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The effects of dietary cation-anion difference and dietary buffer for lactating dairy cattle under mild heat stress with night cooling

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ABSTRACT

The objective of this study was to investigate the interactive effect of DCAD and dietary buffer supply on DMI, ruminal fermentation, milk and milk component yields, and gastrointestinal tract (GIT) permeability in lactating dairy cattle exposed to mild heat stress. A total of 16 lactating Holstein cows, including 8 ruminally cannulated primiparous (80 ± 19.2 DIM) and 8 noncannulated multiparous (136 ± 38.8 DIM) cows, were housed in a tiestall barn programmed to maintain a temperature-humidity index (THI) between 68 and 72 from 0600 h to 1600 h followed by natural night cooling. The experimental design was a replicated 4 × 4 Latin rectangle (21-d periods) with a 2 × 2 factorial treatment arrangement. Diets contained a low DCAD (LD; 17.5 mEq/100g of DM) or high DCAD (HD; 39.6 mEq/100g of DM) adjusted using NH_4Cl and Na-acetate, with low (LB; 0% $CaMg(CO_3)_2$) or high buffer (HB; 1% $CaMg(CO_3)_2$). In addition to measurement of feed intake, ruminal fermentation, and milk and milk component yields, a ruminal dose of Cr-EDTA and an abomasal dose of Co-EDTA were used to evaluate total and postruminal gastrointestinal tract permeability, respectively. Treatments had no effect on DMI, ruminal short-chain fatty acid concentrations, or ruminal pH. Feeding HD improved blood acid-base balance, increased urine volume by 4 ± 1.5 kg/d, and increased milk fat by 0.14 ± 0.044 percentage units and milk fat yield by 36.5 ± 16.71 g/d. HB reduced milk fat percentage by 0.11 ± 0.044 percentage units and had no effect on milk fat yield. The HB treatments reduced urinary excretion of Co by 27% and tended to reduce urinary Cr excretion by 10%. Across all treatments, 72% of the Cr recovery was represented by Co suggesting that much of the permeability responses were postruminal during mild heat stress. Overall, increasing DCAD through greater Na supply during mild heat stress improved blood acid-base balance and may increase milk fat yield. Dietary inclusion of $CaMg(CO_3)_2$ improved postruminal GIT barrier function despite a lack of low ruminal pH. Because there appeared to be a limited interactive effect between DCAD and buffer, increased DCAD and the provision of buffer seem to independently influence physiological and performance responses in lactating dairy cows exposed to mild heat stress with night cooling.

Key words: calcium carbonate, magnesium carbonate, sodium, sodium acetate

INTRODUCTION

Heat stress (**HS**) in lactating dairy cows is a multifactorial disorder that can reduce milk yield by up to 40% (Tao et al., 2018) and negatively affect reproductive success, thereby imposing a large financial burden at the farm level. Economic losses exclusively from reduced milk production are estimated to be US$1.20 billion (Key et al., 2014) and Can$34.5 million (Campos et al., 2022) annually for the American and Canadian dairy industries, respectively (Can$1 = US$0.73 at time of publication). Heat stress occurs when the accumulation of metabolic heat production combined with environmental heat (function of temperature and relative humidity) are greater than the rate at which cattle dissipate excess heat (Kadzere et al., 2002). The temperature-humidity index (**THI**) is used as an indicator to evaluate risk for HS (Armstrong, 1994; Zimbelman et al., 2011); mild HS occurs when THI is between 68 and 71, moderate HS when THI is between 72 and 79, severe HS when THI is between 80 and 90, and lethal HS when THI is above 90 (Zimbelman et al., 2011). Environment Canada meteorological reports indicate that between 2021 and 2023, dairy cattle in the Canadian prairies experienced HS conditions (THI ≥68) on 66% of summer days (Government of Canada, 2024). However, even with milder THI conditions, milk production and milk fat concentration decline during the summer months in Canada (Ouellet et al., 2021). It should be noted that research evaluating chronic exposure to moderate or severe HS may not be

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representative of conditions in more temperate climates, including northern regions of the United States and Canada, that experience mild to moderate daytime HS with night cooling (when THI falls below 68; Ominski et al., 2002). In fact, recent data collected from Canadian dairy farms in Quebec and Ontario reported that milk, fat, and protein yields begin to decline when average daily THI reach 64, 50, and 58, respectively (Campos et al., 2022). These findings suggest that Canadian dairy cattle may be more sensitive to HS, despite night cooling relief, than cattle raised in hotter climates (Vanderzaag et al., 2023). The reduction in lactation performance during mild HS and predicted increases in global temperatures (IPCC, 2022) suggest that HS is an ongoing issue even in temperate climates, and research evaluating the efficacy of intervention strategies to mediate these responses is warranted.

Increasing DCAD (calculated as (Na + K) − (Cl + S); mEq/100 g of DM; Wildman et al., 2007) and provision of dietary buffer supply (Baumgard and Rhoads, 2009) are 2 nutritional strategies commonly implemented to alleviate HS. Heat stressed cattle attempt to thermoregulate by increasing water intake, respiration rates, and sweating, and by decreasing DMI and rumination (Kadzere et al., 2002; Bernabucci et al., 2010). Increasing DCAD by providing more Na or K may help replenish electrolytes lost in sweat, saliva, and urine to maintain blood acid-base balance while stimulating water intake and DMI (Hu and Murphy, 2004). Heat stressed cattle have been reported to be at greater risk for subclinical ruminal acidosis (**SRA**; Golder and Lean, 2024) and hyperpermeability of the gastrointestinal tract (**GIT**; Burhans et al., 2022; Plaizier et al., 2022). More recently, there is evidence to suggest that the intestinal regions of the GIT may be more severely affected during HS exposure than the reticulo-rumen (Burhans et al., 2022; Fontoura et al., 2022). In addition to DCAD, provision of dietary buffers may help to stabilize ruminal pH and reduce the risk of SRA, likely having positive effects on epithelial integrity of the total GIT. Common dietary ingredients used to increase DCAD include $NaHCO_3$, $KHCO_3$, and K_2CO_3 (West et al., 1987). This approach increases DCAD through provision of Na or K while simultaneously providing a ruminal buffer (Bandaranayaka and Holmes, 1976; Burhans et al., 2022). However, the combined strategy of adding a cation source bound to carbonate precludes the ability to decipher whether responses can be attributed to greater cation supply, increased ruminal buffering, or the interaction.

Therefore, the objectives of this study were to determine the effects of DCAD, dietary buffer, and their interaction during exposure to mild HS on DMI, ruminal fermentation, total-tract and postruminal GIT permeability, systemic inflammation, and milk and milk component yields in lactating dairy cattle. We hypothesized that increasing the DCAD, together with provision of a dietary buffer, would synergistically improve DMI, reduce the magnitude of ruminal pH depression, and promote GIT barrier function to stimulate greater milk and milk component yields in lactating dairy cows exposed to mild HS with night cooling.

MATERIALS AND METHODS

This study was conducted between July 2021 and October 2021 at the University of Saskatchewan Rayner Dairy Research and Teaching Facility (Saskatoon, SK, Canada). The procedures for this study were pre-approved by the University of Saskatchewan Animal Research Ethics Board (20200023; Saskatoon, SK, Canada) and followed the guidelines for the humane use of animals by the Canadian Council of Animal Care (Ottawa, ON, Canada). In addition, a protocol describing the research methodology was prepared before study implementation.

Experimental Design and Feeding Management

The study used 16 lactating Holstein cows including 8 primiparous (average ± SD; BW 601 ± 43.5 kg and DIM 80 ± 19.2 at the start of the study) and 8 multiparous (second lactation with an average ± SD; BW 655 ± 51.8 kg and DIM 136 ± 38.8 at the start of the study). Immediately before the start of the study, the milk yields of the primiparous and multiparous cows were on average (mean ± SD) 37 ± 3.6 kg/d and 45 ± 8.3 kg/d, respectively. All primiparous cows were surgically fit with a 7.6-cm ruminal cannula (model 4C, Bar Diamond Inc., Parma, ID). Two weeks after surgery, the cannulas were replaced with a 9-cm ruminal cannula (Robyn Williams, Melbourne, Australia). The number of cows requested was based on power analysis to detect a 2-kg difference in milk yield (n = 16, nominal power of 80%, $\alpha = 0.05$). The experimental design was a replicated 4 × 4 Latin rectangle with a 2 × 2 factorial treatment arrangement. Cows were blocked into rectangles by parity (multiparous vs. primiparous) and DIM. Each period of the Latin rectangle consisted of 21 d, with 12 d for dietary adaptation and 9 d of sample collection.

