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Relationships between gastrointestinal permeability, heat stress, and milk production in lactating dairy cows

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ABSTRACT

Heat stress (HS) is a global issue that decreases farm profits and compromises animal welfare. To distinguish between the direct and indirect effects of HS, 16 multiparous Holstein cows approximately 100 DIM were assigned to one of 2 treatments: pair fed to match HS cow intake, housed in thermoneutral conditions (PFTN, n = 8) or cyclical HS (n = 8). All cows were subjected to 2 experimental periods. Period 1 consisted of a 4 d thermoneutral period with ad libitum intake. During period 2 (P2), the HS cows were housed in cyclical HS conditions with a temperature-humidity index (THI) ranging from 76 to 80 and the PFTN cows were exposed to a constant THI of 64 for 4 d. Dry matter intake of the PFTN cows was intake matched to the HS cows. Milk yield, milk composition, rectal temperature, and respiration rate were recorded twice daily, blood was collected daily via a jugular catheter, and cows were fed twice daily. On d 3 of each period, Cr-EDTA and sucralose were orally administered and recovered via 24 h total urine collection to assess gastrointestinal permeability. All data were analyzed using the GLIMMIX procedure in SAS. The daily data collected in P1 was averaged and used as a covariate if deemed significant in the model. Heat stress decreased voluntary feed intake by 35% and increased rectal temperature and respiration rate (38.4°C vs. 39.4°C and 40 vs. 71 respirations/min, respectively). Heat stress reduced DMI by 35%, which accounted for 66% of the decrease in milk yield. The yields, and not concentrations, of milk protein, fat, and other solids were lower in the HS cows on d 4 of P2. Milk urea nitrogen was higher and plasma urea nitrogen tended to be higher on d 3 and d 4 of HS. Glucose was 7% lower in the HS cows and insulin was 71% higher in the HS cows than the PFTN cows on d 4 of P2. No difference in lipopolysaccharide-binding protein was observed. Heat stress cows produced 7 L/d more urine than PFTN cows. No differences were detected in the urine concentration or percentage of the oral dose recovered for Cr-EDTA or sucralose. In conclusion, HS was responsible for 34% of the reduction of milk yield. The elevated MUN and the tendency for elevated plasma urea nitrogen indicate a whole-body shift in nitrogen metabolism. No differences in gastrointestinal permeability or lipopolysaccharide-binding protein were observed. These results indicate that, under the conditions of this experiment, activation of the immune system by gut-derived lipopolysaccharide was not responsible for the decreased milk yield observed during HS.
Key words: dairy cow, heat stress, leaky gut

INTRODUCTION

Heat stress (**HS**) affects US dairy herds of all sizes and in every region of the country. It is a barrier to US livestock sustainability, and the effects of HS on lactating cows cost the dairy industry $1.2 billion annually (Key and Sneeringer, 2014). Much of the loss in milk production is due to reduced feed intake. However, reduced feed intake only explains about 50% of the observed decrease in milk production (Rhoads et al., 2009; Wheelock et al., 2010; Baumgard et al., 2011), suggesting that HS directly affects milk production. In lactating dairy cows, the HS response markedly alters postabsorptive carbohydrate, lipid, and protein metabolism. These alterations occur through coordinated changes in nutrient supply and utilization across organs and organ systems in a manner distinct from those that occur in animals with a restricted DMI (Baumgard and Rhoads, 2013).

Much of the HS research conducted on lactating dairy cows over the past 2 decades focused attention on skeletal muscle (Koch et al., 2016; Roths et al., 2023) and adipose (Kra et al., 2022; Daddam et al., 2023) metabolism and milk production responses to HS. More recently, attention has turned to the role of the immune system in response to HS (Koch et al., 2019; Fontoura et al., 2022).

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

We and others (Koch et al., 2019; Fontoura et al., 2022) hypothesized that HS in high-producing dairy cows decreases gut integrity, activates the immune system, and partitions nutrients away from milk production.

Evidence to support this hypothesis is building. For instance, Kvidera et al. (2017a) showed that intentional disruption of gut barrier integrity led to increased blood LPS (endotoxin), and increased concentrations of circulating acute-phase proteins, including LPS-binding protein (**LBP**). In a separate experiment with lactating cows, the same research group showed that intravenous administration of LPS induced hypoglycemia and lowered milk yield by 80% and milk lactose by 11% (Kvidera et al., 2017b). The explanation was that LPS administration increased glucose use by immune cells and this immune system activation was prioritized over milk production (Kvidera et al., 2017b). Getting back to HS, Koch et al. (2019) demonstrated that HS directly impaired gut integrity by affecting abundance of tight junction molecules, affected cellular pathways associated with LPS signaling, and also resulted in increased abundance of mucosal and submucosal immune cells in the jejunum. Aligning evidence was also provided by Fontoura et al. (2022), who showed that in lactating cows HS increased total gastrointestinal tract permeability (**GIP**), increased circulating LBP concentration, and decreased milk yield. Further documentation of HS physiology in lactating dairy cows with respect to gut integrity and immune system activation is vital to developing and implementing potential mitigation strategies. Here, we hypothesized that 4 d of HS would lower milk yield and alter milk composition; alter postabsorptive carbohydrate, lipid, and protein metabolism; affect energy balance; and increase GIP, resulting in systemic immune system activation in lactating dairy cows.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (protocol #19–247). This study was conducted in the Metabolic Research Laboratory at the Virginia Tech Dairy Farm (Blacksburg, VA). A total of 16 multiparous Holstein cows (632 ± 46 kg BW; mean ± standard deviation) were purchased from privately owned dairy farms within a 3 h driving distance from Virginia Tech. Requirements for purchase were that each cow be a Holstein, have 4 functional mammary glands, be on their second or greater lactation, be producing minimally 40 kg of milk at approximately 100 DIM, and be visually free from disease. The overall experiment was performed using 2 cohorts with 8 cows per cohort; cohort 1 was on-site from May 11, 2021 to May 25, 2021, and cohort 2 was on-site from July 29, 2021 to August 10, 2021.

Upon arrival of each cohort, cows were randomly placed into 1 of 2 temperature-controlled rooms (n = 4 per room) for 4 d of acclimation where no treatments were applied. Acclimation included immediate adjustment to: a tiestall environment, milking in place, and a common TMR (Table 1); no cows were removed for failure to acclimate. During the acclimation period, cows were housed under constant thermoneutral conditions (18°C, 67% relative humidity (**RH**), temperature-humidity index [**THI**] = 64]. To avoid initial differences in DMI and milk yield between groups, within each cohort, a stratified randomization scheme based on BW on d 4 of the acclimation period (4 levels) was used to assign cows to one of 2 treatments: pair-fed thermoneutral (**PFTN**, n = 8) or HS (n = 8). One HS cow in cohort 1 was removed before imposing treatment due to a nontreatment related shoulder injury and was not replaced. That animal's data were completely removed from all datasets; resultant animal numbers were PFTN, n = 8 with an average BW of 636 kg and HS, n = 7 with an average BW of 629 kg. After treatment assignments were made, all PFTN cows were kept within a single room and all HS cows were kept in a single room. Rooms that were used to implement treatments were kept the same for cohort 1 and cohort 2 to reduce room-to-room variation. The acclimation period for PFTN was extended by 1 d to create a 1 d lag behind HS. Thus, cows within each cohort experienced a 4 to 5 d acclimation period, followed by a 4 d thermoneutral covariate period (**P1**), and then a 4 d treatment period (**P2**). In P1, all cows were housed under thermoneutral conditions (18°C, 67% RH, THI = 64) and had ad libitum access to TMR for 4 d. In P2, HS cows were exposed to cyclical HS (THI = 74 from 1800 to 0600 h and 80 from 0600 to 1800 h) for 4 d. In P2 for PFTN, the THI remained 64 and the previous day's average DMI for the HS room was applied to the PFTN room. At the end of P2, all cows were stunned via penetrating captive bolt and euthanized via exsanguination.

Throughout the experiment, each cow was tethered in a tiestall with sawdust shavings and experienced a 16 h light and an 8 h dark cycle. All cows were milked twice daily (0700 and 1900 h) and the HS cows had ad libitum access to TMR and water through individual feeders. During P2 the PFTN cows had their intake restricted to match the HS cows' intake. All cows were fed a TMR formulated to meet or exceed the NRC (2001) NE_L and MP requirements of a Holstein cow weighing 655 kg, consuming 25 kg of DMI daily, and producing 45 kg/d of milk with 4.0% fat and 3.10% true protein. Cows were fed twice daily (0900 and 1900 h); orts were recorded

daily before the morning feeding and daily DMI data are presented. Samples of fresh TMR were collected on d 2 and 4 of each period and were sent to Cumberland Valley Analytical Services (Waynesboro, PA) for analysis by wet chemistry. Ingredient and average chemical composition of the TMR is shown in Table 1.

Temperature-Humidity Index

Relative humidity and ambient room temperatures were digitally logged every hour for the duration of the experiment. For this, 2 data loggers (model number EL-USB-2+; Lascar Electronics, Whiteparish, Salisbury, UK) were placed at opposite ends of each animal room, for a total of 4 loggers. Each data logger was positioned 145 cm above flooring. Data were downloaded after each cohort finished P2 and were compiled using EL-WIN-USB data logging software (Lascar Electronics). Within each room, RH and ambient temperatures were averaged hourly across the 2 loggers. Furthermore, RH and ambient room temperatures were used to calculate hourly THI with the following equation from Davis et al. (2003): $THI = 0.8 \times T + [RH \times (T - 14.4)] + 46.4$, where T = mean hourly ambient temperature in °C and RH = mean hourly RH divided by 100.

Table 1. Ingredient and nutrient composition of diets[1]

Item	Value
Ingredient, % of DM	
Corn silage	48.3
Grass hay	2.2
Corn grain	18.8
Soybean meal, 48% CP	5.2
Processed soybean meal[2]	11.3
Dried distillers grain with solubles	7.5
Supplement[3]	6.3
Chemical analysis, % ± SD	
CP	17.2 ± 1.2
NDF	32.6 ± 1.3
ADF	18.9 ± 2.5
Crude fat	4.0 ± 0.1
Starch	27.9 ± 0.3
Ash	6.8 ± 3.8
NE_L, Mcal/kg of DM	1.67 ± 0.04

[1]Values listed represent an average of TMR samples collected during the trial. Dietary DM averaged 50.5%.

[2]SoyPlus, cooker-expeller processed soybean meal (Landus Cooperative, Ames, IA).

[3]Each kilogram of supplement DM contained 0.415 kg of ground soybean hulls, 0.119 kg of fat supplement, 0.119 kg of sodium bentonite, 0.148 kg of sodium bicarbonate, 0.059 kg of calcium carbonate, 0.074 kg of sodium chloride, 0.024 kg of magnesium oxide, 0.018 kg of trace mineral premix, 0.005 kg of sodium selenite (0.06% Se), 0.006 kg of vitamin ADE premix, 0.007 kg of vitamin E premix, 0.001 kg of Rumensin (200 g of monensin sodium/kg; Elanco), and 0.006 Clarify larvicide premix (0.67% diflubenzuron; Central Life Sciences).

Clinical Measurements

Clinical measurements were obtained throughout P1 and P2. Rectal temperatures (°C) and respiration rates (respirations/min) were collected twice daily before each milking event. Rectal temperatures were measured using a digital thermometer (Mabis, Waukegan, IL). Respiration rates were determined visually by counting thoracic movement for 15 s and then multiplying by 4. Before analysis, clinical data were averaged by day. Cow BW was measured on d 4 of P1 and P2. The change in BW over P1 and P2 was calculated by subtracting the BW on d 4 of P1 from the initial BW and by subtracting the BW on d 4 of P2 from the BW on d 4 of P1.

Milk Sampling and Analysis

After each milking event in P1 and P2, individual cow milk weights were recorded and representative 50-mL samples were collected and stored at 4°C with a preservative (bronopol tablet; D&F Control System, San Ramon, CA). Milk was stored for a maximum of 3 d before being sent to a commercial laboratory (Lancaster DHI, Manheim, PA) for analysis. Fourier-transform mid-infrared spectrometry was used for analysis of fat, true protein, other solids, and MUN. Somatic cell count was determined by flow cytometry. Individual milk and milk component yields were summed by day. Milk component concentrations were calculated by day by summing the yield of individual components for both milking events and dividing by the total milk yield. Daily ECM and 3.5% FCM were calculated using the following formulas described by Ylioja et al. (2018): $ECM = (0.337 \times \text{milk yield}) + (12.95 \times \text{fat yield}) + (7.65 \times \text{protein yield})$; $3.5\% \text{ FCM} = (0.432 \times \text{milk yield}) + (16.216 \times \text{fat yield})$.

Energy Calculations

Each cow's daily energy intake (Mcal/d) was calculated using actual DMI and average diet energy density for the entire experiment (Table 1). Daily milk energy concentration (Mcal/kg milk) was calculated using each cow's daily milk fat and protein percentages and equation 3–14b in the eighth revised edition of Nutrient Requirements of Dairy Cattle (NASEM, 2021); 4.85% was used for milk lactose. Daily milk energy yield (Mcal/d) was calculated by multiplying daily milk energy concentration by yield in kilograms. Daily maintenance energy (Mcal/d) was calculated using the BW collected during each period and equation 3–13 in the eighth revised edition of Nutrient Requirements of Dairy Cattle (NASEM, 2021). Daily energy balance (Mcal/d) was calculated by subtracting the sum of milk energy and maintenance energy from energy intake.

Blood Measurements and Analyses

Two jugular catheters were placed on the last day of the acclimation period and maintained for the duration of the experiment. Jugular catheterization and catheter maintenance procedures were slightly modified from Estes et al. (2018); here, catheters were placed bilaterally (1 catheter in the left jugular vein and 1 in the right jugular vein) as opposed to ipsilaterally (Estes et al., 2018). The right jugular catheters were used for stable isotope infusion on d 3 of each period as part of an aligning, but separate experimental aim (data not shown). Blood samples (7 mL) were collected from the left jugular catheter once daily at 0900 h during P1 and P2 via syringe. Immediately after collection, 2 small drops of whole blood were analyzed for glucose and BHB concentrations using handheld blood glucose and ketone meters, respectively (Precision xtra; Abbott Diabetes Care Inc., Alameda, CA). The remaining blood was transferred to labeled Vacutainer tubes containing 158 United States Pharmocopeia units of sodium heparin. Plasma was harvested after centrifugation at 1500 × *g* at 4°C and frozen in 1.5 mL aliquots at −20°C until later analysis.

At a later date, thawed blood plasma aliquots were used to measure concentrations of: nonesterified fatty acids (**NEFA**), insulin, LBP, and plasma urea nitrogen (**PUN**). Concentrations of NEFA, insulin, and LBP were analyzed using commercially available kits: NEFA (HR Series NEFA-HR(2), no. 995–34791, 993–35191; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), insulin (10–1201–01, Mercodia AB, Uppsala, Sweden), and LBP (Hycult Biotech, Uden, the Netherlands). Plasma urea nitrogen was determined according to the method of Chaney and Marbach (1962) as modified by Weatherburn (1967). Insulin and LBP were analyzed in duplicate and PUN and NEFA in triplicate. Values were averaged before statistical analysis. In the insulin assay, "low" and "medium" insulin standards manufactured from human serum and insulin were included on each plate (catalog #10–1221–01, Mercodia AB, Uppsala, Sweden). Plasma from a single cow and day was included across all sample plates for determination of interassay variation. Intra- and interassay coefficients of variation were 3.3% and 4.5% for NEFA, 6.2% and 7.4% for insulin, 3.3% and 14.0% for LBP, and 2.6% and 4.3% for PUN.

Urinary Catheter Placement

Urinary catheters were placed as described by Cunningham et al. (1955) at 2100 h on d 2 of P1 and P2, were maintained for approximately 24 h once inserted, and then removed. Briefly, the area directly above the intervertebral space between the fifth sacral vertebrae and the first caudal vertebrae was clipped and then scrubbed 6 times, alternating between 10% povidone-iodine solution (Betadine, Purdue Pharmaceuticals, Stamford, CT) and 70% ethanol. A caudal epidural was administered by inserting an 18-gauge, 1.5-inch (3.81 cm) needle (Becton, Dickinson and Company, Franklin Lakes, NJ) into the sacrococcygeal intervertebral space and injecting 6.5 mL of 2% lidocaine (Covetrus, Dublin, OH). After administering the caudal epidural, the vulva was scrubbed 6 times as previously described. Next, 10 mL of sterile lubricant (Surgilube, HR Pharmaceuticals Inc., York, PA) mixed with 1 mL of 2% lidocaine were manually applied to the vulva. Using sterile technique, a new urinary catheter (All silicone Bard Foley 22 Ch/Fr, 75 cc, medium length, round tip, ribbed balloon, C.R. Bard Inc., Covington, GA) was then inserted into the urethral opening and gently advanced until reaching the bladder. The catheter balloon was then filled with 60 mL of sterile saline. To ensure that urine was collected into a plastic container, one end of a 10 ft (3.048 m) piece of Tygon tubing (0.64 cm inner diameter × 0.14 cm outer diameter; 15.2 m/pack) was attached to the urine drainage port of the catheter and the other end of the tubing was affixed to a collection vessel. To prevent cows from stepping on the tubing and accidentally removing the catheter, we used handmade elastic leg bands with eye hook closures and an elastic loop through which the Tygon tubing was passed before affixing its terminal end to the collection vessel. These leg bands were affixed proximal to each cow's left hock.

GIP Testing

To assess total-tract GIP, a drench solution containing sucralose (2 g/kg of $BW^{0.75}$; Chemical Abstracts Service [**CAS**] no. 56038–13–2; molecular weight [**m.w.**] = 397.64 g/mol; Bulk Supplements, Henderson, NV), D-mannitol (0.6 g/kg of $BW^{0.75}$; CAS no. 69–65–8; m.w. 182.17 g/mol; Acros Chemicals, Vernon Hills, IL), and Cr-EDTA (0.26 g/kg of $BW^{0.75}$) was administered to each cow via an oral-esophageal drench at approximately 2000 h on d 3 of P1 and P2. The drench solution for each cow was mixed immediately before use and the total volume administered to each cow was 4 L. The Cr-EDTA used in the drench solution was produced in 2-L batches up to 14 d before intended use, as described by (Udén et al., 1980). Briefly, 28.4 g of $Cl_3CrH_{12}O_6$ (CAS no. 10060–12–5; m.w. 266.44 g/mol; Sigma-Aldrich, St. Louis, MO) was dissolved in 600 mL of double-distilled water. In a separate flask, 40 g of $C_{10}H_{18}N_2Na_2O_{10}$ (CAS ID: 6381–92–6; m.w. 372.24 g/mol; Sigma-Aldrich, St. Louis, MO) was dissolved into 400 mL of double-distilled water. Once dissolved, the solutions were combined in a 2-L beaker, the beaker was covered with a watch glass, and the solution was brought to and held at 97°C for 1 h while mix-

ing. After 1 h at 97°C, the solution was removed from heat and 4 mL of 1 *M* $CaCl_2$ (CAS no. 10043–52–4; m.w. 110.98 g/mol) were immediately added. The solution was allowed to cool for 2 h and NaOH (CAS no.1310–73–2; m.w. 39.9 g/mol; Sigma-Aldrich, St. Louis, MO) pellets were added to raise the pH of the solution to 6 to 7. Double-distilled water was added to bring the final volume of the Cr-EDTA solution to 2 L. All Cr-EDTA produced was pooled and stored at room temperature until use.

From a 300-mL pooled sample of urine representing 3 h before the oral drench administration, 50 mL were aliquoted for later analysis of baseline sucralose and D-mannitol and 200 mL were aliquoted for later analysis of baseline Cr. Similarly, beginning at the time of oral drenching (approximately 2000 h on d 3 of P1 and P2) and extending thru 24 h, each cow's total urine output was collected and the total volume of urine produced was recorded; after 24 h of continuous urine collection, a 200-mL and a 50-mL representative sample were obtained. Aliquoted urine samples were frozen and stored at −20°C until later analysis of sucralose, D-mannitol, and Cr-EDTA concentrations.

Frozen 50-mL urine aliquots were delivered to ECC Test Laboratory (Ashland, VA) for D-mannitol and sucralose analysis via HPLC-MS using methods we helped validate (Ellett et al., 2022). Briefly, samples were allowed to thaw at room temperature, vortexed for 30 s, and then centrifuged for 5 min at 3,645 × *g* and 21°C. Samples were run on a triple-quadrupole liquid chromatography machine (Agilent Triple Quad MS [LC/TQ]; Agilent) using a hydrophilic interaction liquid chromatography column (Agilent InfinityLab Poroshell 120 HILIC; Agilent). In all, 15 µL of sample was injected onto the column and eluted with an isocratic mixture of 90% acetonitrile (mobile phase A) and 10% aqueous ammonium hydroxide over the course of a 5-min run. The commercial laboratory used a prepared series of standards for sucralose and D-mannitol using analytical-grade reagents. Sugar markers were detected and identified by their retention times and mass spectral analysis. Resulting data were back transformed to reflect the concentration of sucralose and D-mannitol excreted in the 24 h total collection period. The limit of detection for D-mannitol and sucralose was 100 ppb and the limit of quantification was 25 ppb (Ellett et al., 2022).

For urinary Cr-EDTA analysis, 4 mL of thawed urine and 4 mL of nitric acid were placed in an open MARSXpress Teflon digestion tube (CEM Corporation, Matthews, NC) and left uncovered to incubate overnight at room temperature. Next, 2 mL of 30% H_2O_2 was added to each digestion tube; tubes remained uncovered and were incubated for 1 h at room temperature. Then, all digestion tubes were sealed and placed in a MARSXpress rotor within a MARS6 microwave (CEM Corporation, Matthews, NC). Microwave digestion settings were as follows: 20 min ramp up to 200°C, hold at 200°C for 15 min, cool down for 15 min. All urine samples were digested in duplicate, and each duplicate sample was sent to the Virginia Tech Soils Laboratory (Blacksburg, VA) for inductively coupled plasma MS analysis of total Cr. The Cr and sucralose marker dose and marker dose recovery was calculated using the following equation:

$$\text{Marker dose recovery} = (\text{mass of marker recovered}/\text{mass of marker dosed}) \times 100.$$

Statistical Analysis

Before analysis, data were tested for normality using the Univariate procedure, and outliers were identified and removed based on an absolute studentized residual value >3. Clinical measurements, milk production, energy balance, and blood analyte data for P2 were analyzed using the GLIMMIX procedure in SAS, version 9.4 (SAS Institute Inc., Cary, NC). Treatment (HS or PFTN), day (1 to 4), cohort (1 or 2), and their 2-way interactions were fixed model effects; cow nested within treatment by cohort was the random term. Initial cow BW and average daily response variable data collected during P1 were used as covariates where covariate $P < 0.05$. All cows lacked MUN data for P1 due to an error requesting this analysis; hence baseline MUN values were not tested as a covariate in the analysis of P2 MUN.

Change in BW, urine volume, and GIP data were analyzed using the GLIMMIX procedure in SAS using a similar model as described above with the exclusion of day in the model; cow (treatment × cohort) was the random term. Covariates considered in this model were initial cow BW and single-time-point data collected during P1. The urine analysis data set included data from 13 cows (HS, n = 7; PFTN, n = 6); in addition to the HS cow mentioned previously, 2 PFTN cows were removed from the urine data set due to urinary catheter malfunctions. The correlation procedure of SAS was used to determine relationships of selected variables. Least squares means with their SE are reported, and fixed effects were considered significant when Bonferroni-adjusted $P < 0.05$.

