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Effects of rumen-protected choline supplementation in Holstein dairy cows during electric heat blanket-induced heat stress

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ABSTRACT

Dairy cows experiencing heat stress (HS) attempt to thermoregulate through multiple mechanisms, such as reducing feed intake and milk production and altering blood flow to increase heat dissipation. Effects of choline on energy metabolism and immune function may yield it a viable nutritional intervention to mitigate negative effects of HS. The primary objective of this experiment was to determine if supplementation of rumen-protected choline during, or before and during, an increased heat load would ameliorate the negative effects of HS on production and immune status. Heat stress was induced via an electric heat blanket model with a 3-d baseline period and 7-d HS period for all cows. Multiparous mid-lactation (208 ± 31 days in milk) Holstein cows were fed the same basal herd diet, blocked by pre-experiment milk yield, and randomly assigned to receive one of the following: (1) no rumen-protected (RP) choline (n = 7); (2) RP choline (60 g/d) via top-dress during the HS period (n = 8); or (3) RP choline (60 g/d) via top-dress during the baseline and HS periods (n = 8). Imposing HS via electric heat blanket raised respiration rate with all cows surpassing the HS threshold of 60 breaths/min. The increase in respiration rate tended to be ameliorated with either schedule of RP choline supplementation. Milk yield tended to increase when RP choline was supplemented in both the baseline period and during HS. Supplementation of RP choline tended to reduce blood fatty acid and triglyceride and tended to increase the revised quantitative insulin sensitivity check index. The role of RP choline supplementation to partially ameliorate the effects of HS should be further explored as a potential nutritional strategy to mitigate the negative consequences of HS on health and production.

Key words: heat blanket, choline, heat abatement, hyperthermia, nutritional strategy

INTRODUCTION

Heat stress (**HS**) reduces productivity in dairy cattle directly and indirectly via reduced DMI and physiological adaptations (Baumgard and Rhoads, 2013). Even with advances in heat abatement practices, production is still negatively affected by HS (Bernabucci et al., 2014; Ouellet et al., 2019). The total annual economic losses for the US dairy industry were estimated at $1.5 billion in 2003 (St-Pierre et al., 2003) and even with implementing optimal heat abatement strategies, St-Pierre et al. (2003) predicted an $897 million effect of HS. These losses highlight the need for practical strategies to alleviate the financial burden of HS on dairy farms.

The mechanism of action for HS to affect productivity may provide insights into potential nutritional strategies. Baumgard and Rhoads (2013) estimated that 35 to 50% of the reduction in milk yield is explained by reduced intake, suggesting that other effects of HS, including the energetically expensive immune system activation, may also contribute to the negative effect on productivity. Dairy cows under HS may alter blood flow away from internal organs to reduce heat load (McGuire et al., 1989; Hall et al., 2001), but this can damage the gastrointestinal barrier by increasing permeability and allowing LPS to leak into circulation (Hall et al., 2001; Lambert et al., 2002; Pearce et al., 2013). The inflammatory response in part explains the altered state of energy metabolism during HS including hypoglycemia, hyperinsulinemia, and absence of the typically observed increase in circulating fatty acids (**FA**) supply during a state of negative energy balance (Rhoads et al., 2009; O'Brien et al., 2010; Wheelock et al., 2010). Thus, a nutritional intervention that ameliorates the immune response associated with HS could improve the health and productivity of HS animals. Several nutritional approaches have been suggested to potentially mitigate inflammation in dairy cattle.

Given the effect of HS on inflammation and energy metabolism, and the role of rumen-protected (**RP**) choline to influence lipid and energy metabolism and

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mitigate inflammatory response (McFadden et al., 2020), supplementation of RP choline during heat challenge is of interest as a potential mitigation strategy. Focus on RP choline feeding in dairy cows has primarily been during the transition to lactation period with emphasis on the role in preventing liver lipid accumulation through very-low density lipoprotein export; however, recent research has demonstrated it mitigates inflammation and improves immune function in transition dairy cows (Zenobi et al., 2020), improves immune function in calves (Zenobi, 2018), and alters the immune cell responses to LPS ex vivo (Garcia et al., 2018). Furthermore, connections have been made between lipid metabolism and intestinal function with choline deficiency, demonstrated to lead to impairments in intestinal morphology and lipid metabolism (da Silva et al., 2015), and evidence that chylomicrons can protect against endotoxin-induced lethality (Harris et al., 1993) in rodents. Although choline has not been examined as a nutritional intervention during HS in dairy cows, supplementation of RP choline to buck goats during the hot season improved growth and feed conversion (Habeeb et al., 2017). We hypothesized that RP choline supplementation during HS would improve milk energy output through the mitigation of the HS-associated immune response and altered energy metabolism. Additionally, we wanted to determine if supplementation was required before and during, or only during the heat insult, for any potential effects to be observed. On-farm, 2 strategies would be realistic: either feeding RP choline during the heat insult or feeding RP choline a few days before a forecasted heat insult. Therefore, we implemented 2 practical RP choline supplementation strategies using an electric heat blanket (**EHB**) model to test our hypothesis: (1) supplementation of RP choline exclusively during a period of HS or (2) supplementation of RP choline beginning 3 d before and during a period of HS.

MATERIALS AND METHODS

Animal Use, Treatments, and Handling

All animal use and handling protocols were approved by the University of Wisconsin-Madison College of Agricultural and Life Sciences Animal Care and Use Committee. Twenty-three multiparous (mean ± SD: 2.7 ± 0.4 parity), pregnant, lactating Holstein cows (mean ± SD: 44.3 ± 5.9 kg/d milk yield; 208 ± 31 DIM; 800.9 ± 58.0 kg of BW) were housed in individual tie stalls at the University of Wisconsin-Madison Dairy Cattle Center (Madison, WI) in the spring of 2019. The dairy uses an evaporative cooling system without fans or sprinklers directly on the cows. Cows were milked twice daily (0500 and 1700 h) in a parlor and fed a TMR once daily (0800 h).

An increased heat load was applied to all cows by a previously described HS protocol (Al-Qaisi et al., 2018; Pate et al., 2020) using an EHB (Thermotex Therapy Systems Ltd. Calgary). Our experiment consisted of 3 d of baseline measurements followed by 7 d of HS. Cows were enrolled in 3 blocks that were balanced by pre-experiment milk yield: 9 cows in block 1 (milk yield mean ± SD: 41.0 ± 5.4 kg/d); 5 cows in block 2 (42.1 ± 3.3 kg/d); and 9 cows in block 3 (48.8 ± 4.7 kg/d). Within each block, cows were randomly assigned to 1 of 3 treatments (**TRT**). Cows assigned to the control group (**CTL**; n = 7) were fed a basal diet with no RP choline throughout the trial. Cows assigned to the RP choline TRT groups were fed the same diet as CTL but with 17.3 g of supplemental choline chloride by top-dressing 60 g of RP choline product (ReaShure, Balchem Corp.) daily during baseline and HS periods (**RPC+**; n = 8) or HS period only (**RPC**; n = 8). It has been estimated that this RP choline formulation will allow 60 to 80% of the choline in the product to escape ruminal degradation (Brusemeister and Sudekum, 2006; Elek and Husvéth, 2007), whereas almost all unprotected choline would be degraded in the rumen (Sharma and Erdman, 1988). All 3 TRT were represented in all 3 blocks and had equal representation in blocks 1 and 3. In block 2, a cow died before enrollment, and a suitable replacement could not be reasonably enrolled; therefore, block 2 included 1 CTL cow, 2 RPC cows, and 2 RPC+ cows.

The total experimental period was from April 8 to May 17, 2019, and the 3 blocks were April 8 to 26, April 16 to May 4, and April 29 to May 17 for blocks 1, 2, and 3, respectively. To maintain the consistent chemical composition of the basal diet throughout the experiment, the inclusion rate of forages was adjusted, and dry ground corn was added partway through the experiment to compensate for an unavoidable change in corn silage. The basal diet was formulated using the Cornell Net Carbohydrate and Protein System software V6.5.5 with the formulation and chemical composition presented in Table 1. The estimated supply of MP was 2,645 g/d based on the average baseline intake of cows in the experiment (24.48 kg). The estimated metabolizable supplies of lysine and methionine were 6.17 and 2.13% of MP, respectively, resulting in a 2.90 lysine-to-methionine ratio.

Cows were housed in thermoneutral conditions [daily mean ± SD: 15.9 ± 2.6°C; 51.8 ± 9.4% relative humidity; 59.8 ± 3.4 temperature-humidity index (**THI**)] with temperature and relative humidity recorded every 10 min using a data logger (EasyLog EL-USB-2-LCD+,

Table 1. Formulation and composition of the basal diet

Item	Mean	SD
Ingredient offered, % of diet DM		
Corn silage	27.0	8.5
Alfalfa silage	23.4	1.3
Cottonseed	6.0	1.4
Concentrate[1]	37.7	0.1
Cracked corn	5.9	8.4
Nutrient composition		
DM, %	56.2	2.6
CP, %	17.0	0.0
ADL, %	3.5	0.1
NDF, %	25.2	2.0
Starch, %	30.5	3.3
Ether extract, %	5.0	0.2
Ash, %	7.2	0.6
NFC, %	45.6	1.7
NE_L 3×, Mcal/kg of diet DM	1.70	0.02

[1]Concentrate was formulated to contain (% as-fed): 53.13% ground shell corn; 21.25% canola meal; 7.5% distillers grain; 7.5% exceller meal; 3.75% soy hulls; 2.63% calcium carbonate; 1.88% sodium bicarbonate; 0.88% mineral mix [85.50% NaCl; 2.85% ZnO; 2.38% MnO; 1.90% $CuSO_4$; 1.90% Rumensin 90 (Elanco Animal Health); 0.95% mineral oil; 0.95% Se (0.8%); 0.95% biotin (2%); 0.48% $FeSO_4$; 9,480 IU/kg vitamin E; 1,896 kIU/kg vitamin A; 379 kIU/kg vitamin D_3; 0.05% ethylenediamine dihydroiodide (79.5%); 0.01% Co]; 0.75% MaxFat (Sanimax); 0.45% urea (46%); 0.3% magnesium oxide (54%).

Lascar Electronics Inc.). The power cord of each EHB was hung from above the cow using a mounted cord reel with automatic rewind to minimize restrictions to cow behavior. Blankets were put on cows at 0700 h on d 4 of the experiment and remained on the cows until the end of the HS period. The EHB were unplugged for approximately 1 h during milking in the parlor (total of 2 h/d). All blankets were kept at the same setting (off, low, or high), which was chosen by the research team and herd veterinarian based on the physical response of each individual cow (e.g., panting, open-mouth breathing, drooling) to the heat insult to maintain an excessive heat load as the environmental temperature fluctuated. For example, when cows began open-mouth breathing with an extended tongue and excessive drooling, the EHB were turned down one setting (e.g., high to low; low to off) until the behavior subsided. Cows were monitored for these signs daily during the HS period at 0600, 1300, and 1800 h. Blankets were kept at the highest setting possible that did not result in open-mouth breathing, with extended tongue, and excessive drooling, as it is a risk factor for fatality (Sullivan and Mader, 2018). The EHB were kept on the high setting for 366 h, the low setting for 48 h, and the off setting for 90 h throughout the entire experiment.

Sample Collection and Analysis

Samples of TMR were collected daily and dried by forced air oven at 105°C for 24 h to determine DM content for DMI calculations. Individual TMR ingredients were collected weekly and a subsample was subjected to the same drying procedure to adjust the diet for DM changes. A different subsample of the weekly ingredient samples was dried at 55°C for 48 h and ground to pass a 1-mm screen (Wiley Mill, Arthur H. Thomas). The first 3 wk and the last 2 wk of the study were composited separately, and both composited samples were sent to a commercial laboratory (Dairyland Labs, Arcadia, WI) for subsequent analysis (Table 1). The analysis included CP (AOAC International, 2012, method 990.03), ADF (AOAC International, 1996, method 973.18), NDF (AOAC International, 2005, method 2002.04), lignin (AOAC International, 1996, method 973.18), ether extract (AOAC International, 2012, method 920.39), ash (AOAC International, 2012, method 942.05), and water-soluble carbohydrates (Deriaz, 1961); starch was determined according to the modified procedure from Bach Knudsen (1997) in which glucose was analyzed by YSI 2700 (YSI Biochemistry Analyzer, YSI Inc.).

Milk yield was recorded at each of the 2 daily milkings throughout the experiment. The HS period began after the morning milking on d 4 of the experiment; therefore, throughout the study, the p.m. milking and the subsequent day's a.m. milking were paired to represent a milking day. Feed offered and refused was weighed and recorded daily to determine DMI. Milk samples from the last 4 milkings of each period were collected and preserved with 2-bromo-2-nitropropane-1,3-diol (Advanced Instruments Inc.) for analysis of milk composition and SCC. Analysis of milk samples was performed at a commercial laboratory (AgSource, Menominee, WI) for milk composition of fat, protein, lactose, and MUN by Fourier transform infrared spectrometry using the FOSS MilkoScan FT6000 (FOSS Analytical) and milk SCC by Fossomatic FC (FOSS Analytical). The daily component yield was calculated by multiplying the respective milk composition and yield of each milking and summing component yields of p.m. milking and subsequent a.m. milking.

Rectal temperature was determined using a digital thermometer (Cotran Corporation) and the respiration rate was determined by counting flank movements for 15 s and multiplying by 4 to determine breaths per minute; both variables were recorded daily at 0600 and 1800 h. Body weight was measured at 0800 h on d 1 and 11 via a stationary scale calibrated before initiation of the experiment. Blood samples were collected from the coccygeal vessel at 0600 h on d 1 and 11 in tubes containing potassium oxalate and sodium fluoride (BD Vacutainer) for plasma isolation and in tubes without additives (BD Vacutainer) for serum isolation. Plasma and serum were separated from whole blood as follows: plasma tubes were kept on ice until plasma was

isolated by centrifugation at 2,500 × *g* at 4°C for 15 min, and serum tubes were kept at room temperature until serum was isolated by centrifugation at 3,500 × *g* at 15°C for 15 min. Serum aliquots were stored at −20°C. Serum was used for quantification of aspartate aminotransferase (**AST**) and alanine aminotransferase (**ALT**) activities; and quantification of albumin, BUN, BHB, and triglyceride (**TG**) concentrations. Additional serum aliquots were stored at −80°C for quantification of insulin, tumor necrosis factor α (**TNFα**), and LPS-binding protein (**LBP**) concentrations. Plasma aliquots were stored at −20°C for quantification of glucose and FA concentrations. The activity of AST (Catachem; Product no.: V154–0A) and ALT (Catachem, V164–0A), as well as the concentration of albumin (Catachem, V244–12) and BUN (Catachem, V264–12), were quantified using the Catachem Chemwell-T analyzer (Catachem) utilizing the specified Catachem reagents as previously described (Caputo Oliveira et al., 2020). Concentrations of serum BHB (Catachem, V444–0B), plasma glucose (Catachem, C124–12), serum TG (Catachem, V116–12), and plasma FA (Catachem, V514–0B) were also quantified using the Catachem Chemwell-T analyzer utilizing the specified Catachem reagents as previously described (Pralle et al., 2021). Serum insulin (Mercodia Immunoassays and Services; 10–1201–01) and TNFα (Cusabio Technology LLC; CSB-E12020B) concentrations were quantified using bovine ELISA kits according to the manufacturer's protocol. Serum LBP concentration was quantified using the LBP, various species ELISA kit (Hycult Biotech, Uden, The Netherlands; HK503) as directed in the manufacturer's protocol. Two cows (1 CTL and 1 RPC+) had hemolyzed blood samples on d 11 and thus were excluded from the analysis of blood analytes. Interassay coefficients of variation were 6.3, 0.9, 1.1, 1.0, 1.7, 1.7, 8.4, 18.8, 5.2, 6.6, and 6.6% for ALT, AST, albumin, BHB, BUN, glucose, insulin, LBP, FA, TG, and TNFα, respectively. Intra-assay coefficients of variation were all <10%.

Calculations and Statistical Analysis

Body surface area was calculated using the equation body surface area = $0.14 \times BW^{0.57}$ (Brody, 1945), as it is preferable for estimating the body surface area of Holstein cows (Berman, 2003). Body surface areas were ordered sequentially and categorized as lowest third (mean ± SD: 9.5 ± 0.1 m^2; 8 cows), middle third (9.9 ± 0.2 m^2; 8 cows), or maximal third (10.4 ± 0.3 m^2; 7 cows) for use as a covariate in data analysis. The formula for calculating ECM was ECM, kg/d = [0.3246 × milk yield (kg/d)] + [12.86 × fat yield (kg/d)] + [7.04 × protein yield (kg/d)] (Bernard, 1997; Tyrrell and Reid, 1965). Feed efficiency (**FE**) was calculated as kg of milk yield/kg of DMI or kg of ECM yield/kg of DMI. Insulin units were converted (Abuelo et al., 2012) and a revised quantitative insulin sensitivity check index (**RQUICKI**; Holtenius and Holtenius, 2007) was calculated as follows: RQUICKI = 1/[log(plasma glucose mg/dL) + log(serum insulin μIU/mL) + log(plasma FA mmol/L)].

All response variables were analyzed using the mixed procedure in SAS 9.4 (SAS Institute, Inc.). To account for differences in individual cow production before the start of the experiment, milk yield averaged over the week before the experiment start and the milk components from the previous milk test were used as a covariate, after ensuring they were not different by assigned treatment (milk yield, $P = 0.92$; milk components, $P > 0.29$). For BW and blood metabolites, values from the first day of the experiment, before the start of treatment, was used as a covariate. The linear predictor for milk yield, milk composition variables, DMI, FE measures, BW, respiration rate, and rectal temperature variables included the fixed effects of parity (milk yield, milk composition variables, DMI, FE measures, and BW) or body surface area (respiration rate and rectal temperature), TRT, period, the interaction of TRT and period, and the random effects of block and cow nested within TRT. The linear predictor for blood variables included the fixed effects of body surface area and TRT, and the random effects of block and cow nested within TRT.

The covariance of repeated measures was also modeled. Repeated measures were days or samples measured over time within a cow. The final covariance structure was selected independently for each variable by testing all possible structure types and choosing the structure resulting in the lowest Akaike information criterion. Possible structure types included first-order autoregressive, compound symmetry, unstructured, Toeplitz, variance components, first-order heterogeneous autoregressive, heterogeneous compound symmetry, and heterogeneous Toeplitz. The structure most often selected was first-order autoregressive.

Homogeneity of residual variances was analyzed using a "fitted values versus studentized residuals" plot and plots of different model effects versus studentized residuals. If heterogeneity was suspected, covariance structures were modeled separately by model effects and remained in the final model when the Bayesian information criterion was improved. Studentized residuals were analyzed for normality utilizing the univariate procedure's (SAS 9.4) Shapiro-Wilk test and by visual interrogation of a Q-Q plot, to ensure the assumptions of mixed models were reasonably met. Potential

outliers were detected for ECM yield-based FE, and models were run with and without outliers. The results obtained did not differ between models, so the model without outliers that met the model assumptions for residuals was used for final analysis. The following data transformations were used to meet model assumptions: lactose yield, SCC, and insulin were $\log_{10}$ transformed.

Preplanned contrasts were used to analyze differences between TRT, including CTL versus RPC and RPC+, and RPC versus RPC+. Means were compared when $P \leq 0.1$ for main effects. Post hoc contrasts were adjusted by multiplying the P-value by the number of contrasts, and mean comparisons for main effects were Bonferroni adjusted to prevent inflation of the type I error due to multiplicity (Bello and Renter, 2018). Evidence in mean comparison tests was considered significant if $P \leq 0.05$ and a tendency when $0.05 < P \leq 0.1$. Data are provided as least squares means with 95% confidence interval limits, back-transformed when applicable, and presented as least squares means (lower confidence limit, upper confidence limit).

RESULTS AND DISCUSSION

Confirmation of Electric Heat Blanket Induction of Heat Stress

Across all treatments, the EHB raised ($P < 0.01$) respiration rate by 25.2 breaths/min [LSM (lower 95% CI limit, upper 95% CI limit): 41.7 (31.6, 51.8) vs. 66.9 (56.6, 77.1) breaths/min, baseline vs. HS]. All cows reached the respiration rate threshold for HS of 60 breaths/min (Toledo et al., 2020) within 60 h of wearing the EHB. The hyperthermic respiration rate response was observed within 12 h as the average respiration rate at 1800 h on the first d of HS was about 64 breaths/min. Respiration rates in other EHB studies have ranged from 50 to 96 breaths/min, and change in respiration rate observed in this experiment was similar to 2 other EHB experiments (+27 and +13.7 breaths/min, respectively; Al-Qaisi et al., 2018; Pate et al., 2020) but less than another (+42 breaths/min; Al-Qaisi et al., 2020). There was no evidence that the EHB increased ($P = 0.21$) rectal temperature compared with baseline [38.4 (38.3, 38.5) vs. 38.4 (38.3, 38.6) °C, baseline vs. HS] compared with temperatures reported in other EHB experiments (38.5 to 40.2°C; Pate et al., 2020; Al-Qaisi et al., 2018, 2020).

Across all treatments, DMI decreased with EHB-induced HS [25.2 (14.8, 35.6) vs. 24.0 (14.2, 33.8) kg/d, baseline vs. HS]. The 1.2-kg decrease in DMI observed in the current study was not as severe as in previous experiments (−6.0, −12.9, and −3.2 kg, respectively; Al-Qaisi et al., 2018, 2020; Pate et al., 2020). Across all treatments, milk yield decreased ($P < 0.01$) by 1.3 kg during EHB-induced HS [38.0 (35.5, 40.5) vs. 36.7 (34.0, 39.4) kg/d, baseline vs. HS]. The average milk yield decrease during the HS period in this experiment was greater than one EHB experiment (−0.9 kg; Pate et al., 2020) but less than 2 others (−4.1 and −12.1 kg, respectively; Al-Qaisi et al., 2018, 2020). The EHB also decreased ($P < 0.01$) milk fat yield by 0.12 kg/d, ECM yield by 3.2 kg/d, and 3.5% FCM yield by 3.2 kg/d across treatments. Likewise, EHB decreased ($P < 0.01$) milk protein, lactose, and SNF yield by 0.1, 0.15, and 0.27 kg/d, respectively.

Decreased milk yield, along with raised respiration rate are hallmarks of naturally occurring HS (Kadzere et al., 2002; Baumgard and Rhoads, 2013) and EHB experiments (Al-Qaisi et al., 2020; Pate et al., 2020). These responses observed in this experiment suggest HS was successfully induced; however, the severity of HS may be different when compared with other EHB experiments as evidenced by the variation in the magnitude of responses between experiments. The EHB used in this experiment covered an estimated 50% of the cows (Al-Qaisi et al., 2018) and did not cover the abdomen, neck, head, or posterior, which are exposed to the thermal environment of the barn, allowing for heat exchange between the cow and environment. The average daily thermal environment in the barn during this experiment was 60 THI compared with other EHB experiments that had thermal environments of 66 (Al-Qaisi et al., 2018), 70 (Al-Qaisi et al., 2020), and 61 (Pate et al., 2020) THI, respectively. The EHB was not removed for milking but was disconnected from power 2 times/d for milking in this experiment and 3 times/d by Pate et al. (2020), compared with continuous heating by Al-Qaisi et al. (2018, 2020) where cows were housed and milked in box stalls. Therefore, the thermal environment of the barn and whether or not the EHB is disconnected from power for milking may be determinants of the severity of HS achieved via EHB. The heat applied to cows by EHB was reduced when cows were showing signs of extreme HS, and this may have resulted in a milder state of HS. The successful induction of HS allows for proper interrogation of the potential effects of RP choline supplementation during HS.

Effects of Rumen-Protected Choline Supplementation

The effects of nutritional treatment during baseline and EHB-induced HS on production are shown in Table 2. There was an interaction ($P = 0.01$) of TRT and period on respiration rate with a tendency for reduced ($P = 0.09$) respiration rate during the HS period in both RP choline treatments compared with control. The in-

Table 2. Estimated mean and associated 95% CI for milk yield, corrected milk yield, milk component yield, milk composition, DMI, feed efficiency, respiration rate, rectal temperature, and BW in dairy cows treated with control or 2 rumen-protected choline (RPC) feeding strategies during a 3-d baseline period and subsequent 7-d heat stress period induced by electric heat blanket

							P-value				
	Control		RPC[1]		RPC+[2]		Main effect[3]			Contrast[4]	
Item	Baseline	Heat stress	Baseline	Heat stress	Baseline	Heat stress	T	P	T × P	a	b
Milk yield, kg/d	36.4 (33.7, 39.0)	35.7 (33.1, 38.3)	37.8 (35.3, 40.2)	35.9 (33.5, 38.3)	40.0 (37.5, 42.5)	38.5 (36.1, 41.0)	0.08	<0.01	0.51	0.24	0.08
Corrected milk yields											
ECM,[5] kg/d	39.8 (36.6, 43.0)	35.5 (32.4, 38.7)	39.5 (36.6, 42.4)	36.7 (33.8, 39.6)	42.5 (39.5, 45.5)	39.9 (36.9, 42.9)	0.15	<0.01	0.43	0.13	0.12
3.5% FCM, kg/d	40.9 (37.2, 44.6)	36.2 (32.5, 40.0)	40.2 (36.8, 43.7)	38.0 (34.6, 41.4)	43.7 (40.1, 47.2)	40.9 (37.3, 44.4)	0.23	<0.01	0.25	0.14	0.23
Milk component yields											
Fat, kg/d	1.52 (1.37, 1.66)	1.33 (1.19, 1.47)	1.52 (1.38, 1.65)	1.45 (1.31, 1.58)	1.57 (1.43, 1.71)	1.46 (1.32, 1.60)	0.53	<0.01	0.26	0.12	0.88
Protein, kg/d	1.21 (1.08, 1.33)	1.10 (0.98, 1.22)	1.19 (1.07, 1.31)	1.08 (0.96, 1.20)	1.24 (1.12, 1.36)	1.16 (1.04, 1.29)	0.41	<0.01	0.82	0.67	0.16
Lactose, kg/d	1.81 (1.47, 2.24)	1.63 (1.32, 2.01)	1.80 (1.45, 2.24)	1.64 (1.32, 2.03)	1.88 (1.52, 2.33)	1.78 (1.43, 2.20)	0.39	<0.01	0.57	0.40	0.15
SNF, kg/d	3.37 (2.85, 3.89)	3.07 (2.55, 3.59)	3.36 (2.84, 3.89)	3.07 (2.54, 3.59)	3.51 (2.98, 4.03)	3.30 (2.77, 3.82)	0.42	<0.01	0.77	0.46	0.19
Milk composition											
Fat, %	4.09 (3.79, 4.39)	3.93 (3.63, 4.23)	4.10 (3.82, 4.38)	4.29 (4.01, 4.57)	3.97 (3.69, 4.25)	3.89 (3.61, 4.18)	0.22	0.86	0.19	0.32	0.04
Protein, %	3.23 (3.08, 3.39)	3.22 (3.07, 3.38)	3.18 (3.02, 3.33)	3.13 (2.97, 3.28)	3.15 (3.00, 3.30)	3.13 (2.98, 3.29)	0.19	0.31	0.76	0.07	0.96
Lactose, %	4.86 (4.78, 4.93)	4.82 (4.74, 4.89)	4.82 (4.75, 4.90)	4.80 (4.73, 4.87)	4.89 (4.82, 4.97)	4.87 (4.80, 4.95)	0.28	0.13	0.89	0.62	0.14
SNF, %	8.98 (8.85, 9.11)	8.95 (8.82, 9.08)	8.92 (8.81, 9.04)	8.86 (8.74, 8.97)	8.95 (8.83, 9.08)	8.92 (8.80, 9.05)	0.58	0.12	0.80	0.40	0.41
MUN, mg/dL	11.45 (8.69, 14.21)	11.34 (8.59, 14.10)	11.45 (8.66, 14.24)	11.53 (8.74, 14.32)	10.58 (7.77, 13.38)	11.49 (8.69, 14.29)	0.14	0.75	0.34	0.45	0.90
SCC, cells/1000/mL	89.1 (48.5, 163.9)	93.6 (50.9, 172.0)	59.4 (33.2, 106.4)	91.7 (51.4, 163.8)	50.1 (27.8, 90.2)	73.1 (40.6, 131.6)	0.35	0.03	0.39	0.63	0.47
DMI, kg	24.6 (21.3, 28.0)	23.5 (20.0, 27.1)	26.2 (23.1, 29.3)	23.9 (20.6, 27.3)	24.8 (21.7, 27.9)	24.5 (21.1, 27.8)	0.81	<0.01	0.08	0.62	0.71
Feed efficiency											
Milk yield/DMI[6]	1.55 (1.32, 1.79)	1.63 (1.40, 1.86)	1.51 (1.30, 1.73)	1.53 (1.32, 1.74)	1.61 (1.39, 1.84)	1.63 (1.42, 1.85)	0.76	0.06	0.42	0.72	0.47
ECM/DMI[7]	1.67 (1.49, 1.85)	1.72 (1.54, 1.90)	1.63 (1.46, 1.80)	1.68 (1.51, 1.84)	1.75 (1.58, 1.93)	1.72 (1.54, 1.89)	0.74	0.60	0.58	0.82	0.72
Respiration rate[8]	41.0 (32.0, 50.0)	71.8 (62.8, 80.7)	42.6 (34.1, 51.0)	63.3 (54.9, 71.7)	41.5 (33.1, 50.0)	65.6 (57.2, 73.9)	0.71	<0.01	0.01	0.09	0.62
Rectal temperature, °C	38.4 (38.2, 38.6)	38.5 (38.2, 38.7)	38.4 (38.2, 38.6)	38.4 (38.1, 38.6)	38.4 (38.2, 38.6)	38.4 (38.2, 38.7)	0.85	0.21	0.46	0.55	0.66
BW, kg	797.9 (774.3, 821.5)	787.9 (764.5, 811.4)	787.4 (763.7, 811.2)	784.1 (760.4, 807.9)	800.5 (777.0, 824.1)	787.6 (764.2, 811.0)	0.48	0.01	0.42	0.79	0.67

[1]Cows fed RPC during 7 d of heat stress.

[2]Cows fed RPC during 3-d baseline and 7 d of heat stress.

[3]Main effect: T = the effect of treatment; P = the effect of period (i.e., 3-d baseline period or 7-d heat stress period); T × P = interaction of T and P effects.

[4]Preplanned contrasts: a = control vs. rumen-protected choline (the comparison of any rumen-protected choline supplementation vs. no rumen-protected choline); b = RPC vs. RPC+ (the comparison of RPC supplementation methods).

[5]ECM was calculated as follows (Bernard, 1997): ECM kg/d = (0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield).

[6]Feed efficiency measured as kg of milk yield divided by kg of DMI.

[7]Feed efficiency measured as kg of ECM yield divided by kg of DMI.

[8]Breaths per minute.

teraction of TRT and period tended ($P = 0.08$) to effect DMI, although the contrasts were not significant ($P > 0.62$). Supplementation with RP choline did not alter ($P \geq 0.46$) rectal temperature.

The main effect of TRT tended to increase ($P = 0.08$) milk yield, even during the baseline period in the current study. Dry matter intake and other production parameters were not affected ($P \geq 0.14$) overall by TRT. While not an objective of this study, it is intriguing that supplementation with RP choline increased milk yield in a 3-d period. The observation in this experiment of RP choline's ability to exert its effects within a short period of time are not unique. Pregnant, nonlactating Holstein cows supplemented with RP choline for short periods during or after, feed restriction to induce fatty liver had decreased FA mobilization and altered hepatic lipid metabolism (Cooke et al., 2007). Similarly, supplementation during a 5-d baseline period and a 10-d feed restriction period in pregnant, nonlactating Holstein cows was shown to alter hepatic glucose and lipid metabolism, even before feed restriction (Zenobi et al., 2018). While first being explored in dairy cattle nutrition, it was found that abomasal infusion of choline (40–50 g/d) increased milk yield by 3.2 and 1.6 kg/d in primiparous and multiparous cows (64–150 DIM average), respectively, without altering DMI or milk composition in 2 out of 3 experiments (Sharma and Erdman, 1989). Likewise, a subsequent study feeding increasing proportions of RP choline from 5 to 21 wk postpartum noted a tendency for a linear increase in milk yield and an increase in 3.5% FCM (2.4 and 1.7 kg/d increase over control with 0.156 and 0.234% dietary RP choline) with choline treatment (Erdman and Sharma, 1991). Both milk fat and protein percent responded quadratically in that experiment but DMI remained unaltering (Erdman and Sharma, 1991). Since that time, supplementation of RP choline in dairy cows has been primarily studied during the transition to lactation period, and it has been suggested that there may not be additional benefits of supplementing RP choline after the postpartum period in a longitudinal transition cow study (Bollatti et al., 2020). Although the effect of RP choline in the current study is over a short period of time (3 d) in 8 cows per RP choline treatment, the main effect tendency herein suggests that supplementation of RP choline during mid-lactation should be further explored.

There was no evidence that RP choline supplementation during HS affected BW, FE, milk composition, or yields of lactose or SNF compared with CTL cows (Table 2). Within the HS period, supplementation of RPC+ tended to ameliorate ($P = 0.08$) the loss of milk yield compared with RPC. Supplementation with either RP choline treatments tended to reduce ($P = 0.07$) milk protein percent during HS although the reduction was less than 0.1% and did not result in a milk protein yield difference. Milk fat percent during HS was reduced ($P = 0.04$) with RPC+ compared with RPC supplementation. Decreased fat percent with maintained component, ECM, and FCM yield in RPC+ cows is likely reflective of dilution, as associated with increased milk yield during HS. There was no evidence of increased FE (milk yield/DMI or ECM/DMI) due to RP choline supplementation (Table 2). It should be noted that changes in DMI and feed efficiency may not have been fully captured during the 7-d HS period or that the crude measures of FE utilized may not have revealed potential changes in the efficiency of nutrient utilization.

Recent work has expanded our understanding of choline's action to include direct influence on inflammation and hepatic oxidation, as well as lipid, glucose, and methyl carbon metabolism (Chandler and White, 2017, 2019; Zenobi et al., 2018, 2020; Chandler et al., 2020; McFadden et al., 2020). It is possible that RP choline improves production, or recovers potential production losses, in the presence of inflammatory challenge during parturition and HS; therefore, blood metabolites related to energy metabolism and inflammation were investigated. Response to HS in dairy cows characteristically involves increased insulin without the increased FA associated with feed restriction alone (Wheelock et al., 2010). Blood concentrations of BHB, BUN, insulin, and glucose responded to EHB similarly between CTL and RP choline-supplemented cows, as presented in Table 3. Both RP choline supplementation strategies tended to reduce ($P = 0.09$) FA and TG. Supplementation with RP choline also tended to increase ($P = 0.06$) RQUICKI after EHB-induced HS. As a relative indicator of insulin function in dairy cows (Holtenius and Holtenius, 2007), these data suggest that RP choline may modulate the effect of HS on lipid metabolism. Interactions of choline and choline-containing lipid moieties with insulin sensitivity are not fully understood and should continue to be elucidated (Zenobi et al., 2018; McFadden and Rico, 2019; McFadden, 2020).

Concentrations of AST and ALT are markers of liver damage and have been associated with ketosis (Du et al., 2017), fatty liver (Sevinc et al., 2001), and HS (Berian et al., 2019). Treatment altered AST concentration (main effect $P = 0.04$), although directionality differed by RP choline treatment (contrast of CTL vs. RP choline $P \leq 0.02$; Table 3). It is unclear why RPC decreased, but RPC+ increased the concentration of AST, relative to CTL; however, the resulting ratio of AST to ALT was decreased by both RP choline supplementation approaches ($P < 0.01$). There was no effect ($P \geq 0.18$) of treatment on TNFα, albumin, or LBP. It

