Effects of heat stress and dietary zinc source on performance and mammary epithelial integrity of lactating dairy cows

X. Weng, A. P. A. Monteiro, J. Guo, C. Li, R. M. Orellana Rivas, T. N. Marins, J. K. Bernard, D. J. Tomlinson, J. M. DeFrain, S. E. Wohlgemuth, and S. Tao

ABSTRACT

Dietary Zn and heat stress alter gut integrity in monogastric animals. However, effects of Zn on mammary epithelial integrity in heat-stressed lactating dairy cows have 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 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 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 breaths/min), vaginal temperature (39.03 vs. 39.94℃), and decreased dry matter intake (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 concentrations of milk urea nitrogen (9.07 vs. 11.02 mg/mL). Compared with IOZ, cows fed ZMC had lower, and tended to have lower, plasma lactose concentrations during B and EC, 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 have increased 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 and affects gene expression of proteins involved in mammary epithelial cell barrier integrity; however, feeding a portion of dietary zinc as ZMC improves the integrity of the mammary epithelium.

INTRODUCTION

Heat stress (**HS**) is well known to negatively impact performance of lactating dairy cows. Studies (Shwartz et al., 2009; Wheelock et al., 2010) showed that environmental HS reduced milk production by approximately 30 to 40% in mid-lactation dairy cows. While decreased DMI explains approximately half the reduction in milk production (Rhoads et al., 2009; Wheelock et al., 2010), the specific physiological and cellular mechanisms related to reduced milk synthesis by HS in addition to decreased DMI are still unclear. Milk production of the lactating dairy cow is determined by secretory capacity of the mammary gland including both number and activity of

mammary secretory cells (Capuco et al., 2003). Thus, environmental 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 movement of molecules and ions through paracellular pathways in both endothelium and epithelium, including mammary epithelium (Nguyen and Neville, 1998). During lactation, mammary epithelium forms a highly impermeable barrier to separate milk and interstitial fluid and to maintain optimal milk synthesis and secretion (Stelwagen and Singh, 2014). In contrast, 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 dairy cattle. For example, under in vitro conditions, 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.

Dietary Zn, an essential micronutrient, is important to maintenance of critical body functions, such as immunity and metabolism, thereby 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 by supplementation with Zn-AA complex improved the small intestine integrity of growing pigs under HS conditions. However, the role of Zn in maintaining bovine mammary epithelial integrity is still unknown. Source of dietary Zn may also influence epithelial integrity. Pearce et al. (2015) found that replacing a portion of $ZnSO_4$ with Zn-AA complex mitigated 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 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 versus inorganic sources on maintaining integrity of mammary epithelium in bovine is unclear.

We hypothesized that environmental HS impairs mammary function through increasing the permeability of mammary epithelium of lactating dairy cows, and that replacing a portion of dietary inorganic Zn with Zn-AA complex would improve integrity of mammary epithelium, especially under HS conditions. The objective was to evaluate effect of environmental HS and supplementation of different Zn sources (Zn-MET complex vs. Zn hydroxychloride) on lactating Holstein cows' performance and permeability of 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. Procedures and animal handling were approved by the University of Georgia Institutional Animal Care and Use Committee prior to trial initiation.

Seventy-two multiparous lactating Holstein cows were blocked by parity (2.9 ± 0.3) and DIM (99.7 ± 13.4 d), then assigned randomly to 1 of 4 treatments within block in 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 35ppm Zn as Zn hydroxychloride and 40ppm 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 group due to a leg problem, three cows from IOZNC group due to toxic mastitis and indigestion, one cow from ZMCCL group due to indigestion, and two cows from ZMCNC group 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 dietary Zn sources (Table 1). Dose of supplemental Zn was selected based on Nayeri et al. (2014) who reported that replacing 66.6 mg/kg $ZnSO_4$ with Zn-AA complex in the dry cow diet and 40 mg/kg $ZnSO_4$ with Zn-AA complex in the lactating cow diet (75 ppm total supplemental Zn) increased milk yield and reduced SCC in multiparous lactating cows. This 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 (0.9 m diameter) were located at 6-m intervals over feedlines and freestalls, and provided at least 9.5 km/h wind speed at the cow level. Fans ran continuously when the environmental temperature was ≥ 20°C, and continuous misters were activated whenever the 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

Barn air temperature and RH were monitored by Hobo Pro Series Temp probes (Onset Computer Corporation, Pocasset, MA) every 15 min throughout the experiment, and temperature-humidity index (**THI**) was calculated based on THI = (1.8×T+32) - ((0.55-0.0055×RH) × (1.8×T - 26)), where T = air temperature (°C) (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) with daily feed intake measured using a Calan Broadbent feeding system (American Calan Inc., Northwood, NH) throughout the experiment. Individual

dietary ingredients and TMR were sampled three times each week (Mon-Wed-Fri). Dry matter 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 changes in ingredient DM content. Samples of TMR were composited every 2 wk and ground to pass through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) for nutrient analysis (Table 2) by Dairyland Laboratories (Arcadia, WI). Drinking water was sampled at two and five months after onset of the experiment, at different watering locations within pens, for mineral and hardness analyses (Table 3) by Dairyland Laboratories (Arcadia, WI). Water 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 with 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). 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, and 67 and stored at -20 °C for milk BSA analysis. Milk BSA concentration was analyzed using a colorimetric method with bromocresol green dye (Sigma-Aldrich, St. Louis, MO) described by Lieske et al. (2005) with inter- and intra-assays CV of 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. A 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. BSA concentrations in skim milk were then calculated based on a standard curve. BSA concentrations in whole milk were calculated based on the 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, and 68 from a subset of animals (n=6, 5, 5, 7, for IOZCL, IOZNC, ZMCCL, ZMCNC, respectively). Samples were immediately put 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) with inter- and intra-assays CV of 3.5% and 1.9%, respectively.

Mammary biopsy collections

Mammary biopsies were collected at time of enrollment as baseline samples, and on d 7 and 56 relative to 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 following 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. Procedures 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 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) and covered with an aerosol bandage (Neogen Corporation, Lexington, KY) to prevent infection. Tissue was rinsed with PBS, trimmed of fat, 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 a Turbo DNA-free kit (Invitrogen) according to manufacturer's instructions. The cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA).

Relative quantitative RT-PCR was used to examine 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 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 conditions: 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 ice slurry using a Polytron PT 2100 (Kinematica, Littau-Lucerne, Switzerland). The homogenate was kept on ice slurry for 1 h and

then centrifuged at 3,500 × *g* for 10 min at 4°C. The supernatant was collected and 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 Laemmli Buffer (BioRad) containing 5% of β-mercaptoethanol (Sigma-Aldrich) with a dilution of 11 parts of sample plus 4 parts of buffer, and boiled 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 variation. 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 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, diluted 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, diluted in 5% non-fat dry milk) were used as primary antibodies. All primary antibodies were tested to cross react with bovine. 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, ratio of 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 means ± 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 treatment as the random variable. For milk yield analysis, average milk yield of cows before onset of the experiment was included in the model as a covariate. For analyses of plasma lactose and milk BSA concentrations, data obtained at -86 d were included in the model

as covariates. 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 treatment as the 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.

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 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 rate (Table 5). During the environmental challenge phase, regardless of Zn source, CL cows had lower ($P < 0.01$) vaginal temperature (39.03 vs. 39.94 °C, SEM = 0.05 °C, respectively) and respiration rate (64.8 vs. 73.9 breath/min, SEM = 1.3 breath/min, respectively) compared with NC. These results indicate that cooling was effective in reducing cows' heat load (Table 6). There were no ($P \geq 0.20$) differences observed for Zn or Zn by cooling interactions on vaginal temperature or respiration rate (Table 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 5, 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 5). During the environmental challenge phase, regardless of 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 6). Milk SCS and concentration of protein were not affected ($P \geq 0.80$) by cooling treatment (Table 6). However, due to 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 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 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 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 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 1a). No

cooling effect or Zn by cooling interaction ($P \geq 0.36$) were 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, the plasma lactose concentration tended to be higher ($P < 0.10$) in NC cows at d 3, 5, and 41 (Figure 1b). There was no treatment effect or interaction ($P \geq 0.29$) for milk BSA content throughout the study (Table 5, 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 7, Figure 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 7, Figure 2). Cooling did not affect ($P \geq 0.13$) gene expression of claudin 4 and 8 (Table 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 7, Figure 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 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).

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 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 cooling was effective in reducing heat strain 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 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 possible regulatory effects of Zn on thermal regulation are unclear. In the 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 in the current experiment.

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 not consistently reported. Compared with thermo-neutrality, HS reduced milk protein concentration (Rhoads et al., 2009; Shwartz et al., 2009, Wheelock et al., 2010). In contrast, provision of 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 are offered the similar amount of DM, HS cows have similar milk lactose percentages compared with those under thermo-neutrality (Rhoads et al., 2009), indicating that the reduced milk lactose synthesis by HS may be feed intake dependent. Similar to results reported by Rhoads et al. (2009) and Shwartz et al. (2009) that HS increases plasma urea nitrogen concentrations compared with thermo-neutrality, in the current study, NC cows had higher MUN concentration than CL. 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 SCC 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 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 influences on rumen function compared with sulfate-bound trace minerals. However, direct comparisons between hydroxy trace mineral and mineral-AA complex on bioavability and rumen functions 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 66.6 ppm $ZnSO_4$ and 8.4 ppm Zn-AA complex one month before calving and 35 ppm $ZnSO_4$ and 40 ppm Zn-AA complex from calving until 250 DIM had 4.2% higher milk yield relative to those fed 75 ppm $ZnSO_4$. In contrast, the dietary treatments in the current study started during mid-lactation. Future studies are warranted to examine 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 sources were conducted in temperate climates, and it is also possible that different Zn sources affect cow performance differently under HS compared with thermo-neutral conditions.

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 prevented 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 may alter milk fat synthesis compared with IOZ, especially in cows in an evaporatively cooled environment. 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 indicates that cows under HS may respond differently to diets containing different Zn sources thus affecting 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 junction is an indicator of optimal mammary function, and loss of mammary epithelial integrity, during lactation, is related to reduced milk synthesis and secretion (Stelwagen and Singh, 2014). As reviewed by Shennan and Peaker (2000), plasma concentration of lactose is a preferable indicator to assess 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 mammary epithelial barrier by different mechanisms. Environment had no overall impact on plasma lactose concentration, suggesting that cows under long term exposure to HS largely maintain mammary epithelial integrity. However, NC cows tended to have higher plasma lactose concentrations at the beginning of the environmental challenge phase compared with CL. This indicates that permeability of mammary cell junctions may temporarily increase when exposed to acute HS before returning to basal levels as adaptation to HS occurs. Similarly, Stelwagen et al. (1997) observed a gradual decrease in leakage of milk lactose in 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 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 concentration 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 mammary epithelial barriers. Dietary Zn is a critical micronutrient in maintenance of epithelial barrier integrity. In vitro depletion of zinc in the medium of a human intestinal Caco-2 cell culture significantly increased epithelial permeability, while repletion of Zn into the culture rescued the leaky 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 of 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 a better performance of Zn-AA complex over $ZnSO_4$ to maintain epithelial integrity, possibly due to the higher bioavailability (Spears, 2003).

Nevertheless, results in plasma lactose between Zn source obtained in the current study provide evidence that dietary Zn source also influences mammary epithelial integrity in lactating dairy cows. In addition, replacing 40 ppm of Zn hydroxychloride with Zn-MET reduces permeability of 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 is transported across mammary epithelial cells through a transcytosis pathway in mouse lactating mammary gland (Monks and Neville, 2004). However, when the mammary epithelial barrier is disrupted, milk BSA content is dramatically increased due to influx from blood into milk through the paracellular pathways (Stelwagen and Singh, 2014). Therefore, data in the present study indicate that environmental and dietary treatments only altered permeability of mammary epithelium but did not result in a disruption of mammary epithelial barrier.

The paracellular barrier of the epithelium 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 trans-membrane 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). Adherens junction is adjacent to the tight junction and is critical for cell-to-cell adhesion and polarization (Itoh and Bissell, 2003; 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 gland 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 synthesis of proteins related to paracellular junction. The trend for increased 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 increased gene expression of occludin, claudin 3, and ZO-1 in the jejunum (Pearce et al., 2013). Interestingly, although mammary gene expressions are up-regulated more in NC cows than CL, protein expression of occludin and E-cadherin was not influenced by environments. This 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. Furthermore, these data may suggest there is a net loss of junctional proteins due to HS and that mammary gland of NC cows strives to maintain normal epithelial integrity by synthesizing more proteins relative to CL. Therefore, 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 leading to reduced milk production of HS lactating dairy cows.

Surprisingly, although cows supplemented with ZMC had lower plasma lactose concentration than 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' diets partially alleviated increases in intestinal permeability after 12 h exposure to HS without influencing 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-organization of tight and adherens junctional proteins from epithelial junctions to cytoplasm (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 for 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, different sources of dietary Zn may alter cellular activity of Zn-finger proteins to influence gene expression. The fact that 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 lactation performance of dairy cows, but NC cows largely maintained mammary epithelial integrity compared with CL. The protein expression of occludin and E-cadherin was 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 lactose concentration. These data suggest that dietary Zn-MET complex may enhance mammary epithelial integrity but the cellular mechanism is still unclear.

REFERENCES

Abuajamieh, M., S. K. Kvidera, E. A. Horst, E. J. Mayorga, J. T. Seibert, J. S. Johnson, J. W. Ross, M. A. Al-Qaisi, P. J. Gorden, J. DeFrain, R. P. Rhoads, and L. H. Baumgard. 2016. The effects of zinc amino acid complex on biomarkers of gut integrity and metabolism in heat-stressed steers. J. Anim. Sci.94:564-564. (Abstr.)

Capuco, A. V., S. E. Ellis, S. A. Hale, E. Long, R. A. Erdman, X. Zhao, and M. J. Paape. 2003. Lactation persistency: insights from mammary cell proliferation studies. J. Anim. Sci. 81(Suppl. 3):18-31.

Chan, S. C., J. T. Huber, K. H. Chen, J. M. Simas, and Z. Wu. 1997. Effects of ruminally inert fat and evaporative cooling on dairy cows in hot environmental temperatures. J. Dairy Sci. 80:1172-1178.

Chandan, R. C. 2006. Milk composition, physical and procession characteristics. Pages 347-377. Handbook of Food Products Manufacturing. Y. H. Hui, R. C. Chandan, S. Clark, N. Cross, J. Dobbs, W. J. Hurst, L. M. L. Nollet, E. Shimoni, N. Sinha, E. B. Smith, S. Surapat, F. Toldrá, ed. John Wiley & Sons, Inc., Hoboken, NJ.

Chen, K. H., J. T. Huber, C. B. Theurer, D. V. Armstrong, R. C. Wanderley, J. M. Simas, S. C. Chan, and J. L. Sullivan. 1993. Effect of protein quality and evaporative cooling on lactational performance of Holstein cows in hot weather. J. Dairy Sci. 76:819-825.