RESULTS

The THI data are reported in Figure 1. During P1, THI averaged 64 for both HS and PFTN. Beginning on d 1 of P2 for HS treatment, 4 d of cyclical HS were imposed. During P2, daily THI averaged 78 for HS and 64 for PFTN. As expected, rectal temperature and respiration rate were higher in HS cows compared with PFTN beginning on d 2 of P2 and extending thru the end of the

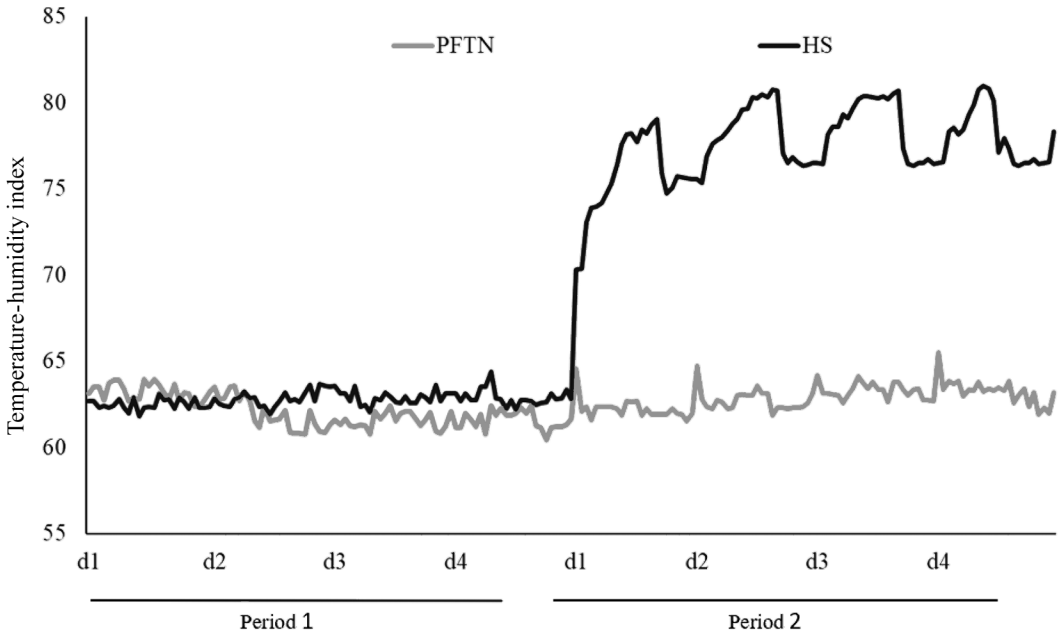

Figure 1. Average hourly temperature-humidity index (THI) during the 4 d of period 1 and period 2, respectively. Temperature-humidity index was calculated using the following equation: THI = 0.8 × T + RH × (T − 14.4) + 46.4, where T represents temperature in °C and RH represents relative humidity. All cows were housed in thermoneutral conditions with ad libitum intake during period 1. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group at the start of period 2.

experiment (treatment × day, $P < 0.01$, Figures 2A and 2B).

Exposure to HS lowered DMI by 35% (day, $P < 0.01$, Figure 3A) and, by design, there were no treatment differences (treatment, $P = 0.49$, Figure 3A). There was a cohort × day interaction ($P = 0.002$, Figure 3A) with cohort 2 eating an average of 0.89 kg of DMI/d more than cohort 1 (data not shown).

Milk yield at the start of the experiment averaged 43 ± 5.6 kg/d for all cows (Figure 3B). Milk yield was not affected by treatment until d 4 of P2, wherein HS cows produced 5 kg/d less milk than PFTN cows (treatment × day, $P < 0.01$, Figure 3B). By d 4 of P2, overall milk yield was reduced 25% from the baseline in the HS cows and 16.5% for PFTN cows. Like daily milk yield, daily yield of milk protein was not affected by treatment until d 4 of P2, when HS cows yielded less daily milk protein (1.05 vs. 0.87 ± 0.04 kg/d, for PFTN and HS, respectively; treatment × day, $P < 0.01$, Figure 3C) than PFTN cows. Similar temporal patterns were observed for decreased daily milk fat yield on d 4 (1.4 vs. 1.25 ± 0.05 kg/d, for PFTN and HS, respectively; treatment × day, $P < 0.03$, Figure 3D). Energy-corrected milk (Figure 3E) and 3.5% FCM (Figure 3F) were lower on d 4 for the HS cows (treatment × day, $P = 0.05$ and $P < 0.01$, respectively).

Milk true protein percent decreased equally in HS and PFTN cows over time from 2.9% ± 0.03% on d 1 of P2 to 2.75% ± 0.03% on d 4 of P2 (Day, $P < 0.01$, Figure 4A). Milk fat percent was not affected by any fixed model effects and averaged 3.8% ± 0.1% for all cows (Figure 4B). Milk other solids yield was lower on d 4 of P2 for HS cows (2.2 vs. 1.9 ± 0.06 kg/d, for PFTN and HS, respectively; treatment × day, $P < 0.01$, Figure 4C). Milk other solids percent was affected by cohort (cohort, $P < 0.01$, Figure 4D). SCC was not affected by any model terms and averaged 250,000 ± 7,000 cell/mL for all cows (Figure 4E). Milk urea nitrogen concentration responded differently over time in PFTN and HS cows; MUN in HS cows exceeded that of PFTN cows by 7.0 mg/dL beginning on d 3 of P2 (treatment × day, $P < 0.01$, Figure 4F).

Daily energy intake and daily energy balance differed significantly by day by cohort (day × cohort, $P < 0.01$, Figures 5A and 5B). Daily milk energy was lower for HS cows on d 4 of P2 (22.9 vs. 26.6 ± 0.44 Mcal/d for PFTN and HS cows, respectively, treatment × day, $P < 0.01$, Figure 5C). There was no difference between treatment groups or cohorts for the change in BW during P2 (data not shown). Final BW averaged 590 kg for HS cows and 603 kg for PFTN cows.

Plasma insulin concentrations were higher for the HS cows on d 4 of P2 (0.48 vs. 0.28 ± 0.08 µg/L for HS and PFTN cows, respectively; treatment × day, $P = 0.02$, Figure 6A) and differed by cohort (cohort, $P = 0.05$, Figure 6A). There was a tendency for PUN to be higher in the HS cows (18.8 vs. 14.7 ± 0.78 mg/dL for HS and PFTN cows, respectively; treatment, $P = 0.07$, Figure

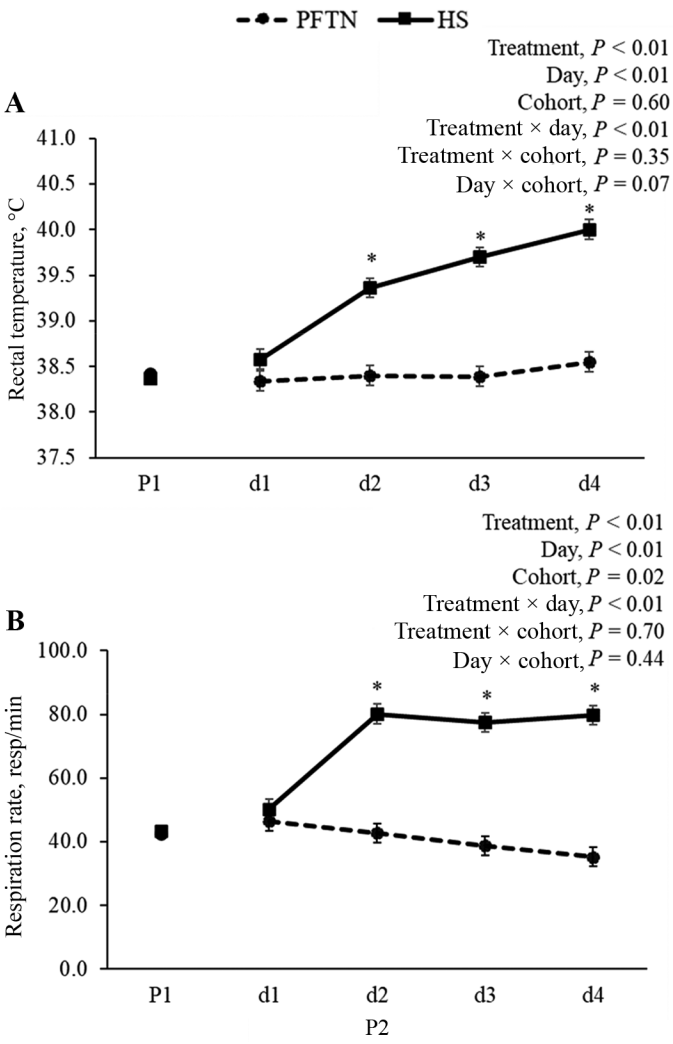

Figure 2. Effects of exposure to heat stress (HS) conditions or pair-feeding in a thermoneutral environment (PFTN) on (A) rectal temperature and (B) respiration (resp.) rate. Solid lines with solid squares represent HS cows, and dashed lines with solid circles represent PFTN cows. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group. Period 1 (P1) represents the average of the baseline data collected in that period where all cows were exposed to thermoneutral conditions with ad libitum intake. Days 1 to 4 represent days the cows were exposed to HS or PFTN. Data are represented as treatment × day LSM ± SEM. The SEM calculations for rectal temperature and respiration rate included 7 cows. The HS treatment group had significantly elevated rectal temperatures and respiration rates on d 2, 3, and 4 of period 2 (P2) when compared with the PFTN treatment group. *Significant treatment × day difference of $P < 0.05$.

6B). Plasma glucose differed by treatment (58 vs. 54 ± 1.03 mg/dL, for PFTN and HS cows, respectively; $P = 0.05$, Figure 6C), by day ($P < 0.01$, Figure 6C), and by cohort ($P < 0.01$, Figure 6C). Plasma BHB differed by day ($P < 0.01$, Figure 6D) and LBP differed by cohort ($P = 0.04$, Figure 6E). Plasma NEFA concentration differed by day ($P = 0.05$, Figure 6F) and cohort ($P = 0.04$, Figure 6F).

The 24-h total urine volume was 7 L higher for the HS cows (Table 2, treatment, $P = 0.01$). Sucralose, D-mannitol, and Cr were undetectable in all baseline samples collected 3 h before dosing GIP markers (data not shown). No difference in oral sucralose dose recovery or the urine concentration of sucralose was observed. The recovery of orally dosed Cr tended to differ between cohorts (Table 2, treatment, $P = 0.08$), and the urine concentration of Cr differed by treatment by cohort (Table 2, treatment, $P = 0.02$). Urinary D-mannitol was undetectable in all urine samples (data not shown).

DISCUSSION

Heat stress in dairy cattle occurs when cattle accumulate heat faster than they can dissipate it. Lactating dairy cattle are especially susceptible to HS because of the increased heat of metabolism and digestion. Ambient temperature and RH work synergistically to influence a cow's ability to dissipate heat. This relationship between ambient temperature and RH can be summarized in a discomfort index known as the THI. When the daily average THI reaches or exceeds 68, HS-related production losses for lactating cattle are commonly observed (Zimbleman et al., 2009). The primary method of heat dissipation observed in dairy cattle is evaporative heat loss via increased respiration rate (Collier and Gebremedhin, 2015). Use of a pair-feeding design allowed for the differentiation between the direct response to HS (such as decreased intake) and indirect (response to decreased intake) effects of HS on milk yield and composition. Numerous reviews (Baumgard and Rhoads, 2013; Collier and Gebremedhin, 2015; Ríus, 2019; Dahl et al., 2020) and studies (Rhoads et al., 2009; Wheelock et al., 2010; Cowley et al., 2015; Gao et al., 2017; Opgenorth et al., 2021) describe the hallmark responses to HS including reduced voluntary feed intake, increased respiration rate and rectal temperature, and a decreased milk and milk solids yield. In our study, cows exposed to a cyclical THI ranging from 74 to 80 during P2 had a 35% reduction in voluntary feed and energy intake, elevated respiration rate (71 vs. 40 respirations/min for HS vs. PFTN, respectively) and rectal temperature (39. 4°C vs. 38.4°C; HS vs. PFTN). These results indicate that HS was successfully induced. We concluded that reduced feed intake was responsible for 66% of the decrease in milk yield observed on d 4, which is in line with the 63% to 75% decrease in milk yield reported by Fontoura et al. (2022). The yield (and not concentration) of milk fat, protein, and other solids (lactose plus ash) were lower in HS cows, aligning with an overall volume reduction. In this experiment, reduction in both protein and fat yields was partially responsible for the reduction in ECM and 3.5% FCM on d 4 in the HS group (Figure 3). We observed a

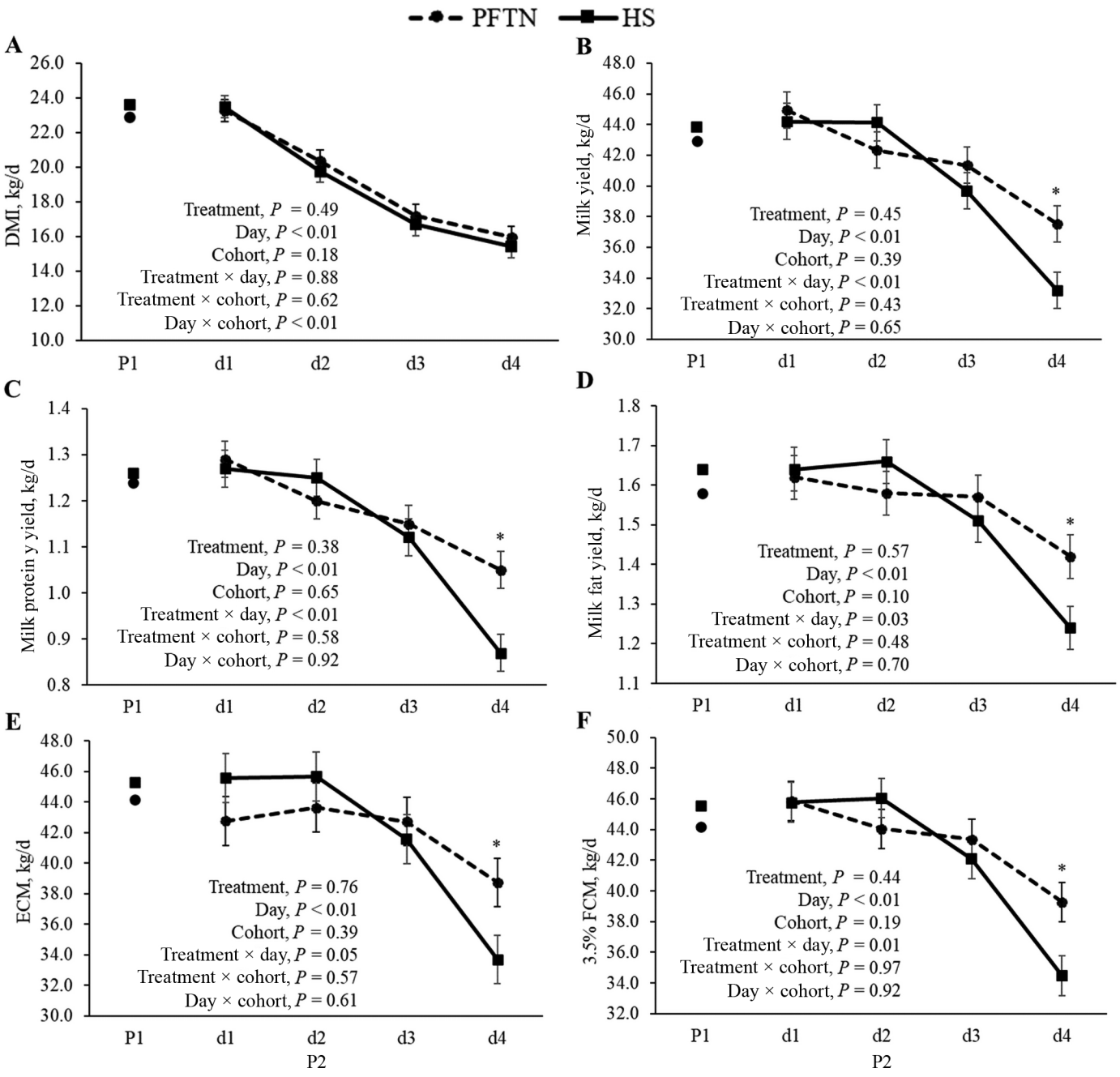

Figure 3. Effects of exposure to heat stress (HS) conditions or pair-feeding in a thermoneutral environment (PFTN) on (A) DMI, (B) milk yield, (C) milk protein yield, (D) milk fat yield, (E) ECM, and (F) 3.5% FCM. Solid lines with solid squares represent HS cows, and dashed lines with solid circles represent PFTN cows. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group. Period 1 (P1) represents the average of the baseline data collected in that period where all cows were exposed to thermoneutral conditions with ad libitum intake. Days 1 to 4 represent days the cows were exposed to HS or PFTN. Data are represented as treatment × day LSM ± SEM. The SEM calculations for DMI, milk yield, milk protein yield, milk fat yield, ECM, and 3.5% FCM included 7 cows. DMI decreased by day, and by design, did not differ between treatment groups. The HS treatment group had significantly lower milk yield, milk protein yield, milk fat yield, ECM, and FCM on d 4 of period 2 (P2) when compared with the PFTN treatment group. *Significant treatment × day difference of $P < 0.05$.

20.6% decrease in milk protein yield on d 4 of HS which is in line with Gao et al. (2017), who reported a 19% decrease in protein yield in HS cows compared with their PFTN counterparts.

Cowley et al. (2015) attributed reduction of milk protein during HS to downregulated protein synthesis within the mammary gland. An alternate explanation is that reduced mammary blood flow during HS may limit

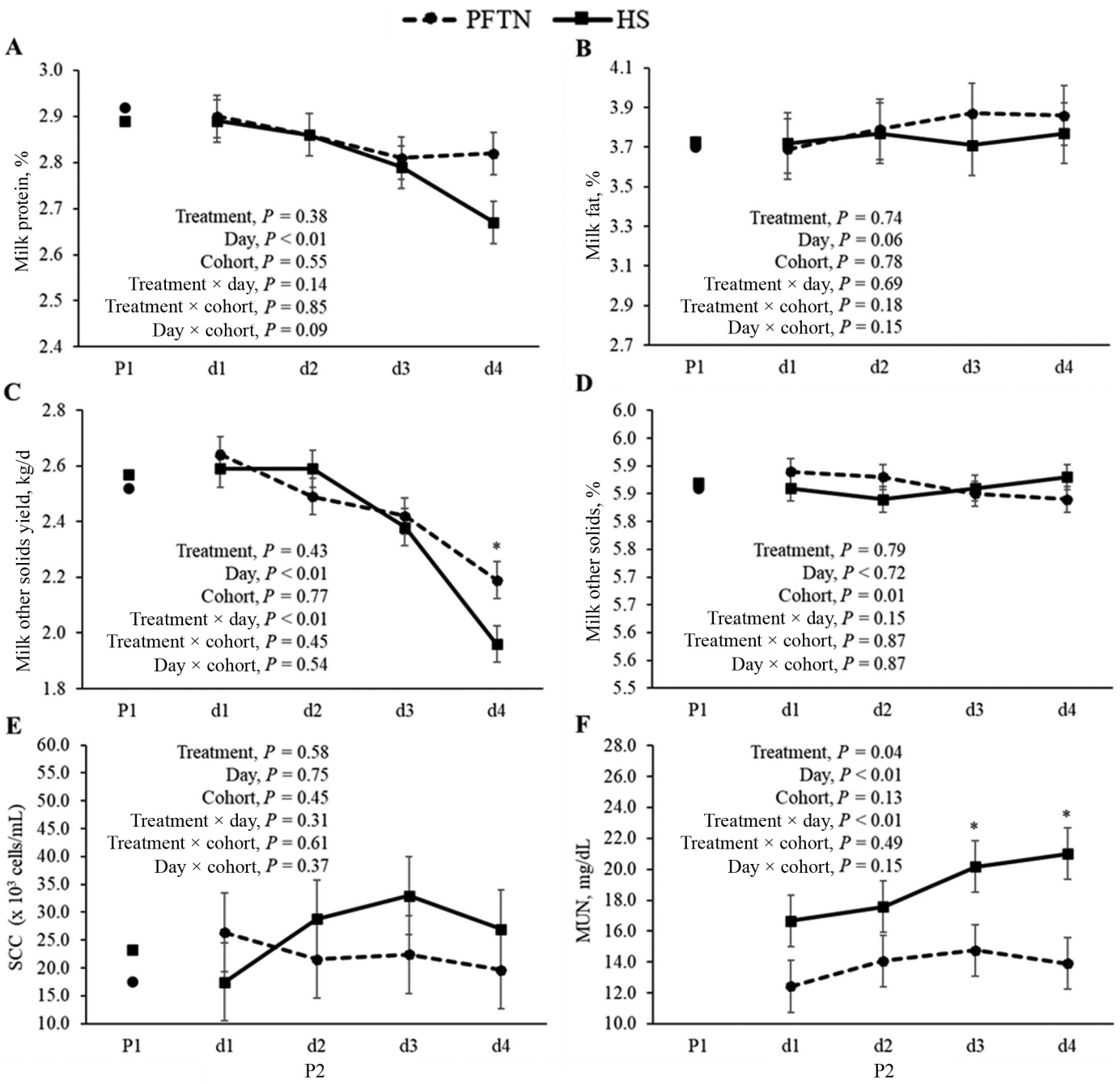

Figure 4. Effects of exposure to heat stress (HS) conditions or pair-feeding in a thermoneutral environment (PFTN) on (A) milk protein %, (B) milk fat %, (C) milk other solids yield, (D) milk other solids %, (E) SCC, and (F) MUN. Solid lines with solid squares represent HS cows, and dashed lines with solid circles represent PFTN cows. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group. Period 1 (P1) represents the average of the baseline data collected in that period where all cows were exposed to thermoneutral conditions with ad libitum intake. Days 1 to 4 represent days the cows were exposed to HS or PFTN. Data are represented as treatment × day LSM ± SEM. The SEM calculations for milk protein %, milk fat %, milk other solids yield, milk other solids %, SCC, and MUN included 7 cows. Milk urea nitrogen for P1 was not recorded and is not included in panel F. The HS treatment group had significantly higher MUN on d 3 and 4 and lower milk other solids yield on d 4 of period 2 (P2) when compared with the PFTN treatment group. *Significant treatment × day difference of $P < 0.05$.

uptake or use, or both, of AA necessary to support protein synthesis (Guo et al., 2018). Gao et al. (2017) reported that 78% of the plasma reduction of AA in HS cows was due to a reduction of gluconeogenic AA, presumably for utilization as gluconeogenic precursors. A common observance in HS experiments across many species at

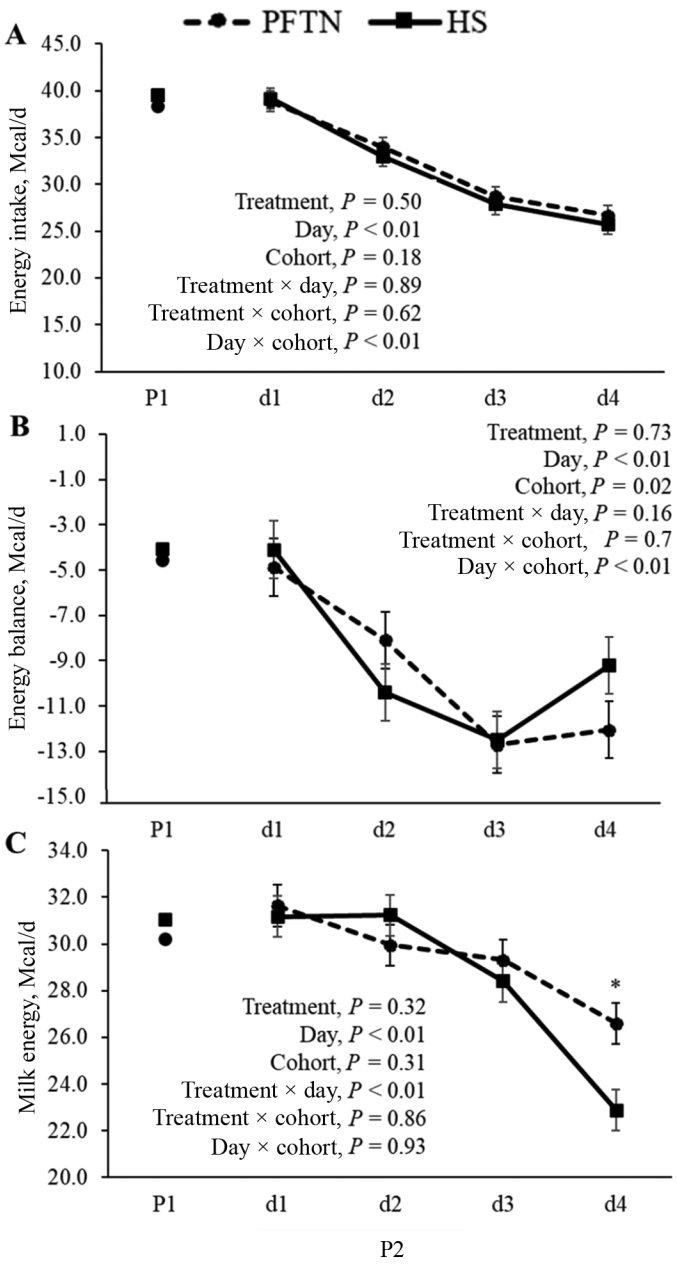

Figure 5. Effects of exposure to heat stress (HS) conditions or pair-feeding in a thermoneutral environment (PFTN) on (A) energy intake, (B) energy balance, and (C) milk energy. Solid lines with solid squares represent HS cows, and dashed lines with solid circles represent PFTN cows. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group. Period 1 (P1) represents the average of the baseline data collected in that period where all cows were exposed to thermoneutral conditions with ad libitum intake. Days 1 to 4 represent days the cows were exposed to HS or PFTN. Data are represented as treatment × day LSM ± SEM. The SEM calculations for energy intake, energy balance, and milk energy included 7 cows. The HS treatment group had significantly lower milk energy on d 4 of period 2 (P2) when compared with the PFTN treatment group. *Significant treatment × day difference of $P < 0.05$.

various physiological stages is elevated PUN (Wheelock et al., 2010; Gao et al., 2017; Opgenorth et al., 2021; Fontoura et al., 2022). We observed a tendency for elevated PUN in HS cows on d 3 and d 4 in P2 and elevated MUN across P2, indicating altered nitrogen metabolism.

The diminished mammary protein synthetic capacity of HS cows observed here could conceivably stem from altered mitochondrial function due to oxidative stress brought about by reduced oxygen delivery to the mammary gland (Guo et al., 2021), and the inhibition of mitochondrial ATP synthesis (Monti et al., 2001; Slimen et al., 2014). This type of altered mitochondrial function leads to accumulation of reactive oxygen species, which can further affect cell function (Guo et al., 2021). Furthermore, increased oxidative stress can lead to tissue-specific insulin resistance in humans (Ceriello and Motz, 2004; Maciejczyk et al., 2019), mice (Yao et al., 2022), and dairy cattle during the transition period (Abuelo et al., 2016; Youssef and El-Ashker, 2017). Paradoxically, insulin has been found to increase casein synthesis within the mammary gland (Menzies et al., 2009; Ríus et al., 2010). Taken together, mammary oxidative stress resulting in reduced insulin sensitivity within the mammary gland may partially explain why elevated circulating insulin levels do not result in an increased milk protein yield. Future work in this area seems warranted.

Elevated circulating insulin concentration is a highly conserved response to HS (Baumgard and Rhoads, 2013) and a major effector of depressed circulating NEFA commonly observed in animals subjected to HS (Baumgard and Rhoads, 2013). Presumably this is because insulin is generally antilipolytic, inhibiting mobilization of fatty acids from adipocytes (Arner et al., 1981). Although we observed a 59.5% increase in circulating insulin on d 4 of HS when comparing the HS cows to the PFTN cows, we surprisingly did not observe a difference in circulating NEFA. Fontoura et al. (2022) reported that HS cows mobilized body fat at a lower rate than thermoneutral pair-fed cows, and Wheelock et al. (2010) report NEFA being 1.63× higher in PFTN cows than HS cows. Circulating NEFA concentration depends, to a large extent, on the amount of subcutaneous fat available for mobilization (Adewuyi et al., 2005). Although BCS before and after heat exposure was not measured in our experiment, it is possible cow adiposity contributed to our lack of treatment differences and BHB. Circulating BHB increased by day but did not differ by treatment. The similar degrees of negative balance observed in both treatment groups is also contributing to the lack of NEFA and BHB response.

Studies show that HS reprioritizes metabolic fuel source utilization in favor of glucose (Wheelock et

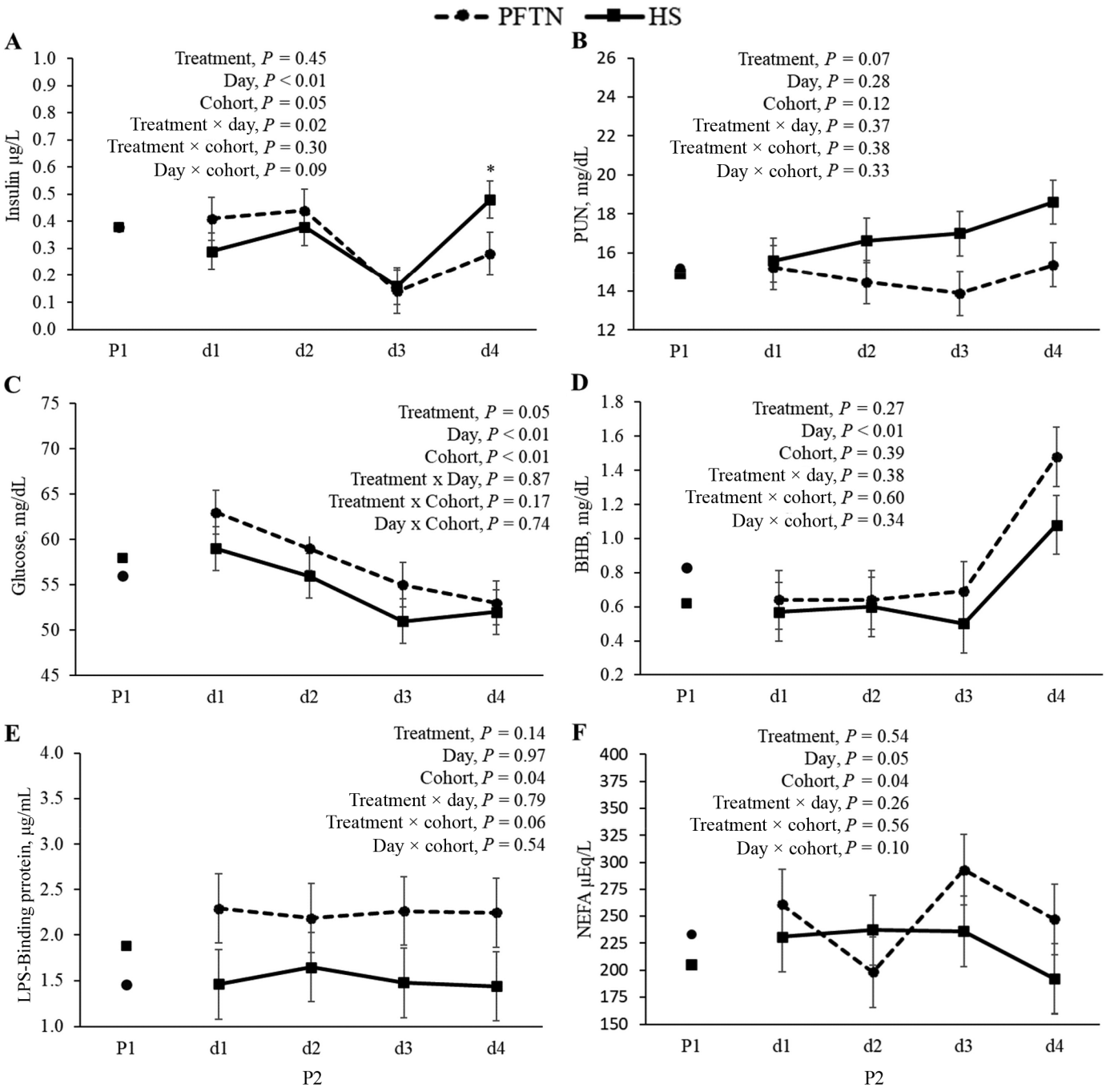

Figure 6. Effects of exposure to heat stress (HS) conditions or pair-feeding in a thermoneutral environment (PFTN) on circulating levels of (A) insulin, (B) plasma urea nitrogen (PUN), (C) glucose, (D) BHB, (E) LPS-binding protein (LBP), and (F) nonesterified fatty acids (NEFA). Solid lines with solid squares represent HS cows, and dashed lines with solid circles represent PFTN cows. All cows were randomly assigned to a HS (n = 7) or a pair-fed thermoneutral (n = 8) treatment group. Period 1 (P1) represents the average of the baseline data collected in that period where all cows were exposed to thermoneutral conditions with ad libitum intake. Day 1 to 4 represent days the cows were exposed to HS or PFTN. Data are represented as treatment × day LSM ± SEM. The SEM calculations for insulin, PUN, glucose, BHB, LBP, and NEFA included 7 cows. The HS treatment group had significantly higher insulin on d 4 of period 2 (P2) when compared with the PFTN treatment group. *Significant treatment × day difference of $P < 0.05$.

al., 2010; Baumgard and Rhoads, 2013; Stewart et al., 2022). It is believed that this is a protective mechanism that evolved to prevent unnecessary metabolic heat production during HS. β-Oxidation of NEFA produces more heat than carbohydrate metabolism (Baumgard and Rhoads, 2013). In line with those findings, we observed a

Table 2. The effect of heat stress (HS) and pair-feeding in thermoneutral conditions (PFTN) on urine production and urine recovery of orally dosed sucralose and chromium

	Period 1[1]		Period 2[2]			*P*-value		
Item	Mean	SD	PFTN	HS	SEM	Treatment	Cohort	Treatment × cohort
Urine volume, L/d	23.2	4.60	16.7[a]	23.7[b]	1.60	0.01	0.38	0.88
Sucralose								
Recovery of oral dose, %	9.00	3.00	11.72	13.45	30	0.69	0.38	0.73
Urine concentration, mg/L	0.98	0.24	0.99	1.23	0.09	0.11	0.16	0.63
Cr								
Recovery of oral dose, %	1.69	0.24	1.62	1.66	0.13	0.80	0.08	0.30
Urine concentration, mg/L	3.66	0.78	4.06	3.68	0.41	0.52	0.02	0.02

[a,b]Differing superscripts within a row indicate a significant difference in period 2 ($P \leq 0.5$).

[1]During period 1, all cows were housed in thermoneutral conditions and were fed ad libitum. Values listed for period 1 represent the baseline mean values and the SD for all cows (n = 12).

[2]During period 2, the cows in the HS treatment group were exposed to cyclical HS conditions (THI range of 74–80). The PFTN cows were exposed to thermoneutral conditions (THI of 64) and their feed intake was reduced to match the intake of the HS cow.

significant decrease in circulating glucose in response to HS. A preference for glucose metabolism in tissues such as skeletal muscle may explain the observed decrease in blood glucose. It is also important to note that HS does not always result in decreased circulating glucose when comparing HS cows to their PFTN counterparts (Rhoads et al., 2009; Wheelock et al., 2010).

Heat stress has been reported to alter intestinal barrier integrity and function (Koch et al., 2019; Opgenorth et al., 2021; Fontoura et al., 2022; Guo et al., 2022). It is hypothesized that the diminished intestinal integrity increases GIP for endotoxins, such as LPS, which activates the immune system and shifts nutrients away from milk component synthesis (Kvidera et al., 2017b). LPS is a bacterial endotoxin found on the cell membrane of gram-negative bacteria that elicits an inflammatory response upon exposure. Once LPS enters systemic circulation it interacts with an accessory protein, LBP (Bannerman et al., 2003). We did not observe a difference in LBP in the present study. Taken with our GIP results, this suggests gut-derived LPS entered into circulation and activated an LBP response at similar rates between treatments.

In a series of separate in vitro rumen fermentation experiments (Ellett et al., 2022), and confirmed in vivo here, we found that D-mannitol (but not sucralose) is rapidly degraded by rumen microbes. This means that sucralose but not D-mannitol is a suitable indigestible marker for functioning ruminants. To our knowledge, this is the first description of oral dosing and urine recovery of sucralose as a measure of total-tract GIP in functioning ruminants. Sucralose and Cr-EDTA were undetectable in all urine samples collected 3 h before dosing GIP markers. This indicates that 3 d between dosing was enough time for the markers to be cleared from the body and that all Cr and sucralose recovered in urine came from the orally dosed GIP marker solution. Importantly, although we were able to recover Cr and sucralose in urine, we did not detect differences in percentage of dose recovered or total yield of either marker. Our results indicate that the degree of GIP was similar across treatments. These findings are in contrast with a recent report where HS increased GIP (Fontoura et al., 2022) and our own hypothesis. Fontoura et al. (2022) assessed GIP by orally dosing a uniform amount of Cr-EDTA with recovery in blood plasma. We orally administered weight-dependent doses of Cr-EDTA (0.26 g/kg of $BW^{0.75}$) and sucralose (2 g/kg of $BW^{0.75}$) and measured dose recovery in urine. Total urine collection allowed for urine pool size to be calculated.

It is worth mentioning that feed restriction is a documented cause of increased GIP (Kvidera et al., 2017c). In our experiment, cows on both treatments experienced a similar degree of feed reduction; it remains possible that feed restriction effects might have uniformly masked an underlying direct effect of HS on GIP. When analyzing our GIP data with period included in the model, there was a tendency for increased percentage of recovery for the orally dosed sucralose in urine during P2 ($P = 0.08$, data not shown), but not for the percentage of recovery for the orally dosed Cr in urine ($P = 0.61$, data not shown). The potential masking effect of equally reduced DMI between treatments apparently did not occur in similar work (Fontoura et al., 2022). Extending total urine collection would have likely increased the percentage of GIP marker recovery and potentially allowed for the detection of a treatment difference. A potential reason why we did not observe any treatment effects in GIP might be attributed to our animal numbers (6 PFTN and 7 HS cows). Fontoura et al. (2022) had 12 cows per treatment. Observed cohort differences are difficult to explain and may be attributed to the source herds for these purchased animals and is compounded by the small sample size. Cohort 2 was enrolled in the trial at the end of July, as opposed to May for cohort 1, and may have been more

resistant to HS conditions due to a longer exposure to ambient summertime conditions before enrollment in our experiment.

Although immune activation may be a component in the reduction of milk yield in lactating dairy cows exposed to mild HS, our results do not support this hypothesis. Under the conditions of our experiment, where mild HS was observed, GIT-derived LPS may not be the driving factor for depressed milk yield. Although our responses to HS appear blunted when compared with other studies, we observed a reduced milk yield on d 4 of HS. Altered blood flow to the mammary gland resulting in hypoxia, a reduction of available preformed fats, and the change in whole-body nitrogen metabolism, could be a significant source of depressed milk production. Insulin resistance within the mammary gland may also limit the rate of protein synthesis.

CONCLUSIONS

Elucidating the physiological response to HS that reduces milk yield and alters milk composition will aid in developing effective and efficient management strategies to mitigate production losses as the global temperature continues to rise. In line with previous reports, we demonstrated that 4 d of HS decreased milk yield in multiparous Holstein cows independently of decreased voluntary feed intake. Sucralose, but not D-mannitol, is a viable alternative marker to assess GIP. Neither the recovery of urinary sucralose nor Cr-EDTA differed between treatment groups, indicating that HS did not increase GIP; lack of treatment difference in LBP corroborates this. Under the conditions of this experiment and contrary to the hypothesis, results suggest that immune system activation through gut-derived LPS did not contribute to the observed decline in milk yield during HS.

NOTES

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Abbreviations used: CAS = Chemical Abstracts Service; GIP = gastrointestinal permeability; HS = heat stress; LBP = LPS-binding protein; m.w. = molecular weight; NEFA = nonesterified fatty acids; P1 = experimental period 1 (thermoneutral with ad libitum feed); P2 = experimental period 2 (heat stress condition treatment); PFTN = pair-fed, thermoneutral housing conditions; PUN = plasma urea nitrogen; THI = temperature-humidity index.

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