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Ruminant Nutrition

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Immune and metabolic effects of rumen-protected methionine during a heat stress challenge in lactating Holstein cows

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Abstract

Multiparous, lactating Holstein cows (n = 32) were randomly assigned to one of two dietary treatments [TMR with rumen-protected Met (**RPM**) or TMR without RPM (**CON**)], and within each dietary treatment group cows were randomly assigned to one of two environmental treatment groups in a split-plot crossover design. In phase 1 (9 d), all cows were fed ad libitum and in thermoneutral conditions (**TN**). In phase 2 (9 d), group 1 (n = 16) was exposed to a heat stress (**HS**) challenge (**HSC**). Group 2 cows (n = 16) were pair-fed (**PFTN**) to HSC counterparts and remained in TN. After a 21-d washout period, the study was repeated (period 2) and the environmental treatments were inverted relative to treatments from phase 2 of period 1, while dietary treatments remained the same for each cow. During phase 1, cows in RPM had greater plasma Met concentration compared with cows in CON (59 and 30 μM, respectively; P < 0.001). Cows in PFTN had a greater decrease (P < 0.05) in plasma insulin than cows in HSC at 4 h (–2.7 μIU/mL vs. –0.7 μIU/mL) and 8 h (–7.7 μIU/mL vs. –0.4 μIU/mL) during phase 2. Compared with cows in PFTN, cows in HSC had an increase (P < 0.05) in plasma serum amyloid A (–59 μg/mL vs. +58 μg/mL), serum haptoglobin (–3 μg/mL vs. +33 μg/mL), plasma lipopolysaccharide binding protein (–0.27 and +0.11 μg/mL), and plasma interleukin-1β (–1.9 and +3.9 pg/mL) during phase 2. In conclusion, HSC elicited immunometabolic alterations; however, there were limited effects of RPM on cows in HSC.

Key words: amino acids, heat stress, insulin, mammary gland, methionine

Introduction

Heat stress (**HS**) causes overt negative effects on the performance of dairy cattle, most notably decreased milk yield and milk component yield. In order to acclimate to HS conditions, dairy cattle undergo a number of acute physiological adaptions, including increased evaporative heat loss via elevated respiration rate (Lemerle and Goddard, 1986; Kadzere et al., 2002), increased sweating rate (McLean, 1963), and increased water intake (Schneider et al., 1988; Silanikove et al., 1998). Additionally, dry matter intake (**DMI**) may decrease as an attempt to reduce heat increment incurred by feed digestion and milk production (Maust et al., 1972; Collier et al., 2017). When acute acclimation to HS is achieved, dairy cattle utilize extensive and complex chronic acclimatization techniques, via

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Abbreviations

AA	amino acids
APP	acute-phase proteins
BW	body weight
CON	TMR without RPM
DMI	dry matter intake
EBAL	energy balance
EHB	electric heat blanket
HS	heat stress
HSC	HS challenge
HSP	heat-shock proteins
IAA	indispensable AA
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MIST1	transcription factors
MP	metabolizable protein
MUN	milk urea nitrogen
NEFA	non-esterified fatty acid
PUN	plasma urea nitrogen
PFTN	pair-fed
RPM	TMR with rumen-protected Met
RQUICKI	revised quantitative insulin sensitivity check index
SAA	serum amyloid A
TMR	total-mixed ration
TN	thermoneutral conditions
TNFα	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling
XBP1	x-box binding protein

homeorhetic mechanisms, to reduce HS by improved efficiency of heat dissipation and decreased heat production (Bernabucci et al., 2010). During times of thermoneutral (**TN**) negative energy balance, insulin concentrations are decreased resulting in increased lipid mobilization and subsequent increase in non-esterified fatty acids (**NEFA**) in the circulation (Bauman and Currie, 1980; Drackley, 1999). However, during times of HS-induced negative energy balance, it has been reported that dairy cattle exhibit increased concentrations of insulin and decreased concentrations of NEFA within the blood (Wheelock et al., 2010; Gao et al., 2017). Furthermore, insulin sensitivity may be tissue dependent and certain tissues, such as adipose, can be equally or more responsive to insulin resulting in a net decrease in lipolysis and fatty acid mobilization by adipose tissue during HS conditions (Baumgard and Rhoads, 2013; Xie et al., 2016). This would explain previously reported decreases in NEFA concentration reported in cows in HS conditions (Wheelock et al., 2010; Xie et al., 2016). Meanwhile, skeletal muscle may exhibit decreased insulin sensitivity during HS, eliciting a catabolic response, as seen by increased plasma amino acids (**AA**) concentrations, increased plasma urea nitrogen (**PUN**) concentration, and increased plasma creatinine concentration (Cowley et al., 2015; Gao et al., 2017). These metabolic changes may in part explain a decreased milk yield response, as nutrient partitioning may be moved away from the mammary gland during times of HS.

Methionine is an indispensable AA (**IAA**), and when diets are properly balanced for IAA, increased milk yield and milk component yield have been reported (Schwab et al., 1976; Armentano et al., 1997; Schwab and Broderick, 2017). Additionally, when rumen-protected Met (**RPM**) is utilized in diets of dairy cows under stress conditions (i.e., transition period), milk yield and milk component yield improve. Previous studies have reported improvements in liver function, inflammation, and oxidative stress when RPM was fed during the transition period (Osorio et al., 2013; Zhou et al., 2016b, 2017; Batistel et al., 2017). However, limited research is available on the effects of RPM feeding during HS on previously mentioned hormonal and metabolic biomarkers in blood, or parameters of inflammation and immune activation in blood. Furthermore, HS has been reported to cause adverse effects on mammary epithelial cells, including increased apoptosis, decreased proliferation and differentiation, and increased inflammatory response mechanisms (Collier et al., 2008; Tao et al., 2018; Salama et al., 2019). However, Met supplementation has been shown to reduce adverse effects of mammary epithelial cell exposure to HS in vitro (Han et al., 2015; Salama et al., 2019). Thus, it is plausible that RPM feeding in dairy cattle exposed to HS may improve mammary cell health and reduce inflammation as well. Therefore, the objectives of this study were to evaluate the effects of a commercially available RPM source (Smartamine M; Adisseo Inc., Antony, France) fed at 1.05 g of RPM/kg of DMI on blood metabolites at two time points postfeeding (4 and 8 h), blood inflammatory biomarkers, as well immunohistochemical parameters in mammary tissue harvested from lactating, multiparous Holstein cows during a heat stress challenge (**HSC**). Our hypothesis was that compared with cows in pair-fed TN conditions, cows undergoing an HSC would experience marked changes in metabolic and inflammatory markers in blood and in mammary tissue. Additionally, we hypothesize that feeding RPM may alter some of these changes, particularly in regard to protein metabolism and synthesis in the mammary gland.

Materials and Methods

Animal care and housing

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (no. 18156). The experimental period occurred from September 2018 to December 2018. Average ambient temperature was 16.4 ± 2.5 °C. Cows were housed in a tie-stall barn and had constant access to water (Pate et al., 2020).

Experimental design and HS procedure

Experimental design and HSC procedure have been previously described in depth by Pate et al. (2020). Additionally, a table of the experimental design is included in the Supplementary Table S1. Briefly, a total of 32 multiparous lactating Holstein [days in milk (mean ± SD) = 184 ± 59 d] cows were randomly assigned to one of two dietary treatments [total-mixed ration (**TMR**) with RPM (Smartamine M; Adisseo Inc., Antony, France; 1.05 g of RPM/kg of DMI; ~30 g/d of Met; ~24 g/d of bioavailable Met according to manufacturer's specifications) or TMR without RPM (**CON**)], and within each dietary treatment group cows were randomly assigned to one of two environmental treatment groups in a split-plot crossover design, where dietary treatment was considered whole-plot and environmental treatment was considered subplot. The study was divided into 2 periods

(period 1 and period 2) with three identical experimental phases (adaption phase, phase 1, and phase 2) within each period. Prior to phase 1 of each period there was an adaption phase (7 d) where cows were fed their respective dietary treatment, and no samples were collected. During phase 1 (baseline phase; 9 d), all cows were in thermoneutral conditions (**TN**) and fed ad libitum. During phase 2 (trial phase; 9 d) group 1 ($n = 16$) was exposed to an HSC, where cows were fitted with an electric heat blanket (**EHB**; Pate et al., 2020). EHBs remained on group 1 cows for the entire duration of phase 2. Meanwhile, group 2 ($n = 16$) remained in TN conditions but was pair-fed (**PFTN**) to their HSC counterparts. After a 14-d washout period, the study was repeated (period 2) and the environmental treatments were inverted relative to environmental treatments from phase 2 of period 1, while the dietary treatments (RPM or CON) remained the same as in period 1 for each cow. The same TMR was fed to all cows throughout the experimental period. During phase 1, all cows were fed once daily at 1300 hours. During phase 2, HSC cows were fed once daily at 1300 hours, while calculated TMR allocation for PFTN cows was divided into 2 and offered at 1300 and 2200 hours in order to minimize the potential effect of slug-feeding. The daily RPM allocation (1.05 g of RPM/kg of DMI) for cows in RPM was mixed with 300 g of dry molasses and top dressed onto the TMR immediately after feeding. Cows in CON were administered a top-dress consisting of 300 g of dry molasses only onto the TMR immediately after feeding. The dosage rate of 1.05 g of RPM/kg of DMI allowed for proper AA balancing of the diet based on relevant literature (NRC, 2001; Schwab et al. 2009; Van Amburgh et al. 2009; Schwab 2012). Based on Cornell Net Carbohydrate and Protein System version 6.5 (Cornell University, Ithaca, NY; Van Amburgh et al., 2015) within AMTS.Cattle.Pro version 4.7 (2017, AMTS, LLC, Groton, NY) predictions, cows in RPM received 2.72 kg of metabolizable protein (**MP**) per day; and 2.57% of MP as Met, 7.01% of MP as Lys, and 2.47% of MP as His with a Lys:Met of 2.73 and a His:Met of 0.96; while cows in CON received 2.71 kg of MP per day; and 2.03% of MP as Met, 7.05% of MP as Lys, and 2.49% of MP as His with a Lys:Met of 3.47 and a His:Met of 1.22. Cows in this experiment were calculated to require 2.68 kg/d of MP, 66.7 g/d of Met, 188.1 g/d of Lys, and 62.7g/d of His. Energy balance (EBAL) was calculated using parameters reported by Pate et al. (2020) with the following equation (NRC, 2001): EBAL = energy intake (Mcal of NEL/d) – [maintenance requirement (Mcal of NEL/d) + lactation requirement (Mcal of NEL/d)]. Diet ingredient composition and nutrient analysis data are available by Pate et al. (2020).

Blood sampling and analysis

Blood samples were taken from the coccygeal vein or artery at 4 and 8 h postfeeding (1700 and 2100 hours, respectively) on days 1, 3, 6, and 9 of phase 1 and days 1, 3, and 6 of phase 2 of each period from each cow using three separate collection tubes coated in heparin, ethylenediaminetetraacetic acid, and silica (serum separator tubes), respectively (BD Vacutainer; BD and Co., Franklin Lakes, NJ). Serum and plasma samples were obtained via centrifugation of the tubes at 2,500 × *g* for 15 min at 4 °C and stored at −80 °C until further analysis was conducted. For phase 1 (baseline), serum and plasma from blood samples collected at 4 and 8 h after feeding were pooled by cow for each period (i.e., one sample per cow per time point for phase 1). Heparinized plasma samples were sent to the University of Illinois Veterinary Diagnostic Laboratory to be analyzed for bovine chemistry profiles (PUN, glucose, NEFA, insulin, and albumin) using the AU680 Beckman Coulter analyzer (http://vetmed.illinois.edu/vet-resources/veterinary-diagnostic-laboratory/clinical-pathology/).

Commercially available assay kits were used to analyze plasma samples for serum amyloid A (**SAA**), lipopolysaccharide binding protein (**LBP**), L-lactate, and interleukin-1β (**IL-1β**), and serum samples for haptoglobin. Glucose, PUN, NEFA, and insulin were analyzed in samples taken at 4 and 8 h postfeeding time points. Haptoglobin, SAA, LBP, L-lactate, and IL-1β were analyzed in samples taken at 8 h postfeeding time points only. Plasma SAA was assessed using the Phase Range Multispecies SAA ELISA kit (Tridelta Development, Ltd., Maynooth, Ireland; detection range: 9.4 to 150 μg/mL), following manufacturer's instructions. Plasma LBP was assessed using the Human Lipopolysaccharide Binding Protein Multispecies Reactive ELISA Kit (Cell Sciences, Newburyport, MA; detection range: 5 to 50 ng/mL), following manufacturer's instructions. Plasma L-lactate was assessed using the L-lactate assay kit (Cayman Chemical, Ann Arbor, MI; detection range 25 μM to 1 mM), following manufacturer's instructions. Plasma IL-1β was assessed using Bovine IL-1β ELISA Reagent Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA; detection range: 7.8 to 2,000 pg/mL), following manufacturer's instructions. Serum haptoglobin was analyzed using Cow Haptoglobin ELISA Kit (Life Diagnostics Inc., West Chester, PA; detection range: 3.9 to 250 μg/mL), following manufacturer's instructions. Insulin sensitivity was calculated using the revised quantitative insulin sensitivity check index (**RQUICKI**; Perseghin et al., 2001) using the following equation:

$$\mathrm{RQUICKI} = \frac{1}{\mathrm{logglucose}\ \left(\frac{\mathrm{mg}}{\mathrm{dL}}\right) + \mathrm{loginsulin}\left(\frac{\mu\mathrm{U}}{\mathrm{mL}}\right) + \mathrm{logNEFA}\left(\frac{\mathrm{mmol}}{\mathrm{L}}\right)}$$

Plasma AA analyses were performed on phase 1 pooled samples and day 6 of phase 2 samples for periods 1 and 2. Briefly, plasma was gravimetrically mixed with universally labeled ^{13}C AA (#CLM-1548, Cambridge Isotope Laboratories) and deproteinized by centrifugation in 500 mM perchloric acid. Free AA were derivatized with the EZ:faast kit (#KG0-7165, Phenomenex, Torrance, CA) and analyzed in a liquid chromatography-mass spectrometry system (LCMS-2020, Shimadzu, Kyoto, Japan) according to the EZ:faast kit instructions. All plasma AA analyses were conducted in the Dr Sebastian Arriola Apelo laboratory (University of Wisconsin, Madison).

Mammary tissue biopsies

Mammary biopsies were performed at ~7 h (post-AM milking) on day 9 of phase 2 of each period using a similar technique to that described by Han et al. (2018). Briefly, a 12 gauge × 16 cm biopsy needle (Bard Magnum; C. R. Bard, Inc., Murray Hill, NJ) was used to remove ~300 mg of mammary tissue via six punctures of ~5 cm in depth. Mammary samples (~50 mg) were immediately placed in 1.5 mL 4% paraformaldehyde solution for 24 h before being transferred to 70% ethanol and sent to the University of Illinois Veterinary Diagnostic Laboratory for paraffin embedding.

Immunohistochemistry: apoptosis, proliferation, and differentiation

Mammary gland tissue samples mounted in paraffin were cut into sections of 4 μm thickness and used for the analysis of cell apoptosis, proliferation, and differentiation. Briefly, samples were deparaffinized by 2× submersion in xylenes for 5 min, submersion in a 50:50 solution of xylenes:EtOH for 3 min, 2× submersion in 100% EtOH for 5 min, submersion in 95% EtOH for 3 min, submersion in 85% EtOH for 3 min, submersion in 75% EtOH for 3 min, submersion in 50% EtOH for 3 min, submersion in 0.85% NaCl solution for 5 min, and submersion in PBS for 5 min. Samples were then immersed in fixative (4% paraformaldehyde solution) for 15 min

at 37 °C. The samples were washed in PBS and then incubated for 15 min at 23 °C in 150 μL of proteinase K solution (AM2546; Invitrogen, Thermo Fisher Scientific, Waltham, MA). Samples were then washed with PBS, placed back in fixative for 5 min at 37 °C, and then washed again with PBS. Apoptotic cells of mammary gland tissue were assessed using terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (**TUNEL**), based on DNA fragmentation detection. Slides were incubated using the Click-iT Plus TUNEL Assay (C10617; Invitrogen, Thermo Fisher Scientific, Waltham, MA) reagents following manufacturer's instructions. Bovine mammary tissue incubated with DNase I (EN0521; Invitrogen, Thermo Fisher Scientific) for 30 min was used for protocol validation. Proliferating cells of mammary gland tissue were assessed using immunohistochemical staining for Ki-67 (9129; Cell Signal Technologies, Danvers, MA). Briefly, samples were blocked with 3% bovine serum albumin for 1 h and then incubated for 16 h at 4 °C in the presence of the primary antibody. After washing in PBS, samples were incubated with an appropriate secondary antibody for 1 h at 23 °C. The mammary gland samples were then counterstained for 15 min with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA). After counterstaining, the samples were mounted with Flouromount Aqueous Mounting Medium (Sigma-Aldrich, Co., St. Louis, MO), and cured for 1 h at 23 °C. Slides were then stored at −20 °C until fluorescence evaluation. Differentiating cells of mammary gland tissue were assessed using immunohistochemical staining for transcription factors **MIST1** (PA5-24145; Invitrogen, Thermo Fisher Scientific) and x-box binding protein 1 (**XBP1**; PA5-25010; Invitrogen, Thermo Fisher Scientific). Briefly, samples were blocked with 3% bovine serum albumin for 1 h then incubated for 16 h at 4 °C in the presence of the primary antibody. After washing in PBS, samples were incubated with an appropriate secondary antibody for 1 h at 23 °C. The mammary gland samples were then counterstained for 15 min with Hoechst 33342 (Thermo Fisher Scientific After counterstaining, the samples were mounted with Flouromount Aqueous Mounting Medium (Sigma-Aldrich, Co.), and cured for 1 h at 23 °C. Slides were then stored at −20 °C until fluorescence evaluation.

The samples were evaluated via fluorescence microscopy under a Leica DMR microscope (Leica Microsystems, Concord, ON, Canada), and images were captured on a MicroPublisher 5.0 RTV (Teledyne QImaging, Surrey, BC, Canada) using a 40× objective lens. Total area (pixels^2) of Hoescht 33342 positive cells, apoptotic cells (TUNEL), and proliferating cells (Ki-67 antigen expressing cells) were measured on five subsections of each sample (n = 48) with the image processing software Axiovision 4.9.3 (Carl Zeiss MicroImaging, Oberkochen, Germany), similar to methods by Sivaguru et al. (2019). Intensity of emission for differentiating cells (MIST1 and XBP1 antigen expressing cells) were analyzed on five subsections of each sample (n = 40) with the image processing software Axiovision 4.9.3 (Carl Zeiss MicroImaging, Oberkochen, Germany), similar to methods by Ryan et al. (2020).

Statistical analyses

Data collected from periods 1 and 2 were analyzed using SAS (v. 9.4, SAS Institute Inc., Cary, NC). Blood data during phase 1 (baseline phase) of each period were averaged and used as baseline to calculate paired-difference (Steel et al., 1997) values for each cow based on the difference between phase 1 baseline means and phase 2 (trial phase) values for each blood variable for each period. Glucose, PUN, NEFA, insulin, and RQUICKI paired-differences values were analyzed for two separate time points postfeeding (4 and 8 h), as well as the average of the paired-difference values from 4 and 8 h postfeeding. Albumin, haptoglobin, SAA, LBP, L-lactate, and IL-1β paired-difference values were analyzed for one time point postfeeding (8 h). The MIXED procedure of SAS was used for paired-difference values of blood variables with multiple measurements within the same period, with day of phase 2 as the repeated effect. The model included sequence [sequence by which the cow received environmental treatments (i. e.; HSC in period 1 and PFTN in period 2, or PFTN in period 1 and HSC in period 2)], environment, diet, environment × diet interaction, environment × day interaction, diet × day interaction, environment × diet × day interaction, and period as fixed effects; cow within sequence as a random effect; and day of phase 2 as the repeated effect using the following model:

$$Y_{ijklmn} = \mu + S_j + E_k + T_l + D_m + (ET)_{kl} + (ED)_{km} + (TD)_{lm} + (ETD)_{klm} + P_n + C_{i(j)} + \varepsilon_{ijklmn}$$

where Y_{ijklmn} = the observations for dependent variables; μ = the overall mean; S_j = the fixed effect of the jth sequence; E_k = the fixed effect of the kth environment (TN or HSC); T_l = the fixed effect of the lth diet (RPM or CON); D_m = the repeated measurement (day) effect; $(ET)_{kl}$ = the interaction of environment and diet; $(ED)_{km}$ = the interaction of environment and day; $(TD)_{lm}$ = the interaction of diet and day; $(ETD)_{klm}$ = the three-way interaction of environment, diet, and day; P_n = the fixed effect of the nth period; $C_{i(j)}$ = the random effect of the ith cow within sequence; and ε_{ijklmn} = the random residual error. Least squares means for diet phase 1 baseline are reported in Tables 1–3.

The MIXED procedure of SAS was used for plasma AA and mammary tissue immunohistochemical data with single measurements in each period. The model included sequence, environment, diet, environment × diet interaction, and period as fixed effects; and cow within sequence as a random effect using the following model:

$$Y_{ijklm} = \mu + S_j + E_k + T_l + (ET)_{kl} + P_n + C_{i(j)} + \varepsilon_{ijklm}$$

where Y_{ijklm} = the observations for dependent variables; μ = the overall mean; S_j = the fixed effect of the jth sequence; E_k = the fixed effect of the kth environment (TN or HSC); T_l = the fixed effect of the lth diet (RPM or CON); $(ET)_{kl}$ = the interaction of environment and diet; P_n = the fixed effect of the nth period; $C_{i(j)}$ = the random effect of the ith cow within sequence; and ε_{ijklmn} = the random residual error. The estimation method was restrictive maximum likelihood and the degrees of freedom method was Kenward–Rogers (Littell et al., 2002). Variables were subjected to five covariance structures: compound symmetry, unstructured, autoregressive order 1, autoregressive heterogeneous order 1, and Toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and was utilized in the model (Littell et al., 2002). Normality and homoscedasticity of residuals distribution was evaluated. Apoptotic cell proportion, proliferating cell proportion, MIST1, and XBP1 were log transformed for better normality and homoscedasticity of residuals. Data presented for these variables were back transformed. Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Results

Energy balance

During phase 1, there was no difference for EBAL between cows in RPM and CON (5.92 Mcal/d and 5.61 Mcal/d; P = 0.82). During

Table 1. Least squares means of phase 1 baseline and least squares means of paired differences and associated pooled SEM for plasma AA (µM) of Holstein cows in different environmental treatments (Env): pair-fed thermoneutral (PFTN) or heat stress challenge (HSC), and fed different dietary treatments as top-dress (Diet): rumen-protected Met (RPM) or no RPM (CON) at 8 h postfeeding on day 6 of phase 2 for periods 1 and 2

	Phase 1[1]		Phase 2[2]									P-value[3]		
			Env		Diet		PFTN		HSC					
Variable[4]	CON	RPM	PFTN	HSC	CON	RPM	CON	RPM	CON	RPM	SEM	Env	Diet	Env × Diet
Total AA	2,029	1,992	+214	−93	+34	+87	+162	+265	−95	−91	137	0.03	0.69	0.71
Total branched-chain AA	511	514	+84	−20	+29	+35	+52	+116	+6	−47	51	0.02	0.91	0.17
Total indispensable AA	974	991	+96	−110	−21	+7	+41	+151	−84	−137	76	0.009	0.70	0.27
Arg	103	95	+7	−3	+5	−2	+8	+5	+3	−10	7	0.14	0.34	0.48
His	71	59	+11	0	+5	+6	+11	+12	−1	+1	5	0.03	0.78	0.92
Ile	162	161	+9	−2	+1	+6	+2	+17	0	−4	12	0.33	0.63	0.41
Leu	173	181	+70	−36	+9	+25	+51	+90	−33	−39	27	<0.001	0.52	0.38
Lys	92	94	+7	−3	+2	+3	+9	+5	−6	+0	8	0.21	0.89	0.54
Met	30	59	+1	−2	−1	+1	−4	+7	+2	−5	10	0.76	0.84	0.33
Met % total AA[5]	1.5	3.1	−0.1	−0.3	−0.1	−0.3	−0.3	0.0	−0.1	−0.7	0.5	0.77	0.63	0.28
Met % indispensable AA[6]	3.1	6.3	−0.5	−0.2	−0.1	−0.6	−0.7	−0.3	+0.4	−0.9	1.0	0.78	0.66	0.39
Phe	51	47	+3	0	+1	+2	+2	+4	−1	−0	2	0.24	0.73	0.89
Thr	66	65	+2	−3	−1	0	+2	+2	−3	−2	4	0.28	0.85	0.96
Trp	63	62	+4	−1	+1	+2	+2	+6	0	−1	3	0.15	0.73	0.44
Val	181	177	+14	−4	+3	+7	+10	+17	−3	−5	12	0.14	0.78	0.70
Total dispensable AA	1,053	999	+119	+33	+73	+79	+130	+108	+16	+49	58	0.12	0.93	0.60
Ala	281	247	+24	+6	+23	+8	+35	+13	+10	+3	24	0.42	0.56	0.74
Asn	36	34	+1	+2	+1	+2	−0	+2	+2	+2	2	0.67	0.72	0.78
Asp	2	2	+32	+33	+33	+32	+32	+33	+35	+31	2	0.82	0.60	0.25
Glu	23	24	−4	0	−2	−2	−4	−4	0	0	1	0.004	0.87	0.92
Gln	246	239	+31	+12	+22	+21	+33	+29	+11	+14	11	0.07	0.98	0.76
Gly	275	271	+64	+18	+34	+49	+66	+61	+1	+36	17	0.007	0.42	0.21
Pro	52	49	+4	+1	+3	+2	+5	+4	+1	+1	2	0.22	0.86	0.76
Ser	75	71	+4	+2	+2	+4	+4	+5	+0	+3	4	0.50	0.62	0.83
Tyr	64	62	−6	−8	−8	−6	−8	−4	−8	−8	45	0.67	0.62	0.52

[1]Least squares means of the data for cows in CON or RPM during phase 1 (baseline) in periods 1 and 2.
[2]Values shown are paired-difference values and were calculated for each cow for each period based on the difference between phase 1 baseline means and phase 2 values for each variable at 8 h postfeeding on day 6 of phase 2 of periods 1 and 2. During phase 2 of periods 1 and 2, environmental treatments were pair-fed thermoneutral (PFTN) and heat stress challenge (HSC), and dietary treatments were rumen-protected Met included with top-dress (RPM) or no rumen-protected Met included with top-dress (CON). Top-dress vehicle was 300 g of dried molasses.
[3]P-values correspond to phase 2 paired differences. No significant sequence effects ($P > 0.10$). A period effect was present for Met as a % of total AA ($P = 0.04$) and Leu ($P = 0.01$).
[4]All analyses were performed on blood plasma (heparinized). All variable units in µM, unless otherwise specified.
[5]Met as a % of total AA = Met (µM)/[total AA (µM) – Met (µM)].
[6]Met as a % of total indispensable AA = Met (µM)/[total indispensable AA (µM) – Met (µM)].

phase 2, there was no difference for EBAL due to environment (PFTN = −4.6 Mcal/d and HSC = −4.7 Mcal/d; SEM = 0.62; $P = 0.68$) or diet (CON = −4.5 Mcal/d and RPM = −4.8 Mcal/d; SEM = 0.62; $P = 0.78$). An environment × diet interaction was present for EBAL. Cows in HSC and RPM had lower EBAL than cows in HSC and CON (−5.3 and −4.1 Mcal/d, respectively; SEM = 0.62; $P = 0.002$).

Serum and plasma

Plasma AA baseline least squares means (phase 1) and least squares means of paired-differences (phase 2) data are in Table 1. During phase 1, cows in RPM had greater plasma Met concentration compared with cows in CON (59 and 30 µM, respectively; $P < 0.001$), greater plasma Met as a percentage of total AA compared with cows in CON (3.1% of total AA and 1.5% of total AA, respectively; $P < 0.001$), and greater plasma Met as a percentage of total IAA compared with cows in CON (6.3% of total IAA and 3.1% of total IAA; respectively; $P < 0.001$). There was no difference for the other plasma AA concentrations in phase 1 ($P > 0.22$). Cows in HSC had a decrease in total AA (−93 µM), total branched-chain AA (−20 µM), and total IAA (−110 µM) compared with cows in PFTN (+214, +84, and +96 µM, respectively; $P = 0.03$, $P = 0.02$, and $P = 0.009$, respectively) during phase 2. Cows in PFTN had an increase in His compared with no change for cows in HSC (+11 and 0 µM; $P = 0.03$). Cows in HSC had a decrease in Leu compared with cows in PFTN (−36 and +70 µM; $P < 0.001$). Cows is PFTN had a greater decrease in Glu compared with cows in HSC (−4 and 0 µM; $P = 0.004$). Cows in PFTN had a greater increase in Gly compared with cows in HSC (+64 and +18 µM; $P = 0.007$). However, there were no effects of diet on concentrations of plasma AA concentrations during phase 2 ($P > 0.35$).

Serum and plasma metabolic markers baseline least squares means (phase 1) and least squares means of paired-differences (phase 2) data are in Table 2. During phase 1, at 4 h postfeeding, cows in RPM had lower plasma NEFA concentrations compared with cows in CON (63 and 72 µM, respectively; $P = 0.02$), and tended to have greater plasma PUN concentrations compared with cows in CON (14.63 and 13.63 mg/dL, respectively; $P = 0.10$). There was no difference for the other plasma metabolic markers

Table 2. Least squares means of phase 1 baseline and least squares means of paired differences and associated pooled SEM for blood metabolites of Holstein cows in different environmental treatments (Env): pair-fed thermoneutral (PFTN) or heat stress challenge (HSC), and fed different dietary treatments as top-dress (Diet): rumen-protected Met (RPM) or no RPM (CON) at two time points postfeeding (4 and 8 h) as well as their average

	Phase 1[1]		Phase 2[2]									P-value[3]			
			Env		Diet		PFTN		HSC						
Variable[4]	CON	RPM	PFTN	HSC	CON	RPM	CON	RPM	CON	RPM	SEM	Env	Diet	Env × Diet	Day
Blood, 4 h postfeeding															
PUN, mg/dL[5]	13.63	14.63	+0.97	+0.62	+0.42	+1.17	+0.68	+1.26	+0.15	+1.08	0.41	0.31	0.10	0.62	0.06
Glucose, mg/dL	65.3	64.8	+0.9	+3.2	+2.2	+1.5	+1.6	+0.2	+4.0	+2.5	1.13	0.02	0.26	0.99	0.09
NEFA, µM[6]	72	63	−4	+2	−2	+2	−6	−10	+2	+4	0.044	0.10	0.64	0.33	0.009
Insulin, µIU/mL	8.9	8.9	−2.7	−0.7	−1.2	−2.3	−2.2	−3.3	−0.1	−1.3	1.0	0.01	0.34	0.95	0.28
RQUICKI[7]	0.400	0.408	+0.03	+0.01	+0.02	+0.02	+0.04[a]	+0.03[a]	0.00[b]	+0.01[b]	0.012	<0.001	0.63	0.05	0.60
Blood, 8 h postfeeding															
PUN, mg/dL[5]	11.11	11.34	−0.66	−0.09	−0.45	−0.27	−0.69	−0.62	−0.25	+0.04	0.47	0.04	0.76	0.70	0.34
Glucose, mg/dL	66.8	66.8	+2.9	+3.1	+3.0	+2.9	+3.3	+2.4	+2.8	+3.4	1.14	0.77	0.91	0.39	0.30
NEFA, µM[6]	100	94	+10	+1	+2	+9	+5	+14	−1	+3	0.031	0.04	0.22	0.51	0.25
Insulin, µIU/mL	8.2	8.3	−7.7	−0.4	−3.8	−4.3	−6.7[a]	−8.7[a]	−0.9[b]	+0.2[b]	1.0	<0.001	0.72	0.04	0.71
RQUICKI[7]	0.388	0.397	+0.06	+0.01	+0.03	+0.04	+0.06	+0.07	+0.01	+0.01	0.009	<0.001	0.81	0.14	0.69
Blood, average[8]															
PUN, mg/dL[5]	12.37	13.01	+0.21	+0.28	+0.04	+0.45	+0.11	+0.31	−0.04	+0.60	0.39	0.80	0.38	0.43	0.17
Glucose, mg/dL	66.0	65.8	+1.8	+3.0	+2.6	+2.1	+2.2	+1.3	+3.0	+3.0	1.02	0.11	0.68	0.51	0.08
NEFA, µM[6]	86	78	+1	+2	−2	+4	−4	+7	+1	+2	0.026	0.89	0.11	0.07	0.02
Insulin, µIU/mL	8.5	8.7	−5.0	−0.5	−2.4	−3.1	−4.2	−5.7	−0.7	−0.4	0.8	<0.001	0.51	0.11	0.31
RQUICKI[7]	0.394	0.401	+0.05	+0.01	+0.03	+0.03	+0.05	+0.05	+0.01	+0.01	0.007	<0.001	0.75	0.99	0.48