Collier, R. J., G. E. Dahl, and M. J. VanBaale. 2006. Major advances associated with environmental effects on dairy cattle. J. Dairy Sci. 89:1244-1253.

Dikmen, S. and P. J. Hansen. 2009. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? J. Dairy Sci. 92:109-116.

Dokladny, K., M. N. Zuhl, and P. L. Moseley. 2016. Intestinal epithelial barrier function and tight junction proteins with heat and exercise. J. Appl. Physiol. 120:692-701.

Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. J. Dairy Sci. 79:543-549.

Finamore, A., M. Massimi, L. Conti Devirgiliis, and E. Mengheri. 2008. Zinc deficiency induces membrane barrier damage and increases neutrophil transmigration in Caco-2 cells. J. Nutr. 138:1664-1670.

Gaughan, J., N. Lacetera, S. E. Valtorta, H. H. Khalifa, L. Hahn, and T. Mader. 2009. Response of domestic animals to climate challenges. Pages 131-170. Biometeorology for Adaptation to Climate Variability and Change. K. L. Ebi, I. Burton, and G. R. McGregor, ed. Springer Netherlands, Dordrecht.

Genther, O. N. and S. L. Hansen. 2015. The effect of trace mineral source and concentration on ruminal digestion and mineral solubility. J. Dairy Sci. 98:566-573.

Hammami, H., J. Bormann, N. M'hamdi, H. H. Montaldo, and N. Gengler. 2013. Evaluation of heat stress effects on production traits and somatic cell score of Holsteins in a temperate environment. J. Dairy Sci. 96:1844-1855.

Itoh, M. and M. J. Bissell. 2003. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. J Mammary Gland Biol Neoplasia 8:449-462.

Kadzere, C. T., M. R. Murphy, N. Silanikove, and E. Maltz. 2002. Heat stress in lactating dairy cows: a review. Livest. Prod. Sci. 77:59-91.

Kellogg, D. W., D. J. Tomlinson, M. T. Socha, and A. B. Johnson. 2004. Review: Effects of zinc methionine complex on milk production and somatic cell count of dairy cows: twelve-trial summary. Prof. Anim. Sci. 20:295.

Knudsen, K. A. and M. J. Wheelock. 2005. Cadherins and the mammary gland. J. Cell. Biochem. 95:488-496.

Lee, S., and S. L. Kelleher. 2016. Molecular regulation of lactation: The complex and requisite roles for zinc. Arch. Biochem. Biophys. 611:86-92.

Lieske, B., A. Jantz, and B. Finke. 2005. An improved analytical approach for the determination of bovine serum albumin in milk. Dairy Sci. Technol. 85:237-248.

Monks, J. and M. C. Neville. 2004. Albumin transcytosis across the epithelium of the lactating mouse mammary gland. J. Physiol. (Lond.) 560:267-280.

Nayeri, A., N. C. Upah, E. Sucu, M. V. Sanz-Fernandez, J. M. DeFrain, P. J. Gorden, and L. H. Baumgard. 2014. Effect of the ratio of zinc amino acid complex to zinc sulfate on the performance of Holstein cows. J. Dairy Sci. 97:4392-4404.

Nguyen, D. A. and M. C. Neville. 1998. Tight junction regulation in the mammary gland. J Mammary Gland Biol Neoplasia. 3:233-246.

NRC. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Oloumi, A., T. McPhee, and S. Dedhar. 2004. Regulation of E-cadherin expression and beta-catenin/Tcf transcriptional activity by the integrin-linked kinase. Biochim. Biophys. Acta 1691:1-15.

Pearce, S. C., V. Mani, T. E. Weber, R. P. Rhoads, J. F. Patience, L. H. Baumgard, and N. K. Gabler. 2013. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. J. Anim. Sci. 91:5183-5193.

Pearce, S. C., M. V. Sanz-Fernandez, J. H. Hollis, L. H. Baumgard, and N. K. Gabler. 2014. Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs. J. Anim. Sci. 92:5444-5454.

Pearce, S. C., M. V. Sanz Fernandez, J. Torrison, M. E. Wilson, L. H. Baumgard, and N. K. Gabler. 2015. Dietary organic zinc attenuates heat stress–induced changes in pig intestinal integrity and metabolism. J. Anim. Sci. 93:4702-4713.

Pereira, P. C. 2014. Milk nutritional composition and its role in human health. Nutrition. 30:619-627.

Rabiee, A. R., I. J. Lean, M. A. Stevenson, and M. T. Socha. 2010. Effects of feeding organic trace minerals on milk production and reproductive performance in lactating dairy cows: a meta-analysis. J. Dairy Sci. 93:4239-4251.

Rhoads, M. L., R. P. Rhoads, M. J. VanBaale, R. J. Collier, S. R. Sanders, W. J. Weber, B. A. Crooker, and L. H. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J. Dairy Sci. 92:1986-1997.

Sanz Fernandez, M. V., S. C. Pearce, N. K. Gabler, J. F. Patience, M. E. Wilson, M. T. Socha, J. L. Torrison, R. P. Rhoads, and L. H. Baumgard. 2014. Effects of supplemental zinc amino acid complex on gut integrity in heat-stressed growing pigs. Animal. 8:43-50.

Shennan, D. B. and M. Peaker. 2000. Transport of milk constituents by the mammary gland. Physiol. Rev. 80:925-951.

Shwartz, G., M. L. Rhoads, M. J. VanBaale, R. P. Rhoads, and L. H. Baumgard. 2009. Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. J. Dairy Sci. 92:935-942.

Spears, J. W. 1989. Zinc methionine for ruminants: relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. J. Anim. Sci. 67:835-843.

Spears, J. W. 1996. Organic trace minerals in ruminant nutrition. Anim. Feed Sci. Technol. 58:151-163.

Spears, J. W. 2003. Trace mineral bioavailability in ruminants. J. Nutr. 133:1506s-1509s.

Stelwagen, K., V. C. Farr, H. A. McFadden, C. G. Prosser, and S. R. Davis. 1997. Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. Am. J. Physiol. 273:R379-386.

Stelwagen, K. and K. Singh. 2014. The role of tight junctions in mammary gland function. J Mammary Gland Biol Neoplasia. 19:131-138.

Tarazón-Herrera, M., J. T. Huber, J. Santos, H. Mena, L. Nusso, and C. Nussio. 1999. Effects of bovine somatotropin and evaporative cooling plus shade on lactation performance of cows during summer heat stress. J. Dairy Sci. 82:2352-2357.

Taylor, R. B., J. T. Huber, R. A. Gomez-Alarcon, F. Wiersma, and X. Pang. 1991. Influence of protein degradability and evaporative cooling on performance of dairy cows during hot environmental temperatures. J. Dairy Sci. 74:243-249.

Tsukita, S., Y. Yamazaki, T. Katsuno, A. Tamura, and S. Tsukita. 2008. Tight junction-based epithelial microenvironment and cell proliferation. Oncogene. 27:6930-6938.

Turner, J. R. 2009. Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. 9:799-809.

Wang, R. L., J. G. Liang, L. Lu, L. Y. Zhang, S. F. Li, and X. G. Luo. 2013. Effect of zinc source on performance, zinc status, immune response, and rumen fermentation of lactating cows. Biol Trace Elem Res. 152:16-24.

Wang, X., M. C. Valenzano, J. M. Mercado, E. P. Zurbach, C. J. Flounders, and J. M. Mullin. 2014. Zinc enhancement of LLC-PK(1) renal epithelial barrier function. Clin Nutr. 33:280-286.

West, J. W. 2003. Effects of heat stress on production in dairy cattle. J. Dairy Sci. 86:2131-2144.

Wheelock, J. B., R. P. Rhoads, M. J. Vanbaale, S. R. Sanders, and L. H. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. J. Dairy Sci. 93:644-655.

Wildman, E. E., G. M. Jones, P. E. Wagner, R. L. Boman, H. F. Troutt, Jr., and T. N. Lesch. 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. J. Dairy Sci. 65: 495-501.

Zimbelman, R. B., R. P. Rhoads, M. L. Rhoads, G. C. Duff, L. H. Baumguard, and R. J. Collier. 2009. A re-evaluation of the impact of temperature humidity index and black globe temperature humidity index (BGHI) on milk production in high producing dairy cows. Page 158-168 in Proc. 24th Southwest Nutr. Man. Conf., Tempe, AZ. University of Arizona, Tuscon.

Table 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 ration DM of cows receiving Zn hydroxychloride and Zn-MET complex, respectively) was hand added to diets.

Table 2. Nutrient content of 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. Composition, pH, and hardness of drinking water on month 2 and 5 after 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 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 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 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

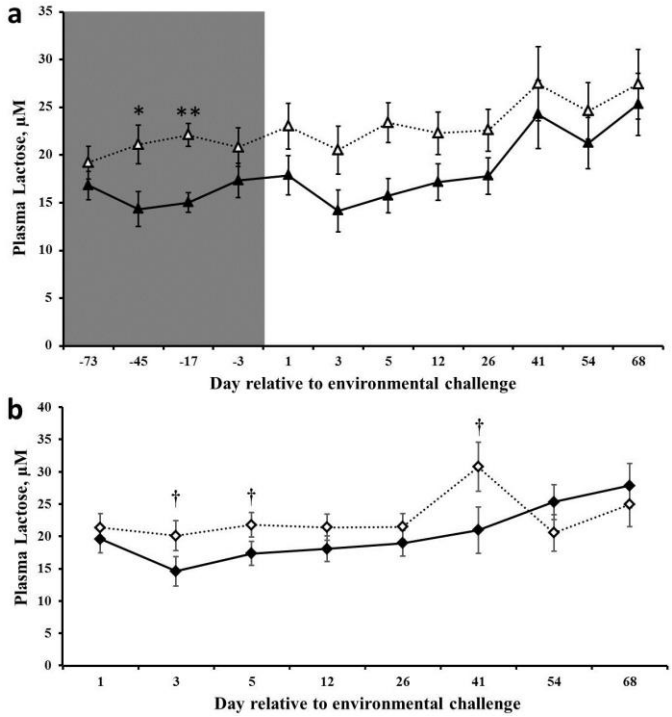
[1]Vaginal temperature
[2]Respiration rate
[3]3.5% FCM = (0.4324 × kg of milk yield) + (16.216 × kg of milk fat yield).
[4]ECM = (0.327 × kg of milk yield) + (12.95 × kg of milk fat yield) + (7.20 × kg of milk protein yield)
[5]Feed efficiency = ECM (kg/d)/DMI (kg/d)
[6]SCS = $\log_{10}(SCC/12.5) / \log_{10}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

Table 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.

Figure 1a. Plasma concentrations of lactose in cows fed diets supplemented with Zn hydroxychloride (open triangles [△], n = 11) or Zn-MET (solid triangles [▲], n = 12) during baseline (shade area) and environmental challenge phases. **Figure 1b.** Plasma concentrations of lactose in cows with (solid diamonds [♦], n = 11) or without cooling treatment (open diamonds [◊], n = 12) during the environmental challenge phase. During 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, †$P \leq 0.10$.



Weng Figure 1

Figure 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). Data are provided based on the significance of gene expression analyses.