has been previously proposed that the negative effects of HS may be partially explained by the activation of the immune response, which is energetically expensive (Baumgard and Rhoads, 2013). Although not significant, it is striking that LBP was numerically lower in RPC+ cows after EHB-induced HS, given that previous research suggests HS sometimes (Cui et al., 2019; Mayorga et al., 2019; Abuajamieh et al., 2018), but not always (Pearce et al., 2015; Gabler et al., 2018; Mayorga et al., 2018), raises blood LBP concentration. The inflammatory response to endotoxemia is partially facilitated via LBP, an acute-phase protein (Lu et al., 2008). The role of choline and phosphatidylcholine in chylomicron formation can protect against endotoxin-induced lethality, intestinal function, and morphology to support intestinal lipid uptake, and mitigate hepatic inflammation in rodents (Harris et al., 1993; da Silva et al., 2015; van der Veen et al., 2017). Through action in the intestine and liver, choline could provide a potential benefit during HS; however, this was not observed as significant differences in the current experiment. The lack of response on blood inflammatory markers herein could be due to insufficient sample size or be because blood samples were not obtained throughout the HS period and inflammatory markers have been shown to be dynamic over time (Kvidera et al., 2017; Horst et al., 2019). Although choline supplementation has been previously noted to mitigate inflammation and improve immune function in transition dairy cows (Zenobi et al., 2020), improve immune function in calves (Zenobi, 2018), alter the immune cell responses to LPS ex-vivo (Garcia et al., 2018), and reduce reactive oxygen species in primary bovine hepatocytes (Chandler and White, 2017; Chandler et al., 2020), the role of choline to modulate immune function during HS should be further explored.

Although a few studies utilizing EHB are now published, at the time this experiment was planned and conducted, no data from EHB experiments with nutritional interventions had been published. Sample size for the current study was based on estimated possible responses and was limited by the number of EHB available. Given the responses reported herein, a sample size of 20 cows/treatment would be needed to detect mean differences (80% power, $\alpha = 0.05$) in milk yield and 28 cows/treatment to detect mean differences in RQUICKI, as examples. This post hoc analysis highlights that sample size was a limitation in the current study and provides insights to guide future nutritional intervention studies using the EHB model to ensure sufficient power for statistical analysis. The data reported in the current experiment support the potential

Table 3. Estimated mean and associated 95% CI for blood analytes in dairy cows treated with control or 2 rumen-protected choline (RPC) feeding strategies during a 7-d heat stress period induced by electric heat blanket

				P-value[3]	
Item	Control	RPC[1]	RPC+[2]	a	b
BHB, mmol/L	0.57 (0.34, 0.79)	0.59 (0.38, 0.80)	0.50 (0.29, 0.72)	0.83	0.39
BUN, mg/dL	12.78 (9.86, 15.69)	12.37 (9.35, 15.39)	12.89 (9.95, 15.82)	0.85	0.55
Glucose, mg/dL	74.35 (67.11, 81.58)	73.82 (67.32, 80.32)	71.69 (64.90, 78.47)	0.60	0.44
Insulin, ug/L	0.41 (0.26, 0.65)	0.49 (0.33, 0.74)	0.34 (0.22, 0.53)	0.98	0.24
FA,[4] mmol/L	0.20 (0.12, 0.28)	0.12 (0.05, 0.19)	0.11 (0.04, 0.19)	0.09	0.86
RQUICKI[5]	0.50 (0.45, 0.55)	0.53 (0.49, 0.57)	0.58 (0.53, 0.63)	0.06	0.16
TG,[6] mg/dL	14.31 (9.87, 18.74)	12.71 (8.10, 17.31)	11.18 (6.84, 15.52)	0.09	0.25
LBP,[7] mg/L	4.47 (2.51, 6.42)	4.52 (2.98, 6.06)	3.29 (1.59, 4.98)	0.63	0.27
TNFα,[8] ng/mL	0.34 (0.20, 0.47)	0.41 (0.27, 0.55)	0.34 (0.21, 0.48)	0.37	0.18
Albumin, g/dL	4.08 (3.89, 4.28)	4.04 (3.84, 4.24)	4.04 (3.85, 4.23)	0.52	0.97
AST,[9] U/L	116.00 (97.35, 134.66)	107.14 (91.67, 122.61)	124.52 (106.13, 142.92)	0.02	0.26
ALT,[10] U/L	28.34 (25.69, 30.99)	28.61 (26.51, 30.72)	29.97 (27.35, 32.59)	0.54	0.42
AST:ALT	4.27 (3.76, 4.78)	3.86 (3.49, 4.23)	4.08 (3.43, 4.73)	<0.01	0.30

[1]Cows fed RPC during 7 d of heat stress.

[2]Cows fed RPC during 3-d baseline and 7 d of heat stress.

[3]Pre-planned contrasts: a = Control vs. rumen-protected choline (the comparison of any rumen-protected choline supplementation vs. no rumen-protected choline); b = RPC vs. RPC+ (the comparison of rumen-protected choline supplementation methods).

[4]Fatty acids.

[5]The revised quantitative insulin sensitivity check index (Holtenius and Holtenius, 2007) was calculated as follows: RQUICKI = 1/[log(plasma glucose mg/dL) + log(serum insulin μIU/mL) + log(plasma FA mmol/L)].

[6]Triglyceride.

[7]LPS-binding protein.

[8]Tumor necrosis factor α.

[9]Aspartate aminotransferase.

[10]Alanine aminotransferase.

role for RP choline to partially mitigate the effects of HS and warrants future exploration.

CONCLUSIONS

During EHB-induced HS, both RPC supplementation approaches tended to ameliorate the effects of HS, as is evident by partially ameliorating the increase in respiration rate. Compared with supplementation during HS, supplementation of RP choline before and during HS, tended to increase milk yield without altering milk component yield. Supplementation of RP choline tended to decrease FA and TG, while tending to increase RQUICKI, compared with control, suggesting that choline modulated lipid metabolism during HS. The potential for RP choline to influence the response to HS should be further explored as a possible nutritional strategy to mitigate the negative effects of HS on health and production.

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