[1]Least squares means of the data for cows in CON or RPM during phase 1 (baseline) in periods 1 and 2.
[2]Values shown are paired-difference values and were calculated for each cow for each period based on the difference between phase 1 baseline means and phase 2 values for each variable. During phase 2 of periods 1 and 2, environmental treatments were pair-fed thermoneutral (PFTN) and heat stress challenge (HSC), and dietary treatments were rumen-protected Met included with top-dress (RPM) or no rumen-protected Met included with top-dress (CON). Top-dress vehicle was 300 g of dried molasses.
[3]P-values correspond to phase 2 paired differences. No significant three-way interaction (Env × Diet × Day), Diet × Day interaction, Env × Day interaction, or sequence effect; $P > 0.10$. A period effect was present for PUN (4 h; $P = 0.006$), glucose (4 h; $P = 0.004$), NEFA (4 h; $P = 0.006$), PUN (8 h; $P < 0.001$), RQUICKI (8 h; $P = 0.03$), PUN (average; $P < 0.001$), and glucose (average; $P = 0.003$).
[4]All analyses were performed on blood plasma.
[5]Plasma urea nitrogen (mg/dL).
[6]Non-esterified fatty acids (mmol/L).
[7]Revised quantitative insulin sensitivity check index (RQUICKI) ={1/[log glucose (mg/dL) + log insulin (µIU/mL) + log NEFA (mmol/L)]} (Perseghin et al., 2001).
[8]Average concentrations of blood metabolites for blood plasma collected at 4 and 8 h postfeeding.

concentrations in phase 1 ($P > 0.16$). At 4 h postfeeding, cows in RPM tended to have a greater increase in PUN compared with cows in CON (+1.17 and +0.42 mg/dL, respectively; $P = 0.10$). At 8 h postfeeding, cows in PFTN had a greater decrease in PUN compared with cows in HSC (−0.66 and −0.09 mg/dL, respectively; $P = 0.04$). At 4 h postfeeding, cows in HSC had a greater increase in plasma glucose compared with cows in PFTN (+3.2 and +0.9 mg/dL, respectively; $P = 0.02$). At 4 h postfeeding, cows in HSC tended to have increased plasma NEFA compared with a decrease in plasma NEFA for cows in PFTN (+2 and −4 µM, respectively; $P = 0.10$); however, at 8 h postfeeding, cows in PFTN had a greater increase in plasma NEFA compared with cows in HSC (+10 and +1 µM, respectively; $P = 0.04$). An environment × diet interaction was present for plasma NEFA for the average of 4 and 8 h postfeeding time points. At 4 h postfeeding, cows in PFTN had a greater decrease in plasma insulin compared with cows in HSC (−2.7 and −0.7 µIU/mL, respectively; $P = 0.01$). Similar plasma insulin results were present at 8 h postfeeding for PFTN compared with HSC (−7.7 and −0.4 µIU/mL, respectively; $P < 0.001$), as well as for the average of 4 and 8 h postfeeding for PFTN compared with HSC (−5.5 and −0.5 µIU/mL, respectively; $P < 0.001$). Additionally, at 8 h postfeeding, an environment × diet interaction was present for plasma insulin ($P = 0.04$). A greater increase in RQUICKI was present for cows in PFTN compared with HSC at 4 h postfeeding (+0.03 and +0.01, respectively; $P < 0.001$), 8 h postfeeding (+0.06 and +0.01, respectively; $P < 0.001$), and for the average of 4 and 8 h postfeeding time points (+0.05 and +0.01, respectively; $P < 0.001$). At 4 h postfeeding, an environment × diet interaction was present for RQUICKI ($P = 0.05$). A day effect was present for plasma NEFA at 4 h postfeeding ($P = 0.009$) and for plasma NEFA for the average of 4 and 8 h postfeeding time points.

Serum and plasma inflammatory markers baseline least squares means (phase 1) and least squares means of paired-differences (phase 2) data are in Table 3. During phase 1, there was no difference for plasma inflammatory markers concentrations ($P > 0.11$). An environment × diet interaction was present for plasma albumin ($P = 0.004$). Cows in HSC had an increase in plasma SAA compared with a decrease in plasma SAA for cows in PFTN (+58 and −59 µg/mL, respectively; $P < 0.001$). Additionally, a tendency for an environment × diet interaction was present for plasma SAA ($P = 0.08$). Cows in HSC had an increase in serum haptoglobin compared to a decrease in serum haptoglobin for cows in PFTN (+33and -3 µg/mL, respectively; $P = 0.02$). Cows in HSC had an increase in plasma LBP compared with a decrease in plasma LBP for cows in PFTN (+0.11 and −0.27 µg/mL,

Table 3. Least squares means of phase 1 baseline and least squares means of paired differences and associated pooled SEM for blood metabolites of Holstein cows in different environmental treatments (Env): pair-fed thermoneutral (PFTN) or heat stress challenge (HSC), and fed different dietary treatments as top-dress (Diet): rumen-protected Met (RPM) or no RPM (CON) at 8 h postfeeding

	Phase 1[1]		Phase 2[2]												
			Env		Diet		PFTN		HSC			P-value[3]			
Variable[4]	CON	RPM	PFTN	HSC	CON	RPM	CON	RPM	CON	RPM	SEM	Env	Diet	Env × Diet	Day
Albumin, g/dL	3.642	3.587	−0.03	−0.05	−0.04	−0.04	−0.05[ab]	0.00[a]	−0.02[ab]	−0.07[b]	0.021	0.19	0.90	0.004	0.83
Serum amyloid A, µg/mL	93	108	−59	+58	−58	+45	−43	−75	+32	+84	42	<0.001	0.84	0.08	0.18
Haptoglobin, µg/mL	37	31	−3	+33	+12	+18	−3	−2	+27	+38	20	0.02	0.78	0.71	0.15
LPS-binding protein, µg/mL	1.64	1.37	−0.27	+0.11	−0.06	−0.11	−0.24	−0.31	+0.12	+0.09	0.17	<0.001	0.81	0.85	0.68
L-lactate, mM/L	0.324	0.423	−0.06	−0.03	−0.07	−0.02	−0.10	−0.02	−0.04	−0.01	0.039	0.36	0.32	0.57	0.54
IL-1β, pg/mL[5]	8.9	18.4	−1.9	+3.9	+0.9	+1.1	−1.4	−2.6	+3.2	+4.6	6.2	0.03	0.98	0.64	<0.001

[1]Least squares means of the data for cows in CON or RPM during phase 1 (baseline) in periods 1 and 2.
[2]Values shown are paired-difference values and were calculated for each cow for each period based on the difference between phase 1 baseline means and phase 2 values for each variable. During phase 2 of periods 1 and 2, environmental treatments were pair-fed thermoneutral (PFTN) and heat stress challenge (HSC), and dietary treatments were rumen-protected Met included with top-dress (RPM) or no rumen-protected Met included with top-dress (CON). Top-dress vehicle was 300 g of dried molasses.
[3]P-values correspond to phase 2 paired differences. No significant three-way interaction (Env × Diet × Day), Diet × Day interaction, or sequence effect; $P > 0.10$. An Env × Day interaction was present for albumin ($P = 0.03$). A period effect was present for all variables ($P = 0.03$).
[4]All analyses were performed on blood plasma, except for haptoglobin (blood serum).
[5]Interleukin-1β (pg/mL).

respectively; $P < 0.001$). Cows in HSC had an increase in plasma IL-1β compared with a decrease in plasma IL-1β for cows in PFTN (+3.9 and −1.9 pg/mL, respectively; $P = 0.03$). Additionally, day effect was present for plasma IL-1β ($P < 0.001$).

Mammary tissue immunohistochemistry

Mammary tissue immunohistochemical data are in Table 4. An environment × diet interaction was present for apoptotic cells (percentage of total cells; $P = 0.03$), as well as for the ratio of apoptotic cells to proliferating cells ($P = 0.005$). Mammary tissue cells from cows in HSC had a greater XBP1 emission intensity compared with mammary tissue cells from cows in PFTN (1,145 and 225, respectively; $P = 0.01$). Additionally, a tendency for an environment × diet interaction was present for XBP1 emission intensity ($P = 0.10$).

Discussion

HS has been shown to alter metabolism in dairy cattle, most notably causing increased insulin concentration and decreased total AA concentration in blood (Wheelock et al., 2010; Gao et al., 2017). Previous research has reported that feeding RPM during stressful periods (i.e., the transition period) resulted in increased Met concentration in the blood (Stella et al., 2018), and improvements in immune function (Osorio et al., 2014; Zhou et al., 2016b). Recently, Pate et al. (2020) reported the performance and physiological variables for cows in the current study fed RPM or not during HS and PFTN conditions. Briefly, cows in HSC had a greater increase in vaginal temperature (+0.2 °C), respiration rate (+13.7 breaths/min), and heart rate (+2.0 beats/min) compared with cows in PFTN (0.0 °C, −1.6 breaths/min, and −0.8 beats/min, respectively). Cows in PFTN had a greater decrease in DMI compared to cows in HSC (−3.9 and −3.2 kg/d, respectively); however, there was no difference in DMI as a percentage of body weight (BW) between HSC and PFTN. Cows in PFTN had a greater decrease in milk yield (-2.6 kg/d) compared with cows in HSC (−0.9 kg/d). Cows in CON had greater decrease in milk protein concentration for PFTN (−0.10% units) and HSC (−0.06% units) compared with cows in RPM for PFTN (0.00% units) and HSC (−0.02% units). Although the effects of HS on dairy cattle performance and physiological parameters have been previously reported, limited research is available on RPM feeding during HS on metabolism and inflammation markers in dairy cattle.

Plasma Met concentration, Met as a percentage of total AA, and Met as a percentage of IAA were assessed during phase 1 in order to understand the efficacy of dietary treatment in the current experiment. Similar to other works that fed RPM to Holstein cows (Toledo et al., 2017; Stella et al., 2018), cows in RPM had an 96% increase in plasma Met concentration compared with cows in CON during phase 1. Additionally, cows in RPM had a 106% increase in Met as a percentage of total AA and a 103% increase in Met as a percentage of IAA compared with cows in CON. These data confirm that the dietary treatment (1.05 g RPM/kg of DMI as top-dress) in the current study adequately increased bioavailable Met for cows in RPM compared with cows in PFTN. It is important to note that cows in RPM and CON had greater Met concentration in plasma (59.4 and 30.4 µM, respectively), compared with previous studies utilizing lactating cows up to 73 d in milk (30.4 and 18.1 µM, respectively; Stella et al., 2018). However, this is likely due to plasma sample timing postfeeding, as Toledo et al. (2017) reported peak Met concentration in plasma at 12 h postfeeding, with similar plasma Met concentration for

Table 4. Least squares means and associated pooled SEM for immunohistochemical markers of mammary gland tissue harvested from Holstein cows in different environmental treatments (Env): pair-fed thermoneutral (PFTN) or heat stress challenge (HSC), and fed different dietary treatments as top-dress (Diet): rumen-protected Met (RPM) or no RPM (CON)

	Phase 2[1]									P-value[2]		
	Env		Diet		PFTN		HSC					
Variable	PFTN	HSC	CON	RPM	CON	RPM	CON	RPM	SEM	Env	Diet	Env × Diet
Apoptotic cells, % of total cells[3,4]	1.3	2.4	2.0	1.8	0.7[a]	2.0[b]	3.3[b]	1.5[ab]	0.9	0.88	0.15	0.03
Proliferating cells, % of total cells[4,5]	0.4	0.4	0.3	0.5	0.3	0.6	0.3	0.4	0.1	0.40	0.39	0.34
Apoptotic cells:Proliferating cells[4]	5.4	8.6	7.9	6.0	2.6[a]	8.2[bc]	13.2[c]	3.9[ab]	3.6	0.91	0.33	0.005
MIST1[6]	1,100	1,500	900	1,700	900	1,400	1,000	2,000	594	0.55	0.13	0.20
XBP1[6]	230	1,150	560	810	110	340	1,010	1,280	408	0.01	0.18	0.10

[1]During phase 2 of periods 1 and 2, environmental treatments were pair-fed thermoneutral (PFTN) and heat stress challenge (HSC), and dietary treatments were rumen-protected Met included with top-dress (RPM) or no rumen-protected Met included with top-dress (CON). Top-dress vehicle was 300 g of dried molasses.
[2]No significant sequence or period effect present ($P > 0.10$).
[3]Apoptotic cells = [total area of apoptotic mammary cells/total area of mammary cells] × 100.
[4]PFTN ($n = 23$), HSC ($n = 25$), CON ($n = 24$), RPM ($n = 24$), PFTN/RPM ($n = 11$), HS/RPM ($n = 13$), PFTN/CON ($n = 12$), HS/CON ($n = 12$).
[5]Proliferating cells = [total area of Ki-67 antigen expressing mammary cells/total area of mammary cells] × 100.
[6]Number of mammary tissue samples used for the analyses are as follows: PFTN ($n = 19$), HSC ($n = 20$), CON ($n = 19$), RPM ($n = 20$), PFTN/RPM ($n = 10$), HS/RPM ($n = 10$), PFTN/CON ($n = 9$), HS/CON ($n = 10$).

cows in RPM and CON (52.4 and 26.0 µM) compared with the current study.

Gao et al. (2017) reported a decrease in total AA concentration for cows in HS compared to cows in PFTN. Others have speculated that the reduction in total AA in the circulation during HS is likely due to increased AA utilization for gluconeogenic purposes, as well as increased AA utilization for inflammatory protein synthesis (Ronchi et al., 1999; Cowley et al., 2015; Gao et al., 2017). In the current experiment, cows in HSC had a decrease in total AA and total IAA concentration 8 h after feeding compared to cows in PFTN. Furthermore, cows in HSC had a lesser decrease in PUN compared with cows in PFTN, which may indicate increased AA deamination in the liver for gluconeogenic purposes or increased circulating acute-phase proteins (APP) such as haptoglobin, which was increased for cows in HSC compared with cows in PFTN, supporting the hypothesis that HS causes increased whole-body AA utilization for gluconeogenesis and APP synthesis, leading to reduced plasma AA concentrations. However, further research is needed to validate this hypothesis. Additionally, a lesser decrease in PUN may be a result of increased microbial protein synthesis via nitrogen incorporation in the rumen; however, this method could not be tested in the current study as ruminal content samples were not collected. Also, data regarding rumen ammonia concentration and microbial crude protein synthesis during HS are inconsistent (Cowley et al., 2015; Gao et al., 2017). Kassube et al. (2017) and Gao et al. (2017) reported decreased Leu concentration in the blood of cows in HS conditions compared with cows in TN conditions. These data are corroborated in the current study, as cows in HSC had a decrease in Leu and total branched-chain AA concentration compared with cows in PFTN. Leucine plays an important role in protein metabolism and insulin responsiveness (Garlick, 2005). Previous studies have reported that increased plasma Leu concentration results in increased protein synthesis in multiple tissues, including skeletal muscle and the mammary gland, and is likely due to Leu direct effects on mTOR activation and increased insulin sensitivity (Escobar et al., 2006; Kim, 2009; Appuhamy et al., 2012; Arriola Apelo et al., 2014). Therefore, reduced Leu concentrations for cows in HSC in the current study may have caused decreased skeletal muscle insulin sensitivity, resulting in overall protein degradation and increased PUN. However, further research on Leu effects during HS conditions is needed.

In the current study, sampling at 4 and 8 h postfeeding yielded divergent paired-difference results for plasma biomarkers (Table 2). For instance, an environment effect was present at 8 h postfeeding, but not at 4 h postfeeding for PUN. Additionally, an environment effect was present at 4 h postfeeding, but not at 8 h postfeeding for blood glucose. It is widely recognized that postprandial blood metabolite concentrations change over time in humans (Takahashi et al., 2018), pigs (Campos et al., 2019), poultry (Buyse et al., 2002), and ruminants (Jenny and Polan, 1975; Chase et al., 1977). In dairy cattle, it has been reported that blood metabolite concentrations are further altered based on physiological and environmental conditions, such as the periparturient period or HS (Wheelock et al., 2010; Zapata et al., 2015; Gärtner et al., 2019). Although it is understood that metabolic changes in blood metabolites are altered during conditions of HS, exact biological mechanisms responsible for these changes are not well defined. While many studies have been conducted to elucidate these mechanisms, it is important to note that blood sample timing among these studies is rather inconsistent. For example, blood sample timing for trials related to HS in dairy cattle have been reported to occur at ~0 to 1 h postfeeding (Kauffman et al., 2018), 2 to 3 h postfeeding (Gao et al., 2017), 12 h postfeeding (Wheelock et al., 2010), and in some cases blood sample timing relative to feeding was not reported (Rhoads et al., 2009; Cowley et al., 2015). The current study shows that postprandial blood sample timing is an important factor to take into consideration when analyzing metabolite concentrations, especially in situations of HS or feed restriction. Although an optimal postprandial blood sampling time point is still to be determined, the authors suggest that standardization of blood sampling time point be considered when utilizing the HS model of research in order to adequately compare blood metabolite results among studies. Additionally, RPM administration method (i.e., top dressed vs. in a TMR) may play a role in AA and other metabolite concentrations in blood. Therefore, it should be taken into consideration when evaluating the present data with the literature. Data on the average of 4

and 8 h postprandial blood samplings were assessed. Averaging sample time point data alter the results of some biomarker concentrations in blood when compared with single time point data (Table 2). For example, an environmental effect was present at 8 h and a tendency for an environment effect is present at 4 h for plasma NEFA; however, no environmental effect is present for the averaged data. Therefore, the practice of averaging data from multiple blood sampling time points should be avoided when measuring blood metabolites postfeeding.

Feeding RPM has been shown to improve liver functionality around parturition (Zhou et al., 2017). During phase 1, decreased plasma NEFA and a tendency for higher PUN concentrations at 4 h postfeeding for cows in RPM than CON could have occurred due to increased liver functionality for cows in RPM. However, the small differences for those markers and the absence of the statistical difference at 8 h postfeeding between RPM and CON suggest that the biological relevance of this finding should be taken with caution. Decreased plasma insulin and no difference in plasma NEFA concentrations at 4 h postfeeding between environmental treatments likely is because PFTN cows at 4 h postfeeding were still in an absorptive state and not considered metabolically restricted. However, a decrease in insulin and a rise in NEFA concentrations at 8 h postfeeding is a typical response for cows in negative energy balance or a feed restricted state, as exhibited by cows in PFTN (Bauman and Currie, 1980; Drackley, 1999). Previous works have described that cows in HS conditions with reduced DMI undergo marked postabsorptive metabolic changes compared with cows with reduced DMI in TN, including increased insulin secretion coinciding with reduced NEFA in circulation, insinuating decreased systemic insulin sensitivity during HS (Itoh et al., 1998; Wheelock et al., 2010; Baumgard and Rhoads, 2013). One method of calculating insulin sensitivity is RQUICKI analysis, which has been extensively used for studies measuring metabolic changes throughout the transition period (Holtenius and Holtenius, 2007; Rico et al., 2015; Gärtner et al., 2019). Although RQUICKI is an indirect method of calculating insulin sensitivity established in humans, and may be variable based on factors such as stage of lactation and pregnancy status (Schoenberg et al., 2012; De Koster and Opsomer, 2013), it may offer valuable information in determining insulin responsiveness in mid-lactation dairy cattle during HS over time postprandial. In the current study, cows in PFTN had a greater increase in RQUICKI (indicating increased insulin sensitivity) at 4 h postfeeding than cows in HSC and an even greater increase in RQUICKI at 8 h. These data, along with reduced insulin, glucose, and PUN, and increased NEFA concentrations suggest that cows in HSC were experiencing reduced insulin sensitivity when compared with cows in PFTN. These data are corroborated by previous research (Rhoads et al., 2009; Wheelock et al., 2010). However, although cows in PFTN had in increase in RQUCKI, they also had an increase in NEFA coinciding with a decrease insulin concentration in plasma, indicating reduced systemic insulin sensitivity. Additionally, cows in PFTN also had a greater decreased in DMI and milk yield, which may confound results related to metabolism (Pate et al., 2020). Therefore, RQUICKI data in this experiment must be evaluated with caution. Additionally, although the pair-feeding TN model is extremely useful in describing metabolic differences between DMI reduction during HS and TN pair-feeding, it is important to note that these metabolic alterations for cows in PFTN may cause confounding effects when measuring differences in lactation performance. Therefore, metabolic data should be used in conjunction with lactation performance data in order to adequately describe differences between cows in HS and pair-fed TN treatment groups.

Cows in HS conditions have an increase in plasma creatinine, urea N, and free AA, as well as increased milk urea nitrogen (MUN), indicating some degree of skeletal muscle breakdown during hyperthermia (Wheelock et al., 2010; Cowley et al., 2015; Gao et al., 2017). Plasma urea N and MUN are correlated and impacted similarly by changes in dietary protein degradation and impacted negatively by increasing intake of energy (Roseler et al., 1993). Larsen et al. (2014) reported that supplying a greater amount of MP during early lactation resulted in a more pronounced negative protein balance, likely due to mammary and tissue need for protein (Larsen et al., 2014). Additionally, there is evidence for increased utilization of glucogenic AA as glucose precursors during periods of negative EBAL (i.e., early lactation; Drackley et al., 2001). It is postulated that skeletal muscle catabolism is used to provide gluconeogenic precursors (i.e., glucogenic AA) to the liver in order to maintain blood glucose homeostasis during HS (Gao et al., 2017; Ríus, 2019). These data collectively could explain why cows under HSC that consumed RPM had lower EBAL than cows in CON in the present work. Additionally, muscle catabolism may play a role in providing AA substrates to the liver for APP synthesis or to bone marrow for leukocyte synthesis during HS-derived inflammation (Bruins et al., 2000, 2002; Hoskin et al., 2016; Ríus, 2019). Although the exact mechanisms behind skeletal muscle breakdown during HS are not fully known, reduced skeletal muscle insulin sensitivity may be the culprit. Furthermore, PUN tended to increase for cows in RPM compared to CON at 4 h postfeeding; however, by 8 h postfeeding there was no difference in PUN among dietary treatments. Also, no MUN difference was detected between cows in RPM or CON (Pate et al., 2020). This suggests that there is minimal, if any, effect of RPM feeding on muscle catabolism or liver AA deamination during HSC. These data are supported by multiple studies in which RPM had no effect on PUN concentration throughout the transition period (Osorio et al., 2014; Zhou et al., 2016a).