Cows were housed in a tiestall barn and milked 3 times per day at 0700, 1400, and 2100 h in a double 6 DeLaval herringbone parlor (DeLaval, Tumba, Sweden). The tiestall facility was equipped with an automated ventilation system enabling control over temperature and humidity (MAXIMUS, Saint-Bruno-de-Montarville, Quebec, Canada). To ensure mild HS conditions were imposed, the ventilation was programmed to maintain a THI between 68 and 72 (Zimbelman et al., 2011) from 0600 h to 1600 h (**HS**; heat stress), and natural night cooling was permit-

ted from 1601 h to 0559 h (**NHS**; nonheat stress). Air temperature and relative humidity were recorded using 4 temperature and humidity data loggers (model R6030; Reed Instruments, Newmarket, ON, Canada) that were 74 cm above the ground and spaced in front of the feed mangers equally throughout the tiestall barn to confirm ambient conditions. The temperature and humidity data were used to calculate THI according to NRC (1971), where T_{db} (°C) is the dry bulb temperature and RH is the relative humidity:

$$\text{THI} = \left(1.8 \times T_{db} + 32\right) - \left(0.55 - 0.0055 \times RH\right) \times \left(1.8 \times T_{db} - 26\right).$$

Within a rectangle, cows were randomly assigned to one of 8 unique treatment sequences, balanced for carry-over effects, to test the effects of DCAD, buffer inclusion, and their interaction. Rectangles had different treatment sequences. The DCAD was calculated using the equation derived by Ender et al. (1971): DCAD = mEq (Na + K) − (Cl + S)/100 g of DM. Dietary treatments (Table 1) were formulated to contain a low DCAD (**LD**; 10 mEq/100 g of DM) utilizing animate (Phibro, Teaneck, NJ), or high DCAD (**HD**; 30 mEq/100 g of DM) achieved with Na-acetate (Macco Organiques Inc., Valleyfield, QC, Canada). The dietary buffer included a low (**LB**; 0% added $CaMg(CO_3)_2$) or high buffer (**HB**; 1% of diet DM added $CaMg(CO_3)_2$ [MIN-AD; Papillon Agricultural Company, Easton, MD]). An individual treatment mix for each diet was prepared at the Canadian Feed Research Center (North Battleford, SK, Canada) and included steam-flaked corn, protein supplements, vitamins, minerals, and additives. All ingredients were manually weighed. Treatment mixes were stored in separate overhead bins and were color coded to blind farm staff to dietary treatments. All diets contained the same forage, protein, grain, mineral, and vitamin sources, except for the ingredients used to manipulate DCAD and buffer. Diets were formulated for a 650-kg lactating cow consuming 26.5 kg of DM producing 40 kg of milk with 4.0% fat and 3.1% true protein using the Nutritional Dynamic System (RUM&N, Reggio Emilia, Italy; Table 2).

Table 1. Ingredient composition of diets formulated to contain low (LD; 10 mEq/100 g of DM) or high DCAD (HD; 30 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$)

Ingredient (% of TMR DM)	HB		LB	
	HD	LD	HD	LD
Barley silage	46.0	46.0	46.0	46.0
Dry rolled barley	15.4	15.3	15.4	15.4
Steam-flaked corn	14.1	14.4	14.2	14.5
Protein blend[1]	9.03	7.82	8.74	8.92
Soybean meal	7.55	6.98	7.16	7.11
Beet pulp[2]	0.97	2.14	0.00	0.99
Beet molasses	1.09	1.00	1.09	1.10
Megalac[3]	0.71	0.73	0.71	0.72
Palmitic acid[4]	1.09	1.10	1.08	1.10
Limestone	0.89	0.86	0.88	0.89
Dicalcium phosphate	0.07	0.01	1.09	1.04
Urea	0.03	0.00	0.10	0.02
MIN-AD[5]	1.00	1.02	0.00	0.00
Sodium acetate	1.21	0.00	2.08	0.47
Magnesium sulfate	0.29	0.00	0.89	0.90
Animate[6]	0.00	1.57	0.00	0.00
White salt	0.00	0.49	0.00	0.24
Mineral mix[7]	0.56	0.56	0.55	0.56

[1]Blend of 28% soymeal and 72% canola meal on an as-fed basis.
[2]Beet pulp was used to substitute ingredients when adjusting for DCAD.
[3]Megalac (Church and Dwight Co. Inc., Princeton, NJ).
[4]Energizer RP-10 (IFFCO, Johor, Malaysia).
[5]MIN-AD (MIN-AD Inc., Winnemucca, NV) is 21.73% calcium and 12.66% magnesium.
[6]Phibro Animal Health Corporation (Teaneck, NJ).
[7]Mineral mix contains 67.5% ground wheat, 15.8% calcium propionate, 11.6% Smartamine methionine (Adisseo Inc., Antony, Frace), and 5.1% vitamins and trace minerals.

Barley silage and dry rolled barley grain were mixed daily for 5 min in a Keenan Mechfiber320 mixer wagon (Alltech Farming Solutions Ltd., Borris, Co. Carlow, Ireland). The barley silage/barley grain mixture and individual treatment mixes were hand weighed on a scale and mixed separately using a Data Ranger (American Calan, Northwood, NH) for 3 min. Feed refused was collected at 0900 h and fresh feed was provided once daily at 0930 h. The weight of the feed offered and refused were measured and recorded daily. The amount of feed provided was targeted to achieve 10% of the weight (as-fed basis) as refusals to ensure ad libitum feed intake. The actual average refusal rates were 13%, 14%, 14%, 14% for LD-LB, LD-HB, HD-LB, and HD-HB, respectively.

BW, BCS, DMI, and Feeding Behavior

Cow BW was measured on an AWS100 DeLaval weigh scale (DeLaval, Tumba, Sweden) on 2 consecutive days at the start and end of each period. The average of the 2 d was used for data analysis. On the same day that BW was measured, 2 observers that were blinded to treatments assessed BCS on a scale from 1 to 5, where 1 = emaciated and 5 = obese (Wildman et al., 1982).

Dry ingredients (treatment supplements and barley grain) were collected once per week, silage samples were collected twice weekly throughout the study, and samples were dried for 96 h in a forced-air oven at 55°C to determine DM. Ingredient DM values were adjusted weekly to ensure diets were mixed accurately on an as-fed basis. Samples of individual feed ingredients were collected from d 16 to 20 of each period, composited on an equal weight basis (0.5 kg/d for dry ingredients and 1.0 kg/d for silage), and stored at −20°C. Refusal samples (20% of total weight refused) were collected from d 17 to 21 and were composited proportionally by cow before be-

Table 2. Chemical composition[1] of experimental diets formulated for low (LD; 10 mEq/100 g of DM) or high DCAD (HD; 30 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$)

	HB		LB	
Item	HD	LD	HD	LD
CP, % of DM	18.4 ± 0.34	18.5 ± 0.53	18.2 ± 0.22	18.5 ± 0.55
Starch, % of DM	25.0 ± 0.31	25.6 ± 1.08	26.0 ± 1.25	25.7 ± 0.71
Ether extract, % of DM	4.2 ± 0.61	4.0 ± 0.49	4.0 ± 0.61	4.0 ± 0.73
aNDFom, % of DM	29.1 ± 0.45	29.4 ± 0.48	28.5 ± 0.27	29.0 ± 0.27
ADF, % of DM	17.1 ± 0.45	17.5 ± 0.22	16.9 ± 0.46	17.5 ± 0.48
Ca, % of DM	1.14 ± 0.063	1.11 ± 0.183	1.00 ± 0.098	1.00 ± 0.097
P, % of DM	0.47 ± 0.010	0.43 ± 0.010	0.69 ± 0.040	0.65 ± 0.013
Mg, % of DM	0.41 ± 0.014	0.45 ± 0.043	0.32 ± 0.005	0.30 ± 0.007
K, % of DM	1.71 ± 0.036	1.69 ± 0.051	1.69 ± 0.037	1.70 ± 0.055
S, % of DM	0.27 ± 0.004	0.30 ± 0.013	0.36 ± 0.005	0.32 ± 0.010
Na, % of DM	0.55 ± 0.025	0.36 ± 0.057	0.73 ± 0.060	0.35 ± 0.047
Cl, % of DM	0.43 ± 0.029	0.98 ± 0.128	0.43 ± 0.027	0.57 ± 0.047
NE_L, Mcal/kg DM	1.66 ± 0.015	1.66 ± 0.013	1.66 ± 0.012	1.67 ± 0.024
DCAD,[2] mEq/100g	38.7 ± 1.38	12.3 ± 0.99	40.4 ± 3.52	22.7 ± 1.80
AACC,[3] mEq H^+/g	20.0	20.0	41.5	41.5

[1]Mean value ± SD.

[2]Calculated as mEq (Na + K) – (Cl + S)/100 g of DM.

[3]Added acid consuming capacity from $CaMg(CO_3)_2$ and $CaCO_3$.

ing stored at −20°C. Feed ingredients and refusals were analyzed in duplicate for particle size distribution with the Pennsylvania State Particle Separator (**PSPS**; Nasco, Newmarket, ON, Canada) and sieve openings of 19, 8, and 4mm, and a pan (Heinrichs, 2013). Thereafter, the sorting index for each fraction was calculated for each cow as actual intake of each screen represented as a percentage of the predicted intake (Leonardi and Armentano, 2003).

A 1.0-kg sample of the feed ingredients and refusal samples was dried in a forced-air oven at 55°C for 96 h to determine the DM before being ground using a hammer mill (Christie-Norris Laboratory Mill; Christie-Norris Ltd., Chelmsford, UK) to pass through a 2.5-mm screen. Ground samples were then sent to Cumberland Valley Analytical Services (**CVAS**; Waynesboro, PA), where they were reground to pass through a 1-mm sieve and analyzed for analytical DM, ash, CP, ether extract, starch, ash-free neutral detergent fiber (**aNDFom**), ADF, undigestible neutral detergent fiber (**uNDF**), Ca, P, Na, K, Mg, Cl, and S. Ash was analyzed according to AOAC International (2000) method 942.05 with the modifications of using 1.5-g sample weight, 4-h ashing time, and using hot weight measurement. The OM was calculated by subtracting the ash concentration from 100%. Crude protein was determined using AOAC (2000) method 990.03 with an FP-528 Nitrogen Combustion Analyzer (Leco, St. Joseph, MI). The ether extract was determined using AOAC International (2006) method 2003.05. Starch was analyzed according to Hall (2009a) with a correction for free glucose and water-soluble carbohydrates according to DuBois et al. (1956). The aNDFom concentration was determined according to Van Soest et al. (1991) except that Whatman 934-AH glass microfiber filters (Sigma-Aldrich, St. Louis, MO) with 1.5-um particle retention were used, and the final filter and sample were then ashed at 535°C in a furnace for 2 h. The ADF concentration was determined using AOAC International (2000) method 973.18 utilizing the same filtration approach as described for aNDFom. The uNDF concentration was calculated as the aNDFom remaining after a 240-h in vitro incubation measured according to Goering and Van Soest (1970). Minerals were analyzed according to AOAC International (2000) method 985.01 with slight modification. Briefly, a 0.35-g sample was dried at 535°C in a muffle furnace for 1 h before being digested in open crucibles for 20 min in 15% nitric acid on a hotplate. Thereafter, samples were diluted to 50-mL and analyzed using inductively coupled plasma optical emission spectroscopy (Perkin Elmer 5300 DV ICP; Waltham, MA).

Feeding behavior was monitored on the 8 cannulated cows using video cameras (GZ-RR440; JVCKENWOOD Canada Inc., Mississauga, ON, Canada) from d 13 to d 16 of each period with retrospective analysis using a 2-min scan sampling approach (Dong et al., 2018). Feeding behavior was monitored before ruminal fluid sampling, fecal sampling, and total urine collection (described below) to avoid behavioral changes provoked by intensive sampling activities. Cow activity at each 2-min interval was assessed and allocated to body posture (standing vs. lying) and activity (eating, ruminating, drinking, or idle). The observed posture and activity were assumed to persist until the next observation time point. Totals of each posture and activity were determined for each cow over the 96-h duration.

Milk Yield, Milk Components, and Milk Fatty Acid Analyses

Cows were milked 3 times daily at 0700, 1400, and 2100 h, and milk yields were recorded at each milking; however, only milk yields collected from d 13 to d 20 were used for statistical analyses. Daily milk samples for each cow (d 13 to 20) were composited proportionally to daily milk yield to create a single sample for each cow/d. The daily composited milk samples were transferred into 2 vials. One (35-mL) vial with a potassium bichromate pellet (Safa et al., 2019) was used for determination of fat, CP, lactose, and SCC concentrations at the Central Milk Testing Laboratory (Edmonton, AB, Canada) within 5 d of collection. Milk fat and protein yield (kg/d) were calculated for the last 7 d of each experimental period based on milk yield and the measured composition.

The second daily composite for each cow was stored frozen until the end of the collection period. Samples were then thawed, mixed, and composited proportionally based on daily milk yield to create a 35-mL sample that was used for milk fatty acid (**FA**) analysis. To determine milk FA composition, a 10-mL sample was transferred into a 15-mL tube and centrifuged at 15,000 × *g* at 4°C for 30 min. The fat at the top of the tube after centrifugation was transferred into a 2-mL microcentrifuge tube and centrifuged at 16,000 × *g* for 20 min at 24°C. Centrifuged samples were then transferred to a wet heating block for 2-min to allow the oil fraction to separate. The oil was transferred into 16-mm glass culture tubes, and the weight was recorded. Tubes were then freeze-dried (Labconco, Kansas City, MO) for 24 h with a gradual temperature decrease. The weight of the freeze-dried samples was recorded, and samples were stored at −20°C until methylation. Freeze-dried milk fat was methylated using sodium methoxide as a base catalyst to form fatty acid methyl esters (**FAME**) according to Yurawecz et al. (1999). Briefly, 0.5 mL of internal standard (c10–17:1, 4 mg/mL hexane) was added to ~40 mg of freeze-dried milk fat and methylated using 2 mL of 0.5 N sodium methoxide in methanol at 50°C for 10 min. Next, 1.5 mL of hexane and 5 mL of water were added, mixed, and the upper organic layer containing FAME were collected and diluted with hexane for FAME profile determined using a CP-Sil 88 column (100 m, 25-µm film thickness; Agilent Technologies, Ramsey, MN) in a TRACE 1310 gas chromatograph (Thermo Scientific) with a flame ionization detector as described by Klopatek et al. (2022). Reference standards no. 463 and 603 from Nu-Check Prep Inc. (Elysian, MN) and a beef fat standard (BF 204; Vahmani et al., 2016) were used for identification of FAME by GC. Calculations used for FAME quantification were based on chromatogram peak area and the internal standard as described by (Wilms et al., 2022).

Ruminal Fermentation

Ruminal pH was measured in the 8 ruminally cannulated cows using indwelling pH systems (Penner et al., 2006) inserted into the ventral sac of the rumen on d 15 and removed on d 19. Ruminal pH systems were standardized the morning before placement using pH 4 and 7 buffer solutions adjusted to 39°C (Fisher Chemical, Fair Lawn, NJ). The location (ventral sac) was maintained with two 900-g weights attached to the bottom of the electrode shroud (Penner et al., 2006). Ruminal pH was recorded at 5-min intervals and the mean, maximum, and minimum pH values, along with the duration (min/d) and area that pH remained below 5.8, were determined daily for each cow. The averages across days within a period for each cow were used for statistical analysis. Indwelling pH systems also recorded ruminal temperature, which was summarized as the mean, maximum, and minimum temperature by day. Rectal temperatures were measured daily at 1530 h from d 16 to d 19 using a digital thermometer (Vicks, North Carolina)

Ruminal digesta collection began on d 16 extending to d 19 with 12-h intervals between consecutive samples and a 3-h offset among days. Collections occurred on d 16 at 2400 and 1200 h, on d 17 at 0300 and 1500 h, on d 18 at 0600 and 1800 h, and on d 19 at 0900 and 2100 h. At each collection, a mixed ruminal digesta sample was collected from the cranial, central, and caudal locations (250 mL/region) at the ruminal fluid–ruminal mat interface. The digesta collected was mixed and strained through 2 layers of cheesecloth. Thereafter, a 10-mL aliquot of ruminal fluid was pipetted into a tube containing 2 mL of metaphosphoric acid (25% wt/vol) for short-chain FA (**SCFA**) analysis. Samples were placed on ice and stored at −20°C. Before analysis, a composite sample was created by combining 1.5 mL of ruminal fluid from each sampling time point for each cow within each period. The SCFA concentrations were analyzed using GC (Agilent 6890 GC Series; Agilent Technologies, Ramsey, MN) with a flame ionization detector as described by (Khorasani et al., 1996). Briefly, the composited ruminal fluid samples were centrifuged at 12,000 × *g* at 4°C for 10 min. Two 1.5-mL portions of supernatant were transferred and centrifuged again at 16,000 × *g* at 4°C for 10 min. The sample (1 mL) was then combined with 0.2 mL of isocaproic acid as the internal standard. The column used was a Phenom FFAP (30.0 m × 320 µm × 0.25 µm; Agilent Technologies, Ramsey, MN) with a maximum temperature of 260°C and using the constant flow mode. The inlet temperature was 170°C with a split ratio of 17:1, and helium was used as the makeup gas. The detector heater was programmed to 250°C.

Ruminal evacuations were performed on d 20 (4 cannulated cows) and d 21 (4 cannulated cows) of each

period at 0700 h (before feeding) to determine the free liquid and DM pools as described by Pursley (2019). Briefly, the ruminal contents were completely removed and stored in an insulated container. Thereafter, digesta was weighed, thoroughly mixed, and a 5-L sample was collected before returning digesta back into the rumen. Each 5-L sample was placed in a mesh bag, and the liquid and solid portions were separated using a wine press (Harvest Bounty Wine Press; Pleasant Hill Grain LLC, Hampton, NE; Karnati et al., 2007). The liquid and solid portions were weighed before and after being dried for 120 h at 55°C in a forced-air oven to determine the DM of the liquid and solid fractions.

Blood, Urine, and Fecal Sampling and Analysis

Blood and fecal samples were collected at the same time points as ruminal fluid collection (described above). Blood samples were drawn from the coccygeal vein into one tube containing 158 IU of Na-heparin for plasma collection and a second tube with silica gel to activate clotting for serum collection (Becton Dickinson, Franklin Lakes, NJ). The vacutainer containing Na-heparin was placed in a cold-water bath and centrifuged immediately at 2,500 × *g* for 15 min at 4°C and plasma was harvested. At each collection, plasma aliquots (0.5 mL) from each sampling time point were added to 2-mL microcentrifuge tubes to create composite samples of plasma for each analyte. These composite samples were stored at −20°C until they were analyzed for plasma glucose and insulin. The second tube containing silica was allowed to clot at room temperature for 30 min before centrifugation at 2,500 × *g* for 15 min at 4°C. Before opening the vacutainer, 1-mL of serum was removed using a syringe and 3.8 cm long 20-gauge needle and the removed sample was injected into a new silica vacutainer for HCO_3 determination by Prairie Diagnostic Services (Saskatoon, SK, Canada). The remaining serum was transferred into 3 microcentrifuge tubes, as described for plasma, and stored at −20°C until they were analyzed for BHB, nonesterified FA (**NEFA**), and serum amyloid A (**SAA**). Plasma and serum composites were created immediately at each collection time point to minimize the number of freeze-thaw cycles.

Serum and plasma composite samples were thawed and adequately vortexed before each analysis. All blood analyses were conducted with standard curves on each microplate and were read on a microplate reader (EPOCH2C; Bio Tek, Santa Clara, CA). Plasma glucose concentration was quantified, in triplicate, via enzymatic determination using a glucose oxidase-peroxidase enzyme (P7119; Sigma-Aldrich, Oakville, ON, Canada) and dianisidine dihydrochloride (D3252; Sigma-Aldrich, Oakville, ON, Canada) with an intra-assay CV of 2.1% and interassay CV of 0.9%. Plasma insulin was determined using Mercodia Bovine Insulin ELISA (10-1201-01; Mercodia, Winston-Salem, NC) in duplicate, resulting in an intra-assay CV of 1.6% and interassay CV of 5.4%. Serum NEFA concentrations were measured enzymatically in triplicate using a commercially available kit (NEFA-HR(2); Wako Chemicals USA, Richmond, VA) with an intra-assay CV of 2.2% and interassay CV of 0.01%. Serum BHB was determined enzymatically using 3-hydroxybutyrate dehydrogenase (3HBDB-RO; Sigma-Aldrich, Oakville, ON, Canada) which catalyzes the oxidation of BHBA to acetoacetate and the corresponding reduction of NAD to NADH. We analyzed BHB in triplicate with an intra-assay CV of 2.1% and an interassay CV of 3.5%. Serum amyloid A was determined using a Tridelta phase range SAA kit (T802; Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) with an intra-assay CV of 5.3% and interassay CV of 1.3%.

Fecal samples were collected directly from the rectum at the same time as ruminal fluid and blood collections. Samples were composited on an equal as-is weight basis (200 g from each collection point) and stored at −20°C. The fecal composites were dried in a forced-air oven at 55°C for 120 h to determine DM content. Dried samples were ground to pass through a 2.5-mm screen using a hammer mill (Christie-Norris Laboratory Mill, Christie-Norris Ltd., Chelmsford, United Kingdom). Subsequently, fecal samples were sent to CVAS, and analyzed for DM, OM, CP, NDF, ADF, starch, ether extract, and uNDF as described for feed and refusal samples to evaluate total-tract digestibility using uNDF as an internal marker to predict fecal output (Huhtanen et al., 1994; Menajovsky et al., 2018)

On d 15 of each period, Foley catheters (24 Fr, 75 mL Bardex Lubricath Catheter; C. R. Bard Inc., Covington, GA) were aseptically inserted and inflated with 80 mL of saline solution. Cows were provided 24 h to acclimatize before collections began the following day. On d 16, catheters were connected to 20-L plastic carboys that contained approximately 500 mL of 37% HCl. The volume of HCl added was recorded but differed among cows based on urine volume and buffering. Urine pH in the carboys was tested daily, and HCl was added to maintain a final urine pH below 3 to prevent nitrogen volatilization. Total urine collection extended for 96 h. Each day, urine was weighed, a representative sample was collected, and the sample was stored at −20°C. During milking, catheters were disconnected from the carboys and clamped shut to prevent loss of urine. Following milking, cows were returned to their stalls and the tubing was cleaned and reconnected to the catheters. Daily urine subsamples were composited based on daily urine output after HCl addition was accounted for.

Total and Postruminal Gastrointestinal Tract Barrier Function

Total-tract and postruminal barrier function were evaluated using a ruminal bolus dose of Cr-EDTA (1 L of a 180 m*M* solution) as described by Zhang et al. (2013) and a simultaneous abomasal bolus dose of Co-EDTA (500 mL of a 360 m*M* solution), respectively (Bertens et al., 2022, 2024). The production of Cr-EDTA and Co-EDTA solutions are described in detail in Bertens et al. (2024) and were prepared according to Binnerts et al. (1968) and Udén et al. (1980), respectively. Before infusion, Cr- and Co-EDTA solutions were subsampled (25 mL). Markers were infused according to Bertens et al. (2024) on d 16 at 1000 h, 30 min after fresh feed delivery. First, Co-EDTA (500 mL) was administered into the abomasum by locating the omasal orifice via the ruminal cannula, followed by the insertion of a vinyl tube (20-mm o.d., length 91 cm; Global Industries, Scarborough, ON, Canada) 20 cm into the omasum. The other end of the tube was attached to a 400-mL container into which the Co-EDTA was poured, followed by 150 mL of water. Immediately thereafter, a cavity was created within the rumen mat into which Cr-EDTA (1 L) was delivered directly. Urine catheters were immediately connected to plastic 20-L carboys, and daily urine subsamples (0 to 24 h and 24 to 48 h) were collected. Stock Cr- and Co-EDTA subsamples and daily urine subsamples were analyzed for Cr and Co concentrations at Prairie Diagnostic Services (Saskatoon, SK, Canada) using inductively coupled plasma emission MS (**ICP-MS**). Samples were prepared with nitric acid and digested in a Multiwave 5000 Microwave (Anton Paar, Graz, Austria). Digested samples were diluted with distilled water and analyzed for Cr and Co (Thermo-Fisher). The Cr and Co concentrations used in the determination of urinary excretion were corrected for the dilutions performed in sample preparation for ICP-MS. Cr-EDTA and Co-EDTA have a similar molecular size and mass (Zubkowski et al., 1995; García-Lafuente et al., 2001), they both are nondigestible and nonmetabolizable, and there is a lack of known transcellular transport mechanisms suggesting they are suitable GIT permeability markers (Bjarnason et al., 1995). Following Cr-EDTA infusion into the rumen and Co-EDTA into the abomasum, the subsequent appearance of markers in urine was measured to indicate total GIT permeability and postruminal permeability, respectively. The concentrations of markers in urine collected 24 and 48 h after infusion were multiplied by the daily urine weight to calculate the total mg of Cr and Co excreted. In addition, the total quantity of Cr and Co excreted in urine after 48 h was calculated as a percentage of the Cr-EDTA and Co-EDTA infused to determine proportional excretion based on unequal molar quantities of Cr-EDTA and Co-EDTA delivered.

Statistical Analysis

Statistical analysis was conducted using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) using a Newton–Raphson optimization with ridging. For all outcomes measured, the model contained the fixed effects of DCAD, buffer, and the 2-way interaction. For production variables including all 16 cows (e.g., DMI, milk yield and components, sorting index, BW, and BCS), the model included the random effects of period and cow within parity. All other response variables for the 8 ruminally cannulated cows included the random effect of period and cow. A compound symmetry residual covariance structure was used to account for negative covariances between cows. Denominator degrees of freedom for comparisons were calculated using the Kenward–Roger correction. Pairwise comparisons of levels of DCAD within buffer and vice versa were tested using the SLICEDIFF option. When an interaction was detected ($P \leq 0.05$), the Tukey–Kramer adjustment was used to compare the LSM. Studentized residuals were used to evaluate normality and homogeneity of variance using the UNIVARIATE procedure. The SCC, duration, and area that pH was ≤5.8 data were not normally distributed and were subsequently analyzed using a log-normal distribution. Sorting activity for each dietary treatment on each sieve of the PSPS was tested for differences from 100% using a 2-tailed *t*-test. Effects of the treatment factors were declared significant at $P \leq 0.050$; and 3 decimal places were shown when $P < 0.100$ and 2 decimal places when $P \geq 0.10$. The video feeding behavior data from period 1 was lost, and therefore only periods 2, 3 and 4 were included in the statistical analysis.

RESULTS

Environmental Conditions

During the imposed daily HS interval, the mean, maximum, and minimum THI (mean ± SD) were 74 ± 1.2, 76 ± 0.9, and 65 ± 2.4, respectively (data not shown). The mean, maximum, and minimum temperature during HS were 26°C ± 0.8, 28°C ± 0.6, and 20°C ± 1.6, respectively. The mean, maximum, and minimum RH were 55 ± 2.3, 68 ± 3.1, and 44 ± 2.8, respectively. During the NHS part of the day, the mean, maximum, and minimum THI were 67 ± 2.5, 75 ± 1.0, and 64 ± 2.4 respectively. The mean, maximum, and minimum temperature during NHS were 21°C ± 1.8, 28°C ± 0.8, and 19°C ± 1.7, respectively. The mean, maximum, and minimum RH were 53 ± 4.3, 64 ± 4.9 and 40 ± 2.9, respectively. Cows were exposed to a THI ≥68 for an average of 15 ± 3.9 h/d over the course of the study. Mean rectal temperatures were 39.1°C ± 0.27

with no differences detected among dietary treatments ($P \geq 0.57$; data not shown).

BW, BCS, DMI, and Feeding Behavior

Average BW and BCS, and BW and BCS change between the start and end of each experimental period were unaffected by dietary treatment (Table 3). We detected no differences in feeding behavior and daily activities among treatments. We found no effects of buffer, DCAD, or buffer × DCAD on DMI, with a mean ± SD of 25.1 ± 0.20 kg/d. We found no effect of DCAD, or the interaction of buffer and DCAD for sorting behavior, regardless of the particle fraction investigated. Cows fed LB sorted against particles retained on the 4-mm sieve more than HB; however, the sorting index values were not different from 100%. Cows fed HB tended ($P = 0.078$) to sort more against particles retained on the 8-mm sieve, although the sorting index values did not differ from 100%.

Milk Yield, Milk Components, and Milk Fatty Acid Composition

We did not identify any effects of buffer, DCAD, or buffer × DCAD on milk yield, milk NE_L, ECM, or 4% FCM (Table 4). We found no buffer × DCAD interaction for milk fat concentration, but there were individual effects of buffer and DCAD. When compared with LD, providing HD resulted in greater milk fat concentration by 0.14 ± 0.044 percentage units and milk fat yield by 36.5 ± 16.71 g/d. Cows fed HB exhibited reduced milk fat percentage by 0.11 ± 0.044 percentage units despite no differences in milk fat yield. We found no effects of buffer, DCAD, or the interaction on milk protein concentration; however, a tendency for an interaction was detected for milk protein yield ($P = 0.095$). Milk lactose concentration was affected by buffer alone, where HB increased lactose percentage by 0.03 ± 0.014 percentage units. Similarly, milk lactose yield tended to be increased with HB by 44.8 ± 26.00 g ($P = 0.092$). High DCAD tended to reduce SCC ($P = 0.062$).

The production of 16-carbon milk fatty acids was increased by 7.4% in HD compared with LD (Table 4). The production of de novo synthesized FA and preformed FA were not affected by buffer, DCAD, or the interaction. A complete set of individual milk fatty acids analyzed are reported in Supplemental Table S1 (see Notes).

Ruminal Fermentation and Digestibility

The mean, maximum, and minimum ruminal pH did not differ among treatments (Table 5), with average

Table 3. The effect of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on BW, BCS, feeding behavior, DMI, and sorting behavior for Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Start BW,[2] kg	633	637	637	634	14.9	0.78	0.88	0.32
Change in BW,[3] kg	5	4	6	6	2.9	0.71	0.86	0.89
Start BCS[2]	2.72	2.70	2.80	2.69	0.069	0.42	0.11	0.23
Change in BCS[3]	−0.06	−0.08	0.00	−0.11	0.046	0.76	0.22	0.35
Behavior, min/d								
Lying	736	733	743	773	23.3	0.30	0.55	0.45
Standing	611	614	605	575	24.3	0.36	0.58	0.49
Idle	545	541	564	563	25.0	0.34	0.96	0.91
Chewing	755	763	732	737	26.4	0.32	0.79	0.96
Ruminating	446	461	428	448	22.6	0.40	0.33	0.87
Eating	310	303	301	291	12.1	0.21	0.30	0.83
Drinking	47	43	55	45	5.0	0.28	0.13	0.53
Milking	84	88	84	88	4.2	0.98	0.44	0.97
DMI, kg/d	25.0	25.4	24.8	25.3	1.03	0.65	0.28	0.88
Sorting index,[4] %								
19-mm sieve	104.1	101.9	105.7*	104.3*	1.77	0.17	0.21	0.79
8-mm sieve	99.1	98.1	99.9	99.7	0.74	0.078	0.43	0.56
4-mm sieve	98.4	99.6	96.8	97.6	0.90	0.049	0.27	0.81
Pan	100.3	103.6*	99.8	99.8	1.56	0.13	0.24	0.24

[1]SEM for the interaction is reported.

[2]Start BW and BCS was measured on the first day of each period.

[3]The change in BW and BCS was calculated as the difference between the start and end of each period. The means presented are not different from zero.

[4]The sorting index was calculated as described by Leonardi and Armentano (2003).

*Treatment mean is different than 100% ($P < 0.05$).

Table 4. The effect of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on milk and milk components for Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Milk yield, kg/d	37.2	37.3	36.1	36.8	1.45	0.12	0.41	0.59
Milk NE_L,[2] Mcal/d	27.7	27.2	27.2	27.3	0.94	0.54	0.53	0.42
ECM,[3] kg/d	37.4	36.8	36.8	37.0	1.30	0.58	0.59	0.38
4% FCM,[4] kg/d	37.8	37.2	37.4	37.3	1.24	0.71	0.35	0.58
Milk composition								
Fat, %	4.14	4.02	4.28	4.12	0.106	0.014	0.002	0.54
Fat, kg/d	1.53	1.49	1.53	1.50	0.050	0.52	0.034	0.64
Protein, %	3.31	3.27	3.28	3.32	0.055	0.69	0.92	0.17
Protein, kg/d	1.23	1.22	1.18	1.22	0.052	0.16	0.23	0.095
Lactose, %	4.57	4.57	4.55	4.53	0.041	0.035	0.50	0.77
Lactose, kg/d	1.70	1.70	1.64	1.66	0.062	0.092	0.55	0.70
SCC,[5] $\times 10^3$ cells/mL	27.5	29.2	24.7	30.9	6.00	0.74	0.062	0.28
Milk fatty acids,[6] g/d								
Total de novo[7]	340.8	340.7	340.6	343.9	14.38	0.77	0.75	0.74
Total mixed[8]	713.3	644.4	709.8	681.0	28.43	0.22	<0.001	0.14
Total preformed[9]	475.5	500.1	482.3	479.0	13.41	0.45	0.26	0.14

[1]SEM for the interaction is reported.

[2]Milk NE_L was calculated according to NASEM (2021) as (0.0929 × fat%) + (0.055 × protein%) + (0.0395 × lactose%).

[3]ECM was calculated according to Sjaunja et al. (1990) as (0.25 × kg milk) + (12.2 × kg fat) + (7.7 × kg CP).

[4]4% FCM was calculated according to Gaines and Davidson (1923) as (0.4 × kg milk) + (15 × kg fat).

[5]Data is log-normal transformed. Back-transformed means and SEM are presented.

[6]Supplemental Table S1 includes all the individual milk fatty acids analyzed.

[7]Total de novo contains milk fatty acids <16C.

[8]Total mixed fatty acids contains all 16C fatty acids.

[9]Total preformed contains >16C fatty acids.

values of 6.38, 6.86, and 5.85, respectively. The average duration and area that pH remained below 5.8 was 10.2 min/d and 2.2 pH × min/d, respectively, and was not affected by buffer, DCAD, or their interaction. Ruminal temperature was unaffected by dietary treatments with average mean, maximum, and minimum temperatures of 39.5°C, 40.5°C, and 36.2°C respectively. We found no differences between treatments for total SCFA concentration or the molar proportions of individual SCFA.

Total, solid, and liquid ruminal digesta weights when presented on an as-is or DM basis were not affected by the buffer × DCAD interaction (Table 6). Cows fed LB tended (P = 0.089) to have 4.5 ± 2.53 kg greater total digesta weight and had a liquid fraction weight that was 4.8 ± 1.79 kg greater than HB. Although the weight of the solid fraction on an as-is or DM basis was not affected, more DM was contained within the liquid fraction for cows fed LB than HB. We found no effects of DCAD on digesta weight or the weights of the solid and liquid fractions.

The daily fecal DM output was not affected by dietary treatment or the interaction among treatments (Table 7). Apparent total-tract digestibility of all measured nutrients was not affected by the buffer × DCAD interaction. However, buffer affected apparent total-tract digestibility of CP ,with HB increasing CP digestibility by 1.7 ± 0.69 percentage units. In addition, HD tended to decrease apparent total-tract digestibility of starch by 1.8 ± 0.94 percentage units compared with LD (P = 0.066).

Blood Metabolites

Plasma glucose and insulin and serum NEFA and SAA were not affected by dietary treatments (Table 8). However, serum BHB tended to be reduced in HB compared with LB (P = 0.070). Serum HCO_3 was affected by DCAD, whereby HD had greater serum HCO_3 concentrations than LD, with a tendency for a buffer × DCAD interaction (P = 0.10) such that HB-LD had lesser HCO_3 concentrations than HB-HD (P = 0.044).

Daily Urine Output and Gastrointestinal Tract Permeability

We observed a tendency for a buffer × DCAD interaction for daily urine output (P = 0.098; Table 9), where LB-HD excreted 6 kg more urine than LB-LD (P = 0.034). We found no buffer × DCAD interaction on total or postruminal gastrointestinal tract permeability, as indicated by the 48-h urinary excretion and % recovery of Cr and Co. Provision of HB tended to reduce total urinary Cr excretion (mg/48 h) by 10% (P = 0.098) and

Table 5. The effects of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on ruminal pH, temperature and SCFA concentrations in lactating Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Ruminal pH								
Minimum pH	5.87	5.76	5.89	5.88	0.118	0.51	0.60	0.64
Mean pH	6.41	6.34	6.41	6.38	0.107	0.81	0.58	0.86
Maximum pH	6.82	6.89	6.87	6.87	0.093	0.90	0.71	0.75
Duration,[2] min/d ≤5.8	7.6	9.9	16.3	7.1	13.35	0.77	0.70	0.45
Area,[2] pH × min/d ≤5.8	2.3	1.5	3.0	1.9	1.78	0.61	0.39	0.99
Rumen temperature, °C								
Minimum	36.2	35.9	36.4	36.5	0.56	0.40	0.78	0.71
Mean	39.4	39.5	39.6	39.4	0.13	0.74	0.86	0.23
Maximum	40.5	40.5	40.7	40.4	0.52	0.63	0.23	0.20
Total SCFA, m*M*	125.8	120.7	122.9	120.2	3.21	0.64	0.30	0.75
Molar proportion, mol/100 mol								
Acetic acid	60.8	62.0	61.2	62.4	1.17	0.73	0.36	0.98
Propionic acid	23.6	22.6	23.3	22.5	1.07	0.86	0.44	0.96
Butyric acid	11.3	11.5	11.1	11.3	0.32	0.58	0.55	0.85
Isobutyric acid	0.83	0.89	0.85	0.86	0.031	0.81	0.30	0.41
Isovaleric acid	1.24	1.36	1.28	1.28	0.065	0.85	0.41	0.40
Valeric acid	1.46	1.41	1.45	1.39	0.059	0.82	0.41	0.90
Caproic acid	0.82	0.30	0.81	0.29	0.345	0.98	0.15	0.99

[1]SEM for the interaction is reported.

[2]Data is log-normal transformed. Back transformed means and SEMs are presented.

Table 6. The effect of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on rumen pool sizes for lactating Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Total digesta weight, kg as-is	82.6	84.5	87.4	88.6	3.81	0.089	0.54	0.89
Solid fraction, kg as-is	35.0	35.4	33.5	36.2	1.85	0.83	0.31	0.46
Liquid fraction, kg as-is	47.6	49.1	53.9	52.4	2.65	0.014	1.00	0.42
Total digesta weight, kg DM	11.2	11.4	10.8	11.8	0.57	0.97	0.18	0.44
Solid fraction, kg DM	9.95	10.16	9.48	10.36	0.548	0.76	0.22	0.44
Liquid fraction, kg DM	1.22	1.27	1.33	1.40	0.058	0.019	0.21	0.83

[1]SEM for the interaction is reported.

Table 7. The effects of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on fecal output and DM, and apparent total-tract digestibility in lactating Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Fecal excretion,[2] kg DM/d	7.17	7.19	7.04	7.33	0.342	0.99	0.45	0.51
Fecal DM, %	16.1	16.1	15.7	16.0	0.32	0.36	0.59	0.62
Digestibility, % of DM								
DM	68.0	68.8	68.1	67.7	0.79	0.42	0.69	0.30
OM[3]	69.5	70.7	69.5	69.7	0.79	0.38	0.24	0.40
CP	71.2	72.4	70.0	70.2	0.97	0.023	0.32	0.45
ADF	46.3	46.1	48.1	49.7	1.71	0.14	0.71	0.60
aNDFom	51.0	51.5	50.3	50.5	1.04	0.28	0.61	0.81
Starch	89.3	91.9	89.2	90.2	1.07	0.35	0.066	0.41
Ether extract	75.8	76.6	76.8	75.8	1.81	0.97	0.96	0.63

[1]SEM for the interaction is reported.

[2]Fecal excretion was predicted using uNDF intake.

[3]Calculated as 100 − ash.

Table 8. The effects of experimental diets containing low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on blood metabolites for lactating Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Plasma metabolite								
Glucose, mg/dL	66.7	63.7	63.1	63.1	2.00	0.34	0.48	0.48
Insulin, mg/L	0.69	0.73	0.65	0.68	0.069	0.25	0.41	0.79
Serum metabolite								
NEFA,[2] mEq/L	119	122	128	111	11.0	0.92	0.56	0.40
BHB, mmol/L	0.62	0.62	0.69	0.68	0.039	0.070	0.82	0.76
SAA,[3] mg/mL	112	104	94	75	34.7	0.35	0.59	0.83
Serum HCO_3, mmol/L	25.4	23.8	24.6	24.4	0.49	0.88	0.030	0.10

[1]SEM for the interaction is reported.

[2]NEFA = nonesterified fatty acids.

[3]SAA = serum amyloid A.

reduced total urinary excretion of Co (mg/48 h) by 27%. Urinary recovery rates of Cr and Co, expressed as a percentage of Cr-EDTA and Co-EDTA infused, showed similar trends as the total amount excreted such that HB reduced the recovery of Co by 24%; however, there was no longer a tendency detected for buffer on Cr recovery. To evaluate the proportion of total-tract permeability that was occurring postruminally, Co recovery was expressed as a percentage of the Cr recovery. As such, the proportion of Co relative to Cr was reduced 16.6 ± 5.50 percentage units for HB compared with LB and was increased 12.6 ± 5.50 percentage units for HD compared with LD. Across all treatments Co recovery represented 72.0% of the Cr recovery.

DISCUSSION

This study was designed to evaluate the independent effects of increased DCAD and provision of a dietary buffer as well as their interaction on DMI, ruminal fermentation, total GIT and postruminal permeability, systemic inflammation, and milk and milk component yields in lactating dairy cattle exposed to mild HS with night cooling. Contrary to our hypothesis, DCAD and buffer inclusion presented no interactive effects on DMI, ruminal pH, GIT permeability, and milk or milk component yields. However, increasing the DCAD enhanced blood acid-base balance and stimulated greater milk fat yield. Alternatively, dietary $CaMg(CO_3)_2$ supplementation reduced postruminal permeability without affecting milk component yields. These findings are interpreted to indicate that DCAD and dietary buffer supply act independently for lactating dairy cattle exposed to mild HS. To corroborate whether HS symptoms are attenuated when consuming high and low DCAD with or without additional buffer supplementation, further research incorporating a thermoneutral control is required.

Table 9. The effect of low (LD; 17.5 mEq/100 g of DM) or high DCAD (HD; 39.6 mEq/100 g of DM) and low (LB; no added $CaMg(CO_3)_2$) or high buffer (HB; 1.0% $CaMg(CO_3)_2$) on urinary excretion and recovery of Cr and Co following a ruminal pulse dose of Cr-EDTA and an abomasal pulse dose of Co-EDTA to evaluate total-tract and postruminal gastrointestinal tract permeability, respectively, in lactating Holstein cows exposed to mild heat stress

	HB		LB			*P*-value		
Variable	HD	LD	HD	LD	SEM[1]	Buffer	DCAD	Buffer × DCAD
Urine output, kg/d	27	26	31	24	1.5	0.73	0.023	0.098
Marker excretion,[2] mg/48 h								
Cr	139.2	150.0	155.0	166.9	12.11	0.098	0.24	0.96
Co	99.8	82.9	126.9	122.7	8.38	<0.001	0.22	0.45
Marker recovery,[3] % of infused								
Cr	1.38	1.43	1.53	1.63	0.132	0.11	0.51	0.79
Co	1.18	0.98	1.43	1.43	0.085	<0.001	0.16	0.18
Co as a % of Cr[4]	71.2	56.3	85.5	75.2	5.20	0.007	0.033	0.68

[1]SEM for the interaction is reported.

[2]Marker excretion is the cumulative urinary excretion 48 h after infusion of Cr-EDTA and Co-EDTA.

[3]Marker recovery was calculated as $\frac{48\text{ h urinary marker excretion (mmol)}}{\text{mmol of marker infused}} \times 100\%$.

[4]The proportion of Co represented as a function of Cr was calculated using marker excretion (mg/48 h).

Environmental Conditions and Dietary Treatments

According to the current HS thresholds proposed for lactating dairy cattle (THI ≥68; Zimbelman et al., 2011 and THI ≥64; Campos et al., 2022), it can be argued that the cows in this study experienced mild to moderate daytime HS. Evidence of daytime HS was supported by elevated rectal and ruminal temperatures (AlZahal et al., 2011) and initiation of behavioral changes (i.e. increased daytime standing and increased nighttime lying to maximize evaporative cooling; Pinto et al., 2020). The conditions imposed in this study emulate natural daily fluctuations in THI, representative of conditions cattle experience in more temperate climates (Zimbelman et al., 2011; Vanderzaag et al., 2023). It appears that cattle in the present study did not fully acclimatize to the HS conditions based on sustained elevated rectal (Abeni et al., 2007) and ruminal temperatures throughout the study.

Experimental diets were formulated to contain similar concentrations of K, Mg, Ca, and S, whereas the Na and Cl concentrations were manipulated using Na-acetate and NH_4Cl to target DCAD values of 10 and 30 mEq/100 g of DM; however, slight differences were observed for the actual mineral concentrations fed. Consequently, the actual DCAD levels achieved were 17.5 mEq/100 g of DM for LD and 39.6 mEq/100 g of DM for HD. We also observed slight differences in DCAD between the HB-LD and LB-LD treatments. However, this did not yield buffer by DCAD interactions, and despite DCAD values being numerically different than targeted, the average difference between HD and LD (22 mEq/100 g of DM) imposed did not compromise the objectives of the experiment.

Interaction Between Buffer and DCAD

Physiological responses to HS include reduced feed intake, increased sweating, and increased panting associated hypersalivation resulting in the loss of electrolytes such as Na, K, Ca, Mg, P, Cl, HCO_3, and HPO_4 to the rumen and systemic circulation (Kadzere et al., 2002; Collier et al., 2019). The limited electrolyte supply to the rumen has been speculated to increase risk for SRA by compromising mechanisms of SCFA absorption that are dependent on intraruminal cation supply (Mooney, 2006; Burhans et al., 2022). In addition, to offset the reduction in DMI response to HS, cattle may alter eating patterns to sort for smaller more fermentable feed fractions and because diets are often formulated to increase energy density (Baumgard and Rhoads, 2009), which inherently increases the risk for ruminal acidosis. However, the data in this study do not support an interactive effect of buffer and DCAD on ruminal fermentation, ruminal pH, regional GIT permeability, or milk and component yields. Increasing luminal Na supply appears to have minimal stimulatory effects on SCFA absorption ex vivo (Bertens et al., 2023), supporting the lack of treatment effect and lack of interactions between DCAD and buffer on ruminal SCFA concentrations and ruminal pH in this study. Although the cattle presented signs of daytime HS, they did not sort for the smaller and more nutrient-dense feed fractions and did not experience low ruminal pH. These results are interpreted to indicate that the modes of action for increased DCAD and provision of dietary buffer in lactating dairy cows during mild HS with night cooling are relatively independent.

Effects of DCAD

Feeding a diet with a positive DCAD postpartum has been shown to restore acid-base status, increase DMI, and drive a favorable milk yield response (Hu and Murphy, 2004; Iwaniuk and Erdman, 2015). Moreover, under HS conditions, it has been postulated that high-producing cows may benefit from a further increase in DCAD to maintain blood electroneutrality via the strong ion theory (Goff, 2018), driving feed and water intake (Wildman et al., 2007). In the present study, cows provided HD had greater blood HCO_3 concentrations, indicating an improvement in acid-base status; however, DMI was not affected. Iwaniuk and Erdman (2015) reported a curvilinear relationship between DMI and DCAD such that DMI increased by 0.61, 0.31, 0.25, 0.19, and 0.13 kg/d as DCAD increased in 10 mEq/100 g of DM increments from 0 to 50 mEq/100 g of DM, and others have demonstrated that the DMI response peaks at 40 mEq/100 of DM (Hu and Murphy, 2004) and plateaus at 20 mEq/100 g of DM (West et al., 1992). These findings highlight that the greatest responses for increased DMI occur when the incremental increases in DCAD are at low DCAD concentrations and that DCAD levels beyond 20 mEq/100 g of DM have minimal added effects. Moreover, although the HD treatments increased blood HCO_3, it could be argued that the HCO_3 concentrations achieved with LD (24.1 mmol/L) were not indicative of metabolic acidosis (Zhang et al., 2022), potentially masking DCAD effects on DMI. Additionally, the cattle in this study presented signs of thermal tolerance rather than thermal acclimatization to the imposed HS conditions, which may have negated potential effects of DCAD during HS.

Increased water intake is an immediate response to HS (McDowell et al., 1969) and increased DCAD (Tucker et al., 1988). Water intake and urine production are highly correlated (West, 2003; Beatty et al., 2006), and although we could not assess water intake, urine output is likely reflective of water intake. Cows consuming HD diets numerically spent more time at the water bowl and excreted 4 ± 1.5 kg more urine than cows fed LD. According to the

free water intake prediction equation (Equation 3) provided by Appuhamy et al. (2016), cows on HD and LD are predicted to have consumed 127 and 121 L/d, respectively. However, prediction estimates were above or at maximal values used to develop the prediction equations (maximum free water intake = 122 L/d). Potential for greater water intake for cows consuming HD treatments was likely driven by greater dietary Na intake (Tucker et al., 1988; Ben Meir et al., 2023). With the assumption that the water consumed was cooler than core body temperature, cows fed HD could theoretically dissipate more heat via conductive heat transfer. However, there were no DCAD effects on ruminal or rectal temperature, challenging whether increased urine output and presumably water intake alleviated mild HS. Although mechanisms of greater conductive heat transfer via increased cold-water intake may not provide additional heat abatement during mild HS, it may be beneficial under more severe HS conditions.

The present study did not detect an effect of DCAD on milk yield. Previous meta-analyses have reported a quadratic relationship between DCAD and milk yield, whereby milk yield peaks at a DCAD of 34 mEq/100 g of DM (Hu and Murphy, 2004; Iwaniuk and Erdman, 2015). This proposed optimal DCAD of 34 mEq/100 g of DM is intermediate to the LD and HD treatments used in this study, which, like DMI, may denote an inadequate DCAD difference between treatments to detect a milk yield response or that the LD did not challenge acid-base status. It has been suggested that the milk yield improvement with elevated DCAD is likely driven by the increase in DMI providing more nutrients to support milk synthesis (Hu and Murphy, 2004; Iwaniuk and Erdman, 2015). Therefore, it is not surprising that we did not detect a DCAD response on milk yield, considering the DMI was also not different. In agreement with our findings, Wildman et al. (2007) exposed lactating dairy cows to HS conditions and provided diets with DCAD values of 28 or 58 mEq/100 g of DM and did not report an increase in DMI or milk yield.

Positive effects of increasing DCAD on milk fat percentage and yield have been previously reported under both thermoneutral (Iwaniuk et al., 2015) and HS conditions (West et al., 1992; Wildman et al., 2007), supporting the findings of the present study. However, previous studies have manipulated DCAD using a cation bound to a carbonate source such as $NaHCO_3$, KCO_3, $KHCO_3$, or Na-sesquicarbonate and attributed the improvements in milk fat to the stabilization of ruminal pH provided by the inclusion of the buffer component rather than the increase in cation supply. In contrast, the present study exclusively detected a DCAD effect on milk fat percentage and yield and not a buffer effect, despite having no effect of buffer or DCAD on ruminal pH or ruminal fermentation outcomes. However, unlike previous studies, we used Na-acetate as the cation source to manipulate DCAD. The use of Na-acetate allowed us to segregate milk fat effects from increased DCAD relative to the provision of dietary buffer. Interestingly, when comparing cation source within the same level of DCAD, Na appears to have a greater stimulatory effect on milk fat percentage and yield than K (West et al., 1992; Iwaniuk et al., 2015). Hence, these findings contradict the notion that improvements in milk fat percentage and yield with increased DCAD are attributed to dietary buffer supply, and rather suggest that other Na dependent mechanisms may be involved.

Although we observed an increase in milk fat with increasing DCAD, it is possible that the use of Na-acetate to manipulate DCAD may have confounded this response. Na-acetate fed at a rate of 2.9% of diet DM has been reported to increase DMI by 2.7 kg/d, increase milk fat yield by 90 g/d, and increase milk fat percent by 0.2% (Urrutia et al., 2019). In the present study, the inclusion of Na-acetate in HB-LD, LB-LD, HB-HD, and LB-HD, was 0.00%, 0.47%, 1.21%, and 2.08% of DM, respectively. As such, it is possible that the increased milk fat concentration and milk fat yield for HD were partly due to the acetate component. In the present study, cows provided HD had increased production of milk fatty acids of mixed origin without any effects on preformed and de novo synthesized fatty acids. These results overlap with the findings of Urrutia et al. (2019), namely that Na-acetate increased production of mixed milk fatty acids but decreased pre-formed FA synthesis. A ruminal infusion of Na-acetate supplying 0, 5, 10, and 15 mol of Na-acetate/d resulted in a dose-dependent increase in milk fat yield (Urrutia and Harvatine, 2017). Although the authors related the greater milk fat response to greater acetate supply, the Na supply was inherently increased at the same time. Therefore, it is not clear whether responses for milk fat observed in this study can be attributed to elevated DCAD via increased Na or the use of Na-acetate.

Effects of Buffer

As mentioned previously, cows in this study did not display evidence for SRA based on relatively high mean ruminal pH and the minimal duration that pH was below 5.8 (Penner et al., 2009; Plaizier et al., 2012). Although HS is often reported to increase risk for SRA (Burhans et al., 2022) the effects of HS on ruminal pH across the literature is contradictory. Some authors have reported a decrease in ruminal pH (Mishra et al., 1970; Collier et al., 1982), whereas others have reported an increase in ruminal pH (Hall, 2009b; Hou et al., 2021). Dietary buffers have potential to resist ruminal pH changes,

with efficacy dependent on solubilization (Le Ruyet and Tucker, 1992) and the pKa of the buffer source. The solubility of $CaMg(CO_3)_2$ is minimal at pH 6.5 and increases as pH decreases (Altland and Jeong, 2016). Therefore, without reductions in ruminal pH, it is unlikely that there would be effects of $CaMg(CO_3)_2$ to resist pH change in the rumen. Considering that the cattle in this study had a mean ruminal pH of 6.38 and a minimum pH of 5.85, this likely resulted in only partial ruminal solubilization of $CaMg(CO_3)_2$, explaining the lack of a buffer effect on ruminal pH. These findings are supported by Crawford et al. (2008) and Razzaghi et al. (2021) who reported limited ability of $CaMg(CO_3)_2$ supplementation to modulate ruminal pH in growing yearling steers and lactating dairy cows fed high-concentrate diets, with reported mean ruminal pH values of 6.0 and 5.8, respectively. Although $CaMg(CO_3)_2$ may have limited ruminal buffering capacity, it will likely solubilize in the abomasum, thereby having the potential to stabilize pH in the lower GIT.

More recently, evidence suggests that HS may induce hyperpermeability of the GIT and that the ensuing immune activation may be the primary cause of compromised growth and performance outcomes not only in ruminants but across livestock species during hot weather (Koch et al., 2019; Mayorga et al., 2020; Burhans et al., 2022). However, the region within the GIT where barrier function is compromised to the greatest extent during HS in ruminants is unknown. It has been speculated that the postgastric regions are more severely affected compared with the pre-gastric regions based on greater structural vulnerability of the lower GIT and histological alterations detecting mast cell infiltration in the jejunum of heat stressed pigs (Koch et al., 2019). To address the challenge of differentiating regional permeability in ruminants, a novel approach utilizing a dual permeability marker technique was used in the present study to evaluate total-tract and postruminal permeability (Bertens et al., 2022; Bertens et al., 2024). Using the dual marker technique, it was observed that postruminal permeability of the GIT as depicted by Co-EDTA recovery in urine represented 72% of the Cr-EDTA recovery in urine. In this study, HB tended to reduce total-tract permeability by 10% and reduced postruminal permeability by 27%. It is plausible that regulation of intestinal pH may have been improved in the HB treatments via greater abomasal solubilization of $CaMg(CO_3)_2$, partially explained by the 21% reduction in intestinal permeability when expressed as a function of total-tract permeability with HB. Unfortunately, fecal pH was not measured, and therefore we cannot confirm whether fecal pH was affected. However, Rauch et al. (2012) reported greater fecal pH in cows supplemented with $CaMg(CO_3)_2$ when compared with cows fed $NaHCO_3$ and control diets. The reduction in the translocation of Co-EDTA across the lower GIT with HB is assumed to imply a reduced probability that other large molecules, bacteria, and endotoxins may cross the epithelium and enter systemic circulation. Recognition of foreign bodies by immune cells within the GIT epithelia, lymphatic, and circulatory systems elicits local and systemic proinflammation to facilitate activation of a robust immune response (Ceciliani et al., 2012). The systemic response to inflammation is activated by the synthesis and release of acute phase proteins (**APP**) in the liver such as SAA (Cray et al., 2009; Trela et al., 2022). Despite improvements in postruminal barrier function with HB, we did not detect a corresponding reduction in systemic inflammation as represented by SAA. The release of APP is a nonspecific response to inflammation and APP have a short half-life (24 to 48 h) in blood following stimulation (Gruys et al., 2005). Hence, the use of a single APP as an indicator of systemic inflammation, as in the present study, may diminish the ability to detect differences in response to a nutritional intervention strategy.

Immune cells use glucose as their primary energy substrate once activated (Palsson-McDermott and O'Neill, 2013). Therefore, during HS-mediated immune activation, glucose is repartitioned toward immune cells and away from the mammary gland thereby limiting nutrient availability for milk synthesis (Kvidera et al., 2017). Rhoads et al. (2009) reported that heat stressed cows secreted 200 g to 400 g less lactose than their thermoneutral counterparts. In the present study, cows consuming HB secreted 45 ± 26.0 g more lactose in milk than cows fed LB. This outcome of greater milk lactose could be linked to the reduction in postruminal permeability, resulting in more glucose available for milk synthesis; however, there was no evidence of altered systemic inflammation in the present study. The increased lactose concentrations and the coinciding greater osmotic pressure of milk (Wheelock et al., 2010) may have driven the numerical increase in milk yield of 0.8 ± 0.50 kg with HB. Given that HB decreased milk fat by 0.11 ± 0.044 percentage units without an effect on milk fat yield or ECM, it is likely that the reduced milk fat concentration was a result of dilution driven by numerically greater milk yield. Additionally, the lack of low ruminal pH in this study likely limited the ability for HB to modulate ruminal pH having minimal positive effects on milk fat synthesis associated with stabilization of ruminal pH (Allen, 1997).

CONCLUSIONS

Provision of dietary buffer and increased DCAD do not seem to interact to affect DMI, ruminal fermentation, GIT permeability, and milk and milk component yields in lactating dairy cattle exposed to mild HS with night cooling. However, increasing DCAD from +17.5 to +39.6 mEq/100 g of DM through provision of Na-acetate

improved blood acid-base balance and increased milk fat percent and yield. Despite the lack of low ruminal pH in the present study, dietary $CaMg(CO_3)_2$ reduced postruminal permeability and tended to reduce total GIT permeability. These findings provide independent opportunities for increased DCAD and provision of dietary buffer to modulate physiological and performance responses of lactating dairy cows under HS. Future studies incorporating a thermoneutral control are required to corroborate whether increased DCAD and dietary buffer supply alleviate production losses associated with HS and to elucidate the specific mechanisms involved.

NOTES

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Nonstandard abbreviations used: ACCC = added acid consuming capacity; aNDFom = ash-free neutral detergent fiber; APP = acute phase protein; CVAS = Cumberland Valley Analytical Services; FA = fatty acid; FAME = fatty acid methyl esters; GIT = gastrointestinal tract; HB = high buffer diet; HD = high DCAD diet; HS = heat stress; ICP-MS = inductively coupled plasma–mass spectroscopy; LB = low buffer diet; LD = low DCAD diet; NEFA = nonesterified FA; NHS = nonheat stress; PSPS = Pennsylvania State Particle Separator; SAA = serum amyloid A; SCFA = short-chain FA; SRA = subclinical ruminal acidosis; THI = temperature-humidity index; uNDF = undigestible neutral detergent fiber.

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