inter- and intraassay coefficients of variation of 8.3 and 3.9%, 4.9 and 2.0%, 14.0 and 3.7%, 14.9 and 3.5%, and 9.1 and 2.9%, respectively.

Weng et al. (2017, 2018) previously reported procedures for mammary biopsy collection, total RNA extraction from mammary tissues, reverse transcription, and primer design. Briefly, mammary biopsies were collected at enrollment before the baseline phase as baseline samples, and d 7 and 56 relative to the onset of

environmental challenge phase from a subset of animals ($n = 8/\text{treatment}$). One cow from the ZMC-NC group developed clinical ketosis during the baseline phase and one-quarter of the other ZMC-NC cows were automatically dried off after first tissue collection. Therefore, these 2 cows were excluded from the following mammary biopsy collection. The average parity and DIM at the onset of environmental challenge phase of the remaining 30 cows were 2.4 ± 0.8 and 170 ± 36 d (mean $\pm$ SD). Total RNA was extracted from mammary biopsies using TRI-reagent solution (Sigma-Aldrich, St. Louis, MO) and PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Waltham, MA). To perform the quantitative real-time PCR, cDNA from 50 ng of purified RNA was used for each reaction with Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 10 min for initial denaturation and enzyme activation, 40 cycles of 95°C for 15 s, and 57°C for 1 min for amplification. The mRNA abundance of proteins involved in preformed fatty acid transport (lipoprotein lipase), de novo fatty acid synthesis (fatty acid synthase, acetyl-CoA carboxylase 1), NADPH synthesis (cytoplasmic isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate 1-dehydrogenase), glucose transportation (facilitated glucose transporter member 1), lactose synthesis (α -LA), and glycolysis (ATP-dependent 6-phosphofructokinase and hexokinase-2) was examined. Sequences of target genes are reported in Supplemental Table S1 (<https://doi.org/10.3168/jds.2020-18380>), and *GAPDH* was used as the housekeeping gene. The mRNA abundance was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where CT is the threshold cycle, with data of baseline samples as calibrator.

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

The intake and milk yield and composition data were reported in detail by Weng et al. (2018). During the environmental challenge phase, daily THI averaged 77.6 (SD = 3.8) and 77.8 (SD = 3.8) in CL and NC pens, respectively. Within a day, the THI in both pens remained similar and ranged from 73 at 0700 h to 82 at 1500 h (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18380>). Cows without evaporative cooling had greater ($P < 0.01$) daily vaginal temperature (39.94 vs. 39.03°C, Supplemental Figure S2, <https://doi.org/10.3168/jds.2020-18380>), and lower ($P < 0.01$) DMI (21.6 vs. 26.7 kg/d), milk yield (24.5 vs. 34.1 kg/d), and BW (664 vs. 711 kg) compared with CL cows. These results suggest that deprivation of evaporative cooling increased heat load and impaired the performance of lactating dairy cows. However, supplemental Zn source had no effect on these measurements ($P > 0.50$). Compared with cows under evaporative cooling, NC cows had lower ($P < 0.01$) milk lactose yet similar ($P = 0.80$) protein concentrations. Relative to other treatments, CL cows supplemented with ZMC had the lowest milk fat concentration (environment by diet interaction: $P = 0.04$).

Relative to CL cows, plasma of NC cows had lower ($P < 0.01$) concentrations of glucose, NEFA, BHB, and triglyceride but greater ($P < 0.01$) insulin concentration (Table 1). Supplemental Zn source had no ($P > 0.25$) effect on plasma metabolites or insulin except that cows supplemented with ZMC had greater ($P = 0.05$) plasma concentration of triglyceride than cows fed IOZ. Environment, dietary treatment, or their interactions had no effect ($P > 0.10$) on mammary mRNA abundance except that NC cows had greater ($P = 0.03$) mRNA abundance of ATP-dependent 6-phosphofructokinase and tended ($P = 0.08$) to have greater mRNA abundance of 6-phosphogluconate dehydrogenase compared with CL cows (Table 2).

Compared with CL cows, lower plasma glucose concentration observed for NC cows may have resulted from lower DMI. In early and mid lactation, heat-stressed and pair-fed thermal neutral cows had similar reductions in plasma glucose concentration (Wheelock et al., 2010; Lamp et al., 2015), suggesting that lower blood glucose concentration of heat-stressed cows is mainly due to a reduction in DMI. Interestingly, despite lower glucose concentrations, plasma insulin concentrations were greater in NC cows compared with CL cows. This may indicate enhanced insulin secretion, delayed insulin clearance, or both. Previous studies (Wheelock et al., 2010; Baumgard et al., 2011; Tao et al., 2012) reported that exposure to heat stress had no effect on insulin

Table 1. The overall LSM and SEM of plasma concentrations of glucose, BHB, nonesterified fatty acids (NEFA), triglyceride, and insulin of cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC) exposed to either evaporative cooling (CL) or not (NC)¹

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

¹Samples were collected on d 1, 3, 5, 12, 22, 26, 41, 54, 61, 68, 75, and 81 relative to the onset of the environmental challenge phase.

response to glucose infusion in lactating and dry dairy cows, reflecting unaltered pancreatic insulin release to glucose stimulation. During the dry period, NC cows had similar insulin clearance after an intravenous insulin infusion compared with CL cows, suggesting that heat stress had no effect on insulin clearance before calving (Tao et al., 2012). The exact mechanisms for increased plasma insulin concentrations by deprivation of evaporative cooling observed in the current study are not clear and require further investigation. Despite reduced DMI, NC cows had lower plasma NEFA concentrations than CL cows, which may be expected because NC cows had greater plasma concentration of insulin. It has been reported that exposure to heat stress blunts adipose tissue mobilization even when cows are under negative energy balance in early or mid lactation (Wheelock et al., 2010; Lamp et al., 2015).