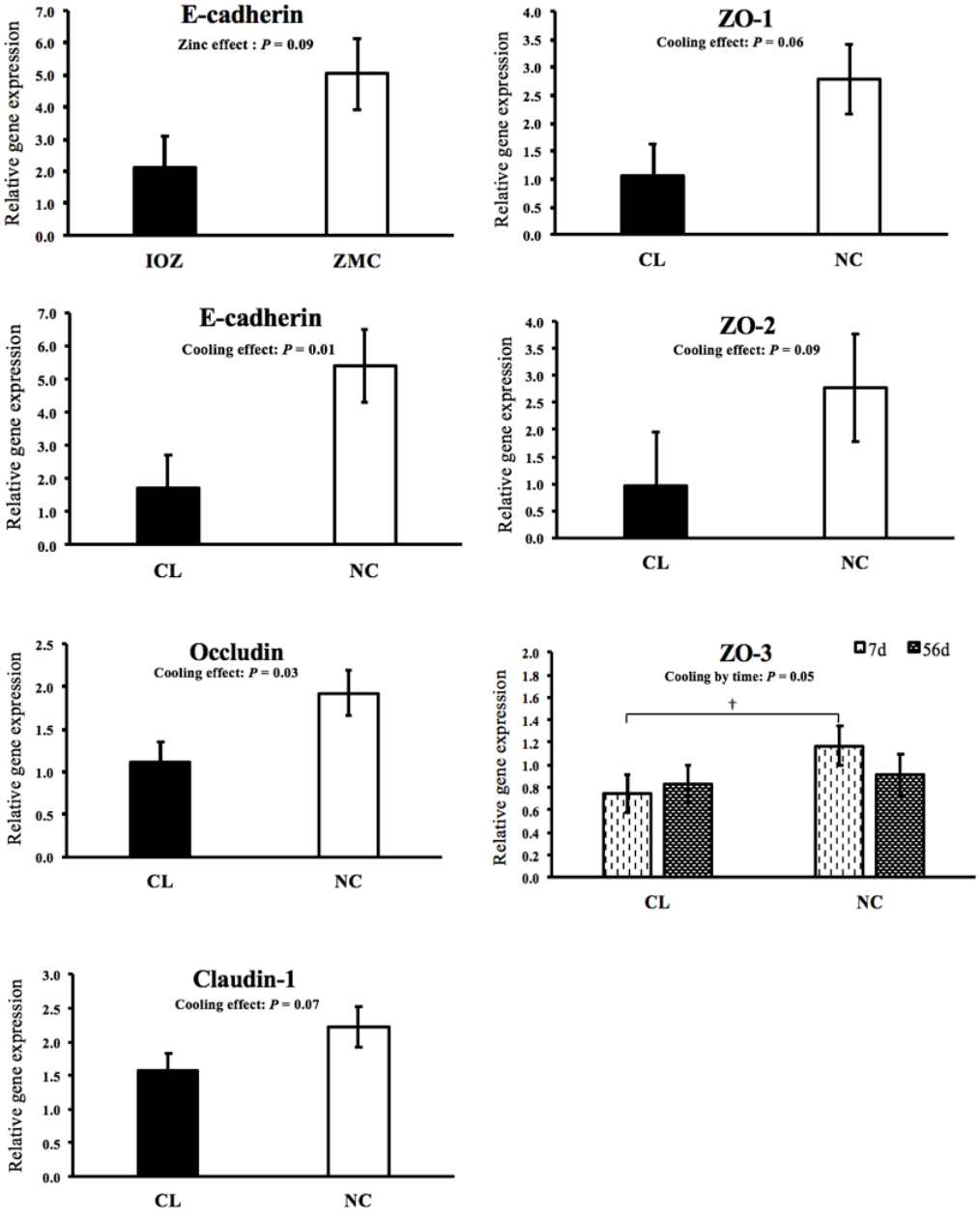


Figure 3. Protein expression of mammary tissue in cows fed diets supplemented with Zn hydroxychloride (IOZ, n = 16) or Zn-MET complex (ZMC, n = 14) exposed to either cooling (CL, n = 16) or not (NC, n = 14). Data are presented as least squares mean ± standard error of mean and displayed as the ratio of 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.