Previous studies and reviews have thoroughly discussed the methods by which HS causes immunoactivation. One mechanism is that HS directly activates cellular heat-shock factors, triggering an increase in the synthesis of heat-shock proteins (**HSP**) which initiate an inflammatory response in innate immune cells (Asea et al., 2002; Collier et al., 2008; Pawar et al., 2014; Zininga et al., 2018). Another mechanism is that HS causes lipopolysaccharide (**LPS**)-induced immunoactivation. Briefly, as animals attempt to dissipate heat to the environment during HS, blood flow to the gastrointestinal tract is reduced causing a reduction in intestinal barrier integrity due to decreased oxygen and nutrient supply (Hales 1973; Pearce et al., 2013). This barrier function degradation is characterized by an influx of bacterial endotoxin (i.e., LPS) into the circulation, thus eliciting a marked immunological response (Baumgard and Rhoads, 2013; Pearce et al., 2013; Koch et al., 2019). In the current study, cows in HSC had an increase in LBP, a positive APP responsible for endotoxin sequestration, transfer, and presentation to macrophage and granulocytes (Ceciliani et al., 2012). These data are in agreement with previous research measuring the effects of HS on LPS and LBP concentration in humans (Selkirk et al., 2008), rats (Hall et al., 2001), pigs (Pearce et al., 2013; Campos et al., 2019), and ruminants (Koch et al., 2019; Zachut et al., 2020), and thus support the LPS-induced immunoactivation model. Furthermore, during immunoactivation (via HSP-induction, LPS-induction, or both) innate immune cells release pro-inflammatory cytokines (i.e., TNFα, IL-1β, and IL-6), which cause the release of positive APP from the liver (Ceciliani et al., 2012; Murphy et al., 2012). Increased plasma concentrations of IL-1β

in cows in HSC indicate immune activation and is supported by previous reports studying the effects of HS on immunoactivation and pro-inflammatory cytokine production. Zachut et al. (2020) reported a 6.7-fold increase in tumor necrosis factor (**TNFα**) for cows in HS, while Min et al. (2016b) report increases in both TNFα and interleukin-6 (IL-6) for cows in HS. Additionally, it has been reported that plasma IL-1β, IL-6, and TNFα are increased during HS in rats (Ji et al., 2014). Additionally, greater increases in haptoglobin and SAA concentrations were present for cows in HSC compared with PFTN. Hamzaoui et al. (2013) reported an increase in plasma haptoglobin when dairy goats were in HS conditions compared with TN conditions. Therefore, when data regarding blood inflammatory markers from the current study are taken together, they indicate that cows in HSC elicited a robust immune response that is consistent with previous literature. Furthermore, as discussed by Pate et al. (2020), degree of HS may vary based on numerous factors; including severity, duration, and HS-induction method. Consequently, understanding the ways in which varying degrees of HS affect dairy cattle is of great importance to the dairy industry. Inflammatory biomarker data provided in the current study, along with other metabolic biomarkers as described here and in previous literature (Baumgard and Rhoads, 2013; Ríus, 2019), may be combined to determine the degree of HS that an animal may be facing via blood sampling.

As previously discussed, increased circulating insulin concentration as well as reduced insulin sensitivity have been reported in lactating cows, and may be a result of HS-induced negative energy balance caused by reduction in DMI, as well as elevated body temperature (Itoh et al., 1998; Wheelock et al., 2010). However, metabolic alterations are likely altered by stage of lactation as well. Tao et al. (2012) reported no changes in insulin, glucose, or NEFA in dry cows succumbed to HS conditions. Mechanisms causing altered postabsorptive metabolism in cows in HS are complex and largely unknown, particularly in regard to immune system orchestration of these events. Baumgard and Rhoads (2013) suggested that the increase in circulating insulin concentration seen in HS lactating cows is likely due to increased pancreatic β-cell secretion of insulin during immunoactivation, rather than a decreased ability for peripheral tissue to uptake insulin. It has thus been theorized that increased insulin secretion by pancreatic β-cells is a pro-inflammatory cytokine response as opposed to a direct response to circulating LPS. This is supported by the fact that LPS challenged cows had neither an increase nor decrease in protein abundance of insulin receptors in muscle, adipose, or liver tissues (Horst et al., 2019). Furthermore, dairy cows and calves challenged with LPS had a delay in insulin concentration increase post LPS challenge, insinuating that a secondary messenger is the cause of increased insulin secretion (Waldron et al., 2006; Baumgard and Rhoads, 2013). Based on data from the study herein, this postulation seems highly plausible as increased plasma insulin concentration is concomitant with increased plasma IL-1β concentration, as well as the fact that previous studies have reported increased cytokine concentrations during HS (Min et al., 2016a, 2016b; Zachut et al., 2020). However, this postulation is speculative, as it is unknown whether the increased plasma cytokine concentration during HS is coincidental or causal in regard to increased insulin secretion. Studies measuring the effects of inflammatory cytokine or chemokine treatments on metabolism biomarkers are inconsistent. Subcutaneous injection of TNF-α (Yuan et al., 2013) and intrauterine infusion of interleukin-8 (IL-8; Zinicola et al., 2019b) in early lactation cows resulted in no alteration in insulin concentrations, while subcutaneous IL-8 administration to calves resulted in increased insulin concentrations and sensitivity (Zinicola et al., 2019a). Therefore, further research is needed regarding pro-inflammatory cytokines effects on metabolism during HS.

Previous studies have determined that HS has marked direct effects on mammary tissue, likely contributing to decreased milk yield and milk component yield, as well as increased SCC (Kaufman et al., 2018; Tao et al., 2018). Collier et al. (2006) and Salama et al. (2019) reported increased expression of cell apoptosis genes and decreased expression of cell proliferation genes in mammary cells incubated in HS conditions, indicating that programmed epithelial cell death may occur during HS. These data are confirmed in the present study, as cows in HSC and CON had a greater percentage of apoptotic mammary cells than cows in PFTN and CON. It has also been reported that gene expression and protein abundance of HSP increase in mammary epithelial tissue incubated at high temperatures in vitro (Collier et al., 2006; Han et al., 2015; Salama et al., 2019), as well in vivo (Orellana et al., 2017), indicating that mammary epithelial cells increase synthesis of cytoprotective proteins during HS. Han et al. (2015) and Salama et al. (2019) both reported increased expression of HSP family A member 1A (HSPA1A) when Met was supplemented to the mammary epithelial cells under HS conditions. Furthermore, Kaufman et al. (2018) and Salama et al. (2019) showed an increase in mRNA expression and protein abundance of IAA transporters in mammary epithelial tissue under HS conditions. When these data are taken together, it is plausible that under HS, mammary epithelial cells have an increased demand for IAA (i.e., Met) in order to synthesize HSP necessary for cellular protection. In the current study, cows in HSC and CON had a greater proportion of apoptotic to proliferating cells than cows in HSC and RPM. **need something here about how RPM increase apoptosis in pftn cows. These data are corroborated by previous works reporting increased expression of genes related to cell proliferation and cell anti-apoptosis, as well as decreased concentrations of oxidative stress indicators in mammary epithelial cells supplemented with Met under HS (Han et al., 2015; Salama et al., 2019). However, these data must be carefully evaluated, as RPM feeding had no effect on percentage of apoptotic cells or percentage of proliferating mammary cells during HSC in the current study. In fact, RPM resulted in greater percentage of apoptotic mammary cells than CON during PFTN conditions. Thus, further research is needed to understand the effects of AA feeding on mammary epithelial tissue, especially during conditions of HS.

Spliced XBP1 isoform is an activated transcription factor involved in the unfolded protein response, and is responsible for the activation of genes related to endoplasmic reticulum biogenesis and development of secretory vesicles, such as MIST1 (Lee et al., 2002; Davis et al., 2016; Cant et al., 2018). Recent studies have proposed that increased XBP1 in mammary cells may enhance protein secretory capacity (Nichols et al., 2019). In the current study, mammary cells from cows in HSC had a greater emission intensity for XBP1 than cows in PFTN, indicating that those mammary cells had an increase in spliced XBP1 isoform protein abundance. This result is interesting, as cows in HS have been previously reported to have decreased milk protein content compared with cows in pair-fed TN conditions; although, decreased milk yield was also detected (Cowley et al., 2015; Gao et al., 2017), dissimilar from the current study. However, there was no difference in milk protein concentration between environmental treatments for cows in this study (Pate et al., 2020). Although XBP1 intensity was increased for cows in

HSC, there was no effect of environmental treatment on MIST intensity. Therefore, these data suggest that there was no overt increase in cell differentiation in mammary cells from cows in HSC. It is also important to note that there was a greater reduction in milk yield for cows in PFTN compared with HSC, which may also confound results detected on a molecular level.

Conclusion

Cows in HSC displayed marked changes in metabolism, most notably increased insulin, decreased NEFA, decreased total AA, and decreased total IAA compared with cows in PFTN. Furthermore, HSC caused immunoactivation, as indicated by increased circulating pro-inflammatory cytokines and positive APP. Additionally, compared with cows in CON, cows in RPM had decreased mammary cell apoptosis to proliferation ratio during HSC. However, compared with cows in CON, cows in RPM had an increase in mammary cell apoptosis to proliferation ratio, meaning that RPM impacted differently cellular protection capacity during PFTN. Finally, data from this experiment indicate that postprandial blood sample timing should be evaluated when assessing metabolic biomarkers and that standardization of sample timing should be considered among studies utilizing HS models in dairy cattle.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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