In the present study, replacing a portion of inorganic source of Zn with Zn-Met complex did not affect plasma concentrations of glucose, BHB, NEFA, and insulin, suggesting that supplemental Zn source had no

substantial effect on systemic metabolism of mid- to late-lactation dairy cows under both CL and NC environments. Nayeri et al. (2014) reported that replacing 66.6 mg of ZnSO₄/kg of DM with Zn-AA complex in dry cow diets and 40 mg of ZnSO₄/kg of DM with Zn-AA complex in lactating cow diets had no effect on plasma glucose and BHB concentrations in early lactation, but increased NEFA concentrations compared with cows supplemented with ZnSO₄. However, the increased NEFA release might be due to lower DMI and greater feed efficiency of cows supplemented with Zn-AA in early lactation rather than direct effects of Zn-AA on adipose tissue mobilization (Nayeri et al., 2014). In heat-stressed growing pigs, dietary supplementation of Zn-AA had no effect on plasma glucose, NEFA, and insulin concentrations compared with those fed diet containing ZnSO₄ (Sanz Fernandez et al., 2014; Pearce et al., 2015). Interestingly, cows fed ZMC had greater plasma concentrations of triglyceride than those fed IOZ regardless of the environment. Few studies have reported the effect of dietary Zn sources on blood

Table 2. The overall LSM and SEM of mRNA abundance of proteins associated with metabolism in mammary tissues collected from cows fed diets supplemented with Zn hydroxychloride (IOZ) or Zn-Met complex (ZMC) exposed to either evaporative cooling (CL) or not (NC)¹

Gene ²	IOZ		ZMC		SEM	P-value		
	CL	NC	CL	NC		Zn	CL	Zn × CL
n	8	8	6	8				
<i>LPL</i>	0.52	0.98	0.86	0.83	0.31	0.74	0.48	0.44
<i>ACACA</i>	1.26	2.53	1.38	0.56	1.07	0.37	0.90	0.31
<i>FASN</i>	2.44	3.71	0.81	0.84	1.83	0.25	0.79	0.81
<i>IDH1</i>	0.98	1.39	0.99	0.82	0.46	0.56	0.82	0.55
<i>G6PD</i>	1.21	1.32	1.49	0.52	0.79	0.62	0.51	0.44
<i>PGD</i>	0.79	1.27	0.89	1.26	0.23	0.82	0.08	0.80
<i>SLC2A1</i>	1.03	1.07	0.83	0.84	0.35	0.55	0.94	0.97
<i>LALBA</i>	0.81	0.71	1.16	0.73	0.27	0.52	0.36	0.58
<i>HK2</i>	0.07	0.81	0.30	0.38	0.33	0.67	0.13	0.20
<i>PFKM</i>	0.72	1.62	0.94	1.47	0.30	0.83	0.03	0.52

¹Mammary tissues were collected on d 7 and 56 relative to the onset of the environmental challenge phase.

²*LPL* = lipoprotein lipase; *ACACA* = acetyl-CoA carboxylase 1; *FASN* = fatty acid synthase; *IDH1* = isocitrate dehydrogenase, cytoplasmic; *G6PD* = glucose-6-phosphate 1-dehydrogenase; *PGD* = 6-phosphogluconate dehydrogenase; *SLC2A1* = facilitated glucose transporter member 1; *LALBA* = alpha-lactalbumin; *HK2* = hexokinase-2; *PFKM* = ATP-dependent 6-phosphofructokinase.

triglycerides in ruminants. In multiparous Sahiwal cows (*Bos indicus*), supplementation of 60 mg/kg of DM of Zn as ZnSO₄ from 2 mo before until 4 mo after parturition had no effect on plasma triglyceride concentration compared with unsupplemented controls (Chandra et al., 2018). The origin of increased plasma triglyceride concentration in cows fed ZMC in the current study is not clear but unlikely due to the increased hepatic secretion as supplemental Zn source had no effect on plasma NEFA concentration. It has been reported that supplementation of Zn-AA complex increased gut integrity compared with inorganic source of Zn under stressed conditions (Sanz Fernandez et al., 2014; Pearce et al., 2015), indicating improved function of intestinal cells. Thus, it is possible that cows supplemented with ZMC had greater fat absorption in the small intestine compared with IOZ cows, supporting increased plasma triglyceride concentrations.

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

d) had no apparent effect on expression of enzymes that mediate triglyceride hydrolysis and de novo fatty acid synthesis, at least at the mRNA level. This is consistent with the similar milk fat percentage between CL and NC cows (Weng et al., 2018).

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

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

ACKNOWLEDGMENTS

The authors thank the staff of the Dairy Research Center of the University of Georgia (Tifton, GA) for animal care and data collection. This project was supported by Zinpro Corporation (Eden Prairie, MN) and the National Institute of Food and Agriculture (Washington, DC) Hatch project (GEO00749). D.J.T. and J.M.D. are employed by Zinpro Corporation. The remaining authors are associated with the University of Georgia and have no other conflicts of interest.

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