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Prepartum heat stress in dairy cows increases postpartum inflammatory responses in blood of lactating dairy cows

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ABSTRACT

Uterine diseases and heat stress (HS) are major challenges for the dairy cow. Heat stress alters host immune resilience, making cows more susceptible to the development of uterine disease. Although HS increases the incidence of uterine disease, the mechanisms by which this occurs are unclear. We hypothesize that evaporative cooling (CL) to alleviate HS in prepartum cows has carry-over effects on postpartum innate immunity. Nulliparous pregnant Holstein heifers were assigned to receive either forced CL that resulted in cool conditions (shade with water soakers and fans; n = 14) or to remain under HS conditions (barn shade only; n = 16) for 60 d prepartum. Postpartum, all cows were housed in a freestall barn equipped with shade, water soakers, and fans. Respiratory rate and rectal temperature during the prepartum period were greater in HS heifers compared with CL heifers, indicative of HS. Although milk production was decreased in HS cows compared with CL cows, the incidence of uterine disease and content of total or pathogenic bacteria in vaginal mucus on d 7 or d 21 postpartum was not affected by treatment. Whole blood was collected on d 21 and subjected to in vitro stimulation with lipopolysaccharide. Lipopolysaccharide-induced accumulation of IL-1β, IL-10, and MIP-1α was greater in blood collected from HS cows compared with CL cows. Our results imply that prepartum HS during late pregnancy has carry-over effects on postpartum innate immunity, which may contribute to the increased incidence of uterine disease observed in cows exposed to prepartum HS.

Key words: heat stress, innate immunity, uterine disease

INTRODUCTION

Heat stress (**HS**) occurs when elevated environmental temperature causes an imbalance between the heat accumulation and heat dissipation mechanisms of an animal (Yousef, 1985). Animals under HS display physiological and behavioral changes that attempt to reduce heat production and optimize heat loss to maintain a thermoneutral body temperature. Elevated environmental temperature combined with elevated humidity make it more difficult for an animal to dissipate heat to the environment and achieve homeostasis. A well described metric for thermal stress that combines ambient temperature and relative humidity is the temperature-humidity index (**THI**). When the average THI is above 68, dairy cows exhibit signs of HS including increased respiratory rate, increased rectal temperature, and reduced milk yield (Zimbelman et al., 2009; Collier et al., 2011). In the subtropical state of Florida, the average THI is above 68 for 257 d of the year (70% of the year; Ferreira et al., 2016). Cows exposed to HS have less DMI and milk production, increased number of inseminations per pregnancy, and have greater number of days open compared with thermoneutral cows (García-Ispierto et al., 2007; Ouellet et al., 2020). Moreover, HS is a risk factor for retained placenta and metritis (DuBois and Williams, 1980; Gernand et al., 2019; Molinari et al., 2022).

Elevated temperatures trigger a stress response characterized by activation of the hypothalamic-pituitary-adrenal axis and subsequent cortisol secretion (Abilay et al., 1975). The effect of a stress response to the immune system is controversial, with reports showing stimulation or suppression of the immune response depending on several factors including the type of stressor, length of exposure to the stressor, and the status of the animal at the time of stress (Dhabhar, 2009; Tao and Dahl, 2013). Humans, pigs, and rats subjected to HS have elevated concentration of proinflammatory cytokines in peripheral blood including IL-1β, IL-6, TNFα, and IFNγ (Bouchama et al., 1993; Ju et al., 2014). In parallel, peripheral lymphocytes isolated from dairy cows

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exposed to HS during the dry period have impaired proliferative potential compared with lymphocytes isolated from cooled cows (do Amaral et al., 2011). There is also evidence suggesting a carry-over effect of HS on innate immune function; particularly, neutrophils from cows exposed to prepartum HS and returned to cooling conditions postpartum have impaired phagocytosis and oxidative burst up to 20 d after the resolution of HS (do Amaral et al., 2011).

Uterine disease affects up to 40% of postpartum cows and is characterized as an infection of the uterus by pathogenic bacteria including *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, or *Prevotella melaninogenica*. To prevent the development of uterine disease, cows mainly rely on limiting the damage caused by pathogens (tolerance), and eliminating pathogens (resistance; Sheldon et al., 2020). Mechanisms of tolerance are not well characterized in the endometrium but are thought to include protection against bacterial cytolysins that damage endometrial cells during uterine infections (Ormsby et al., 2021). Resistance mechanisms in the endometrium are better characterized and depend on the innate immune system initially recognizing pathogen-associated molecular patterns. These pathogen-associated molecular patterns, such as LPS from gram-negative bacteria, are recognized by specific receptors, including Toll-like receptors, present on innate immune cells and endometrial cells (Cronin et al., 2012; Turner et al., 2014). Activation of the innate immune response in the endometrium results in the secretion of proinflammatory cytokines (such as IL-1β and IL-6) and chemokines (such as IL-8 and MIP-1α), which attract and activate immune cells, induce secretion of acute phase proteins required to eliminate pathogens, and are considered resistance mechanisms (Takeuchi and Akira, 2010; Healy et al., 2014; Sheldon et al., 2019; Ormsby et al., 2021)

Given the potential of HS to affect innate immunity, and the increased incidence of uterine disease during summer, we hypothesized that HS affects innate immunity, predisposing cows to uterine disease. Hence, we investigated the effect of prepartum HS on postpartum innate immunity by evaluating bacterial content of the vagina and immune responses of blood in primiparous Holstein cows exposed to controlled HS conditions.

MATERIALS AND METHODS

Animal Enrollment

This experiment was conducted at the University of Florida Dairy Research Unit from June to November, 2019, and was approved by the Institutional Animal Care and Use Committee at the University of Florida (protocol number 201910738). Nulliparous pregnant Holstein heifers were blocked by BCS and PTA for milk yield, housed in the same freestall barn, and assigned to receive forced evaporative cooling (**CL**) that resulted in cool conditions (shade with water soakers and fans; n = 14) or to be subject to HS conditions (barn shade only; n = 16) for 60 d prepartum before their expected calving date. There were 2 HS and 2 CL pens, and animals were randomly assigned to pen within treatment. To account for potential pen effects, the heat and cooling pens were switched midway through treatments. All postpartum cows were housed in the fresh pen of a freestall barn equipped with shade, water soakers, and fans. Incidence of retained placental membranes, fever, ketosis, lameness, metritis, mastitis, diarrhea, and displaced abomasum were recorded for the first 21 DIM. Thermoregulatory measurements, diet information, and subsequent milk yield are reported in detail by Davidson et al. (2021).

Vaginal Mucus and Blood Sampling

Vaginal mucus was collected on d 7 and 21 postpartum from each cow. To collect vaginal mucus, the vulva was thoroughly cleaned with 70% ethanol and dried with paper towel before the insertion of a sterile Metricheck tool (Simcro), consisting of a stainless-steel rod with a rubber collection cup. Once the tool was inserted into the vagina, consistent movements were performed to sample the whole vaginal canal. After removal of the tool, the collected mucus was graded and placed in a sterile bijou tube (Thermo Fisher Scientific). The mucus was graded as follows based on the proportion of pus in the sample: grade 0 = clear vaginal mucus, grade 1 = mucus with flecks of white pus, grade 2 = mucus with less than 50% white pus, grade 3 = mucus with more than 50% white pus, and grade 4 = sanguinopurulent mucus (adapted from Sheldon et al., 2009). Mucus samples were stored at −80°C for further analysis.

Whole blood was collected on d 21 postpartum from coccygeal vessels into evacuated tubes (Vacutainer, Becton Dickson) containing lithium-heparin anticoagulant. Samples were maintained on ice and transported to the laboratory for further processing.

Bacterial DNA Isolation and Quantification of Total Bacterial Load

Isolation of total bacterial DNA from vaginal mucus samples was performed using the DNeasy Power Soil kit (Qiagen) as previously described in (Piersanti et al., 2019). Briefly, samples were thawed on ice and then homogenized by vortexing for 5 min. Samples were

Table 1. The PCR primers used for real-time PCR (F = forward; R = reverse)

Bacteria	Target gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Source
Escherichia coli	*16S*	F–GTTAATACCTTTGCTCATTGA R–ACCAGGGTATCTAATCCTGTT	53.5	Malinen et al. (2003)
Trueperella pyogenes	*plo*	F–GGCCCGAATGTCACCGC R–AACTCCGCCTCTAGCGC	64.5	Belser et al. (2015)
Fusobacterium necrophorum	*ikta*	F–GATTGGGGGATAGCGGTAAT R–GAGCCTCCACATTTAGTCGC	63.0	Cunha et al. (2018)
Prevotella melaninogenica	*phyA*	F–ACAAAGAGGCAAACCAAGCG R–TACGAAGCATCCGTTCAGGG	55.0	In-house

weighed and 250 mg of each sample was added to a tube containing garnet particles and guanidine thiocyanate. Samples were homogenized using 3 cycles of a tissue homogenizer (30 s at 6,000 × *g*, 60 s pause, 30 s at 6,000 × *g*; Precellys 24, Bertin Instruments) with a 5-min incubation on ice between each cycle. Supernatants were collected and added to the DNeasy Power Soil spin columns for purification of DNA following the manufacturer's instructions. Purified DNA was subjected to total bacterial 16S rRNA quantification using the Femto Bacterial DNA Quantification Kit (Zymo Research). Briefly, real-time quantitative PCR was performed using the provided primer mix containing SYTO 9 fluorescent dye and primers targeting 16S rRNA in a 20-μL reaction with 2 μL of total extracted DNA in each well. A CFX Connect Real-Time PCR System (Bio-Rad Laboratories) was used for a 3-step protocol consisting of initial denaturation at 95°C for 10 min, amplification with 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. A standard curve provided with the kit was performed in parallel with the samples to quantify 16S rRNA. Reactions were performed in duplicate and no-template controls were included. Results for total bacterial content are described as nanograms of 16S rRNA per milligram of vaginal mucus.

Detection of Recognized Bacteria in Vaginal Mucus

Quantification of pathogenic bacteria associated with uterine disease was performed using DNA isolated from vaginal mucus samples. Specific primers for *E. coli*, *T. pyogenes*, *F. necrophorum*, and *P. melaninogenica* were designed using the National Center for Biotechnology Information primer-design tool, or were previously published and verified by BLAST (Table 1). Primers were validated for amplification efficiency before analysis and followed MIQE guidelines consisting of a Pearson correlation coefficient $R^2 > 0.98$ and efficiency between 90 and 110% (Bustin et al., 2009). Quantitative real-time PCR was performed in 20-μL reactions containing 18 μL of SYBR Green Master Mix (Bio-Rad Laboratories), 300 n*M* of each forward and reverse primer, and 2 μL of template DNA. A polymerase chain reaction was performed using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories) with a 3-step protocol consisting of enzyme activation at 95°C for 3 min, amplification with 40 cycles of denaturation at 95°C for 10 s, annealing for 10 s (temperature for each primer in Table 1), and extension at 72°C for 30 s. Each reaction was performed in duplicate, and no-template controls and melt curve were included for each assay. For each pathogen, a standard curve with purified DNA was prepared and run in parallel with the samples for pathogen quantification. Total DNA from purified *E. coli* MS499 (Goldstone et al., 2014b), *T. pyogenes* MS249 (Goldstone et al., 2014a), and *F. necrophorum* (supplied by Klibs Galvão, University of Florida) was obtained by extraction of live bacterial cultures using the DNeasy Power Soil kit, as described above. Purified DNA of *P. melaninogenica* was purchased from ATCC (Manassas, VA; #25845). Results were normalized by weight of vaginal mucus and are expressed as cfu/μL per mg of vaginal mucus for *E. coli* and *T. pyogenes* and pg/μL per mg of vaginal mucus for *F. necrophorum* and *P. melaninogenica*.

Whole Blood LPS Challenge Assay

Whole blood collected on d 21 postpartum was challenged with LPS. Briefly, ultrapure *E. coli* O111:B4 LPS (Invivogen) was diluted in warm Dulbecco's modified Eagle medium (Thermo Fisher Scientific) to a 10 μg/mL concentration immediately before each challenge. Two aliquots of 980 μL of whole blood from each cow were transferred to 2-mL snap lock tubes (Eppendorf). Aliquots were treated with either 20 μL of warm Dulbecco's modified Eagle medium as a negative control or with 20 μL of ultrapure LPS for a final concentration of 200 ng/mL. We chose this concentration of LPS to better reflect the physiological exposure of blood during infection, opposed to supraphysiological concentrations (5,000 to 20,000 ng/mL) routinely used by others in

this assay (Jahan et al., 2015; Amadori et al., 2018; Vailati-Riboni et al., 2017, 2019). Samples were placed into a rotating tube holder and maintained at 38.5°C for 4 h. Following incubation, samples were centrifuged at 8,500 × *g* for 10 min at 4°C to collect plasma, which was stored at −80°C for further analysis.

Detection of Inflammatory Mediators in Whole Blood

The plasma from the whole blood challenge assay was analyzed using the Milliplex Bovine Cytokine/Chemokine 15-plex kit (BCYT1–33K, EMD Millipore) according to the manufacturer's instruction. The kit allowed for the simultaneous quantification of IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα, and VEGF-A. Samples were read using a MAGPIX instrument with xPONENT software (Luminex). Samples that had a concentration above the range of detection for any analyte were diluted 1:5 in assay buffer and the assay was repeated. Quality control values for each marker were consistently within the range indicated by the manufacturer.

In addition to the multiplex detection of inflammatory mediators, additional quantification of IL-1β was evaluated using a commercial bovine IL-1β ELISA kit (ESS0027; Thermo Fisher Scientific) according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using mixed models in SPSS Statistics v26 (IBM Corporation). To analyze the effect of prepartum CL on total bacterial load and specific pathogen load, linear mixed-effects models were used that included the fixed effects of pen, treatment (CL vs. HS), day (7 vs. 21 d postpartum), and the interaction between treatment and day. Cow nested with treatment was used as a random effect. We found no effect of pen on total bacterial load and specific pathogen load and was, therefore, excluded from further analysis. To analyze the association between vaginal mucus grade and bacterial load, linear mixed-effects models were used that included the fixed effects of mucus grade (0 to 4), treatment (CL vs. HS), and the interaction between mucus grade and treatment. Cow nested within treatment was used as a random effect. Concentration of cytokines were analyzed using a linear mixed-effects model with the fixed effects of treatment (CL vs. HS), LPS exposure (LPS vs. medium), and the interaction between treatment and LPS exposure. Cow nested within treatment was used as a random effect. Statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.08$. Data are reported as mean ± standard errors of mean.

RESULTS

Prepartum HS Decreased Milk Yield, But Did Not Affect Incidence of Postpartum Uterine Disease

Details data pertaining to thermoregulatory measurements and subsequent milk yield of cows are reported by Davidson et al. (2021). The average daily THI for each pen was not different between HS and CL pens during the prepartum treatment period (77.34 vs. 77.33 ± 0.20). Respiratory rate (44.3 vs. 60.0 ± 1.6 breaths/min) and rectal temperature (38.7 vs. 38.8 ± 0.04°C) were greater in HS nulliparous heifers during the prepartum period when heifers did not have access to fans or water soakers. Gestation length was shorter in HS nulliparous heifers compared with CL heifers (272.7 vs. 276.4 ± 1.4 d). When all postpartum cows had access to fans and water soakers, milk production was decreased in HS cows relative to CL cows (35.8 vs. 31.9 ± 1.4 kg/d), consistent with the induction of HS in the HS cows (Collier et al., 2017).

The clinical incidence of retained placental membranes, fever, ketosis, lameness, metritis, mastitis, diarrhea, and displaced abomasum in the first 21 DIM was not different between HS and CL cows (Table 2).

The average vaginal mucus grade of cows was higher on d 7 compared with d 21 postpartum (3.08 ± 1.65 vs 0.15 ± 0.05). However, we found no effect of the prepartum treatment on the average vaginal mucus on d 7 (CL, 1.9 ± 3.1 vs. HS, 1.8 ± 3.1) or d 21 postpartum (CL, 0.3 ± 0.1 vs. HS, 0.1 ± 0.1). The proportion of cows with a vaginal mucus grade ≥3 on d 7 (CL, 62.5% vs. HS, 46.2%) or d 21 postpartum (CL, 12.5% vs. HS, 15.4%) was also not significantly affected by prepartum HS.

Prepartum HS Did Not Affect Postpartum Bacterial Content of Vaginal Mucus

Others have shown differences in bacterial diversity and pathogen abundance between metritis and healthy cows using similar numbers of animals used here (Jeon et al., 2015; Ma et al., 2018; Jones et al., 2022). Total bacterial content in the vaginal mucus was not affected by prepartum HS; however, it tended to be greater ($P = 0.08$) for samples collected on d 7 compared with those of d 21 (3.08 ± 1.65 ng/mg of mucus vs. 0.15 ± 0.05 ng/mg of mucus; Figure 1A). No significant interaction was observed between day

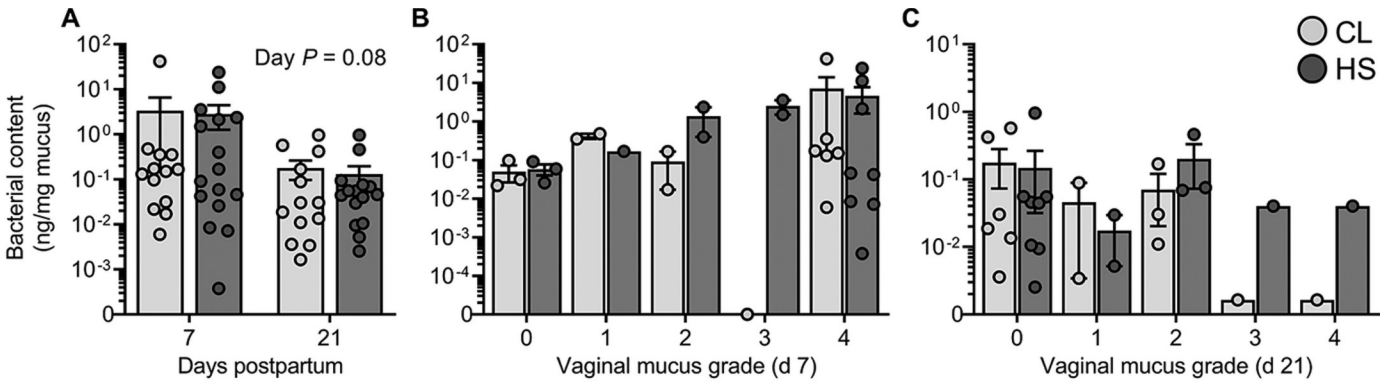

Figure 1. Total bacterial content of vaginal mucus. (A) Total bacterial content of vaginal mucus from cows housed under evaporative cooling (CL) conditions or heat stress (HS) conditions during the prepartum period was quantified by measuring bacterial 16S rRNA collected on d 7 and d 21 postpartum. (B) Total bacterial content on d 7 according to vaginal mucus grade. (C) Total bacterial content on d 21 according to vaginal mucus grade. Bars represent the mean ± SEM, and each dot represents an individual cow. Data are expressed as nanograms of 16S rRNA per milligram of vaginal mucus and were analyzed with a model that included the fixed effect of treatment, day of collection, vaginal mucus grade, and the interactions between treatment and day, as well as treatment and vaginal mucus grade.

and treatment for bacterial content in vaginal mucus. Total bacterial content in vaginal mucus was not affected by vaginal mucus grade, or the interaction between treatment and vaginal mucus grade on d 7 or d 21 (Figure 1B-C).

The presence of vaginal *E. coli* DNA was detected in 89.3% of cows on d 7 and 82.1% of cows on d 21 (Figure 2A). The presence of vaginal *F. necrophorum* DNA was detected in 69.0% of cows on d 7 and 28.6% of cows on d 21 (Figure 2B). The presence of vaginal *P. melaninogenica* DNA was detected in 100% of cows on d 7 and on d 21 (Figure 2C), whereas the presence of vaginal *T. pyogenes* DNA was detected in 96.6% of cows on d 7 and 78.6% of cows on d 21 (Figure 2D). Vaginal content of *E. coli*, *F. necrophorum*, and *P. melaninogenica* were not affected by treatment, day, or the interaction between treatment and day (Figure 2). Vaginal content of *T. pyogenes* on d 7 tended to be greater ($P = 0.08$) compared with d 21 (266.8 ± 147.6 cfu/μL per mg of mucus vs. 2.1 ± 9.1 cfu/μL per mg of mucus; Figure 2D); however, vaginal content of *T. pyogenes* was not affected by treatment or the interaction between treatment and day.

Prepartum HS Increases Postpartum Inflammatory Responses to LPS in Blood

Accumulation of IL-1α, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IFNγ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα, and VEGF-A were evaluated in blood challenged with medium alone or medium enriched with 200 ng/mL of LPS for 4 h (Figure 3 and 4). Challenging whole blood with LPS increased the accumulation of the cytokines IL-1α, IL-1β, IL-6, IL-10, IL-17A, IL-36RA, IFNγ, TNFα, and VEGF-A compared with control ($P < 0.01$; Figure 3), whereas LPS challenge increased the accumulation of the chemokines IP-10, MIP-1α, and MIP-1β relative to control ($P < 0.01$; Figure 4). Lipopolysaccharide-induced ac-

Table 2. Clinical disease incidence in the first 21 DIM[1]

Health condition	Cooling condition[2]	Heat stress condition[2]
No disease	5/14 (35.7)	9/16 (56.3)
Retained placental membranes	0/14 (0.0)	0/16 (0.0)
Fever	0/14 (0)	1/16 (6.3)
Ketosis	8/14 (57.1)	6/16 (37.5)
Lameness	0/14 (0.0)	0/16 (0.0)
Metritis	1/14 (7.1)	1/16 (6.3)
Mastitis	0/14 (0.0)	0/16 (0.0)
Diarrhea	0/14 (0.0)	1/16 (6.3)
Displaced abomasum	0/14 (0.0)	0/16 (0.0)

[1]Clinical diseases were evaluated in all cows in the first 21 DIM.

[2]Data shown are the absolute number of cows in each group and the proportion (%) of cows with each condition in parentheses. No effect of treatment was observed for any clinical condition evaluated.

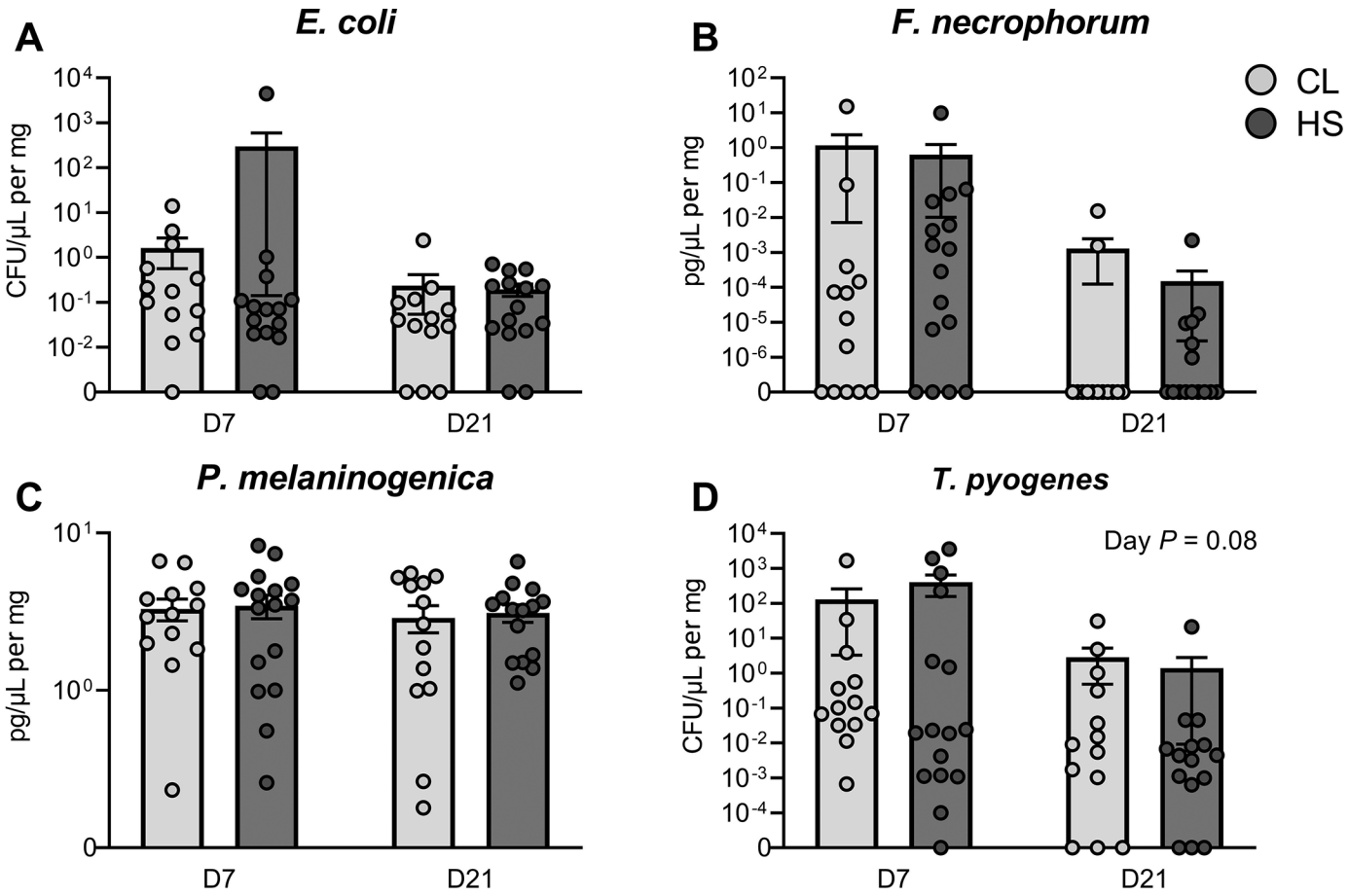

Figure 2. Specific pathogen content of vaginal mucus. Vaginal mucus from cows housed under evaporative cooling (CL) conditions or under heat stress (HS) conditions during the prepartum period was collected on d 7 and d 21 postpartum. Contents of *Escherichia coli* (A), *Fusobacterium necrophorum* (B), *Prevotella melaninogenica* (C), and *Trueperella pyogenes* (D) were quantified in vaginal mucus using quantitative real-time PCR. Data for *E. coli* and *T. pyogenes* are expressed as cfu/µL per milligram of vaginal mucus, and data for *P. melaninogenica* and *F. necrophorum* are expressed as pg/µL per milligram of vaginal mucus. Bars represent the mean ± SEM, and each dot represents an individual cow. Data were analyzed with a model that included the fixed effect of treatment, day of collection, and the interaction between treatment and day of collection. Vaginal mucus was not collected for 2 CL cows, 1 on d 7 and 1 on d 21.

cumulation of IL-1β, IL-10, and MIP-1α was greater ($P < 0.01$) in blood collected from HS cows compared with CL cows, whereas LPS-induced accumulation of IL-1α tended ($P \leq 0.08$) to be greater in blood of HS cows relative to CL cows (Figures 3A, 3B, 3D, and 4D). Regardless of LPS challenge, blood from HS cows tended ($P < 0.08$) to have greater accumulation of IL-1β, IL-10, and MIP-1α after culture compared with CL cows (Figures 3B, 3D, and 4D). Concentrations of IL-4 were undetectable in all samples.

A commercial bovine ELISA for IL-1β was used to confirm the findings of the multiplex IL-1β assay (Figure 3J). Data obtained from ELISA showed that treatment of whole blood with LPS increased ($P = 0.03$) the accumulation of IL-1β compared with blood treated with medium alone. In parallel with the multiplex assay, data obtained from ELISA showed that LPS-induced accumulation of IL-1β was increased ($P < 0.01$) in blood from HS cows compared with CL cows. A simple linear regression of IL-1β concentration in samples measured using ELISA and the multiplex assay showed a linear association between the 2 assay methods ($P < 0.01$, $R^2 = 0.69$; Figure 3K); however, the values obtained using the ELISA were less than those reported using the multiplex assay.

DISCUSSION

The incidence of uterine disease and retained placenta is associated with increased THI and HS in the dairy cow (DuBois and Williams, 1980; Gernand et al., 2019). We have demonstrated that uterine disease incidence is increased in hotter months; however, this occurs in the absence of increased bacterial content of vaginal mucus (Molinari et al., 2022). The following question therefore remains: if greater bacterial con-

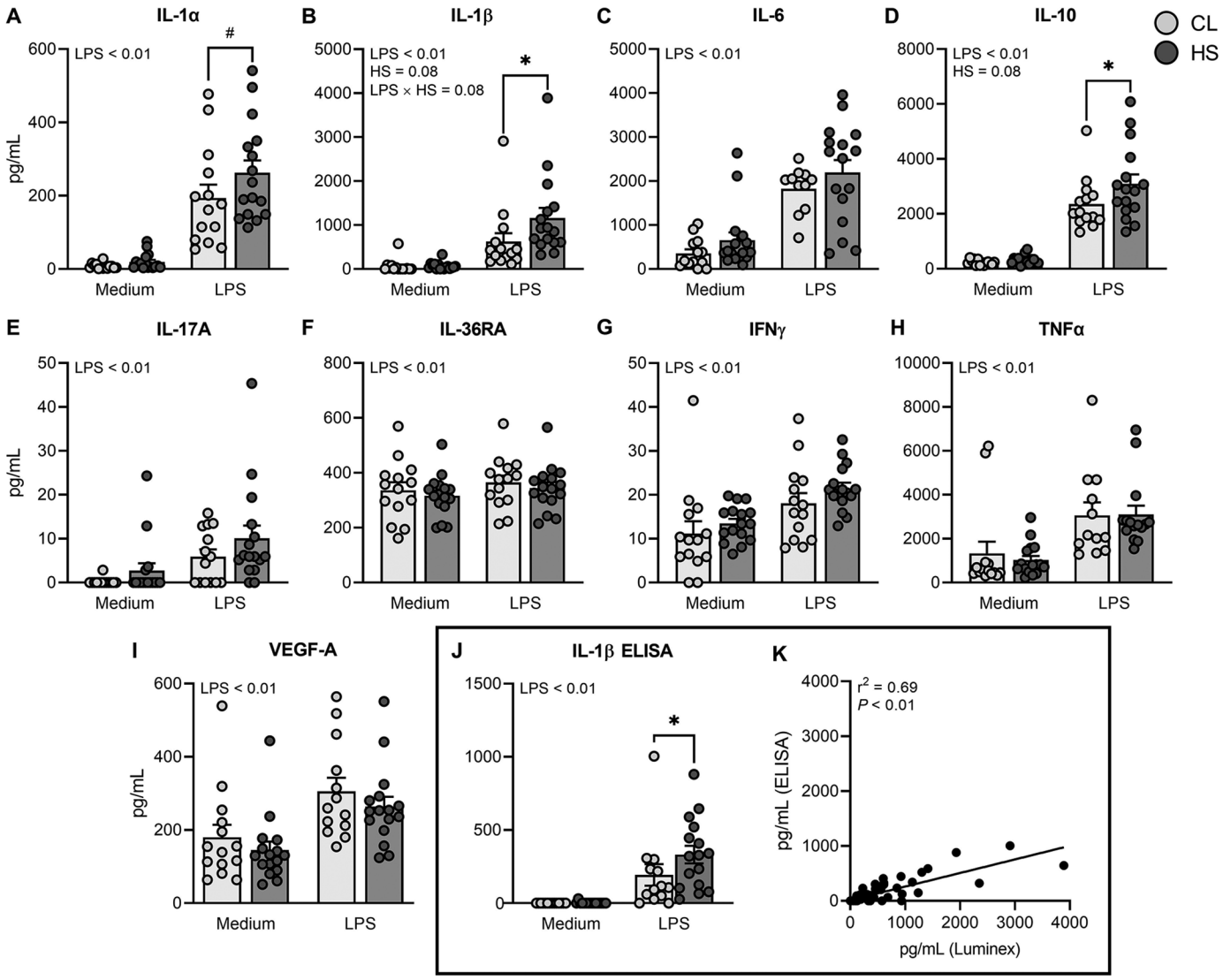

Figure 3. Accumulation of inflammatory mediators following a whole blood LPS challenge. Whole blood from cows housed under evaporative cooling (CL) conditions or under heat stress (HS) conditions during the prepartum period was collected on d 21 postpartum and challenged with 200 ng/mL of LPS or medium alone for 4 h. Accumulation of (A) IL-1α, (B) IL-1β, (C) IL-6, (D) IL-10, (E) IL-17A, (F) IL-36RA (IL-1F5), (G) IFNγ, (H) TNFα, and (I) VEGF-A were evaluated using a multiplex assay. (J) Accumulation of IL-1β was evaluated using a commercial ELISA kit. Bars represent the mean ± SEM and each dot represents an individual sample. Data were analyzed with models that included the fixed effects of cow treatment, LPS challenge, and the interaction between cow treatment and LPS challenge. (K) Data acquired from ELISA assay were compared with data acquired from the multiplex assay using identical samples. Each dot represents an individual sample. Data were analyzed using a simple linear regression. $^*P \leq 0.05$ and $\#P \leq 0.08$.

tent of the reproductive tract is not responsible for increased uterine disease during the summer, could HS affect host immunity and effectively reduce immune resilience to pathogens? Herein, we investigated how prepartum HS affected bacterial content of vaginal mucus and production of inflammatory mediators in whole blood following an in vitro LPS challenge. Heifers in the HS group had increased prepartum rectal temperature and respiration rate and decreased milk yield postpartum compared with CL heifers, indicating that HS heifers experienced HS in the prepartum period. This experimental paradigm allowed for the study of immune responses to pathogens postpartum when all the cows received HS remediation. Our results suggest that exposure to prepartum HS during late pregnancy has minimal effect on bacterial content of vaginal mucus postpartum or the development of postpartum uterine disease. However, prepartum HS has carry-over effects on postpartum host immune function, whereby blood of HS cows challenged with LPS had increased accumulation of pro-inflammatory (IL-1 α and IL-1β) cytokines, and anti-inflammatory (IL-10) cytokines

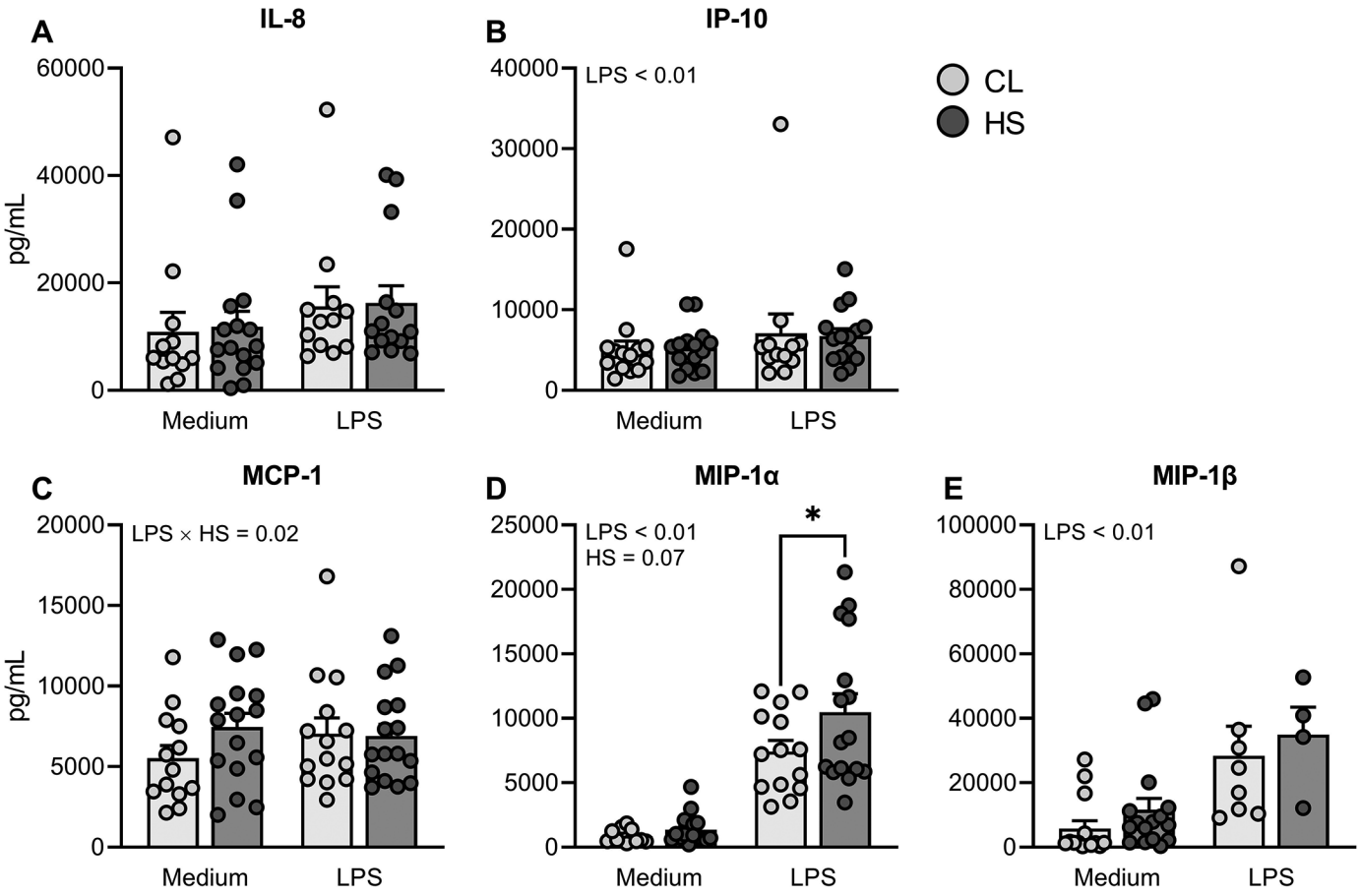

Figure 4. Accumulation of chemokines following a whole blood LPS challenge. Whole blood from cows housed under evaporative cooling (CL) conditions or under heat stress (HS) conditions during the prepartum period was collected on d 21 postpartum and challenged with 200 ng/mL of LPS or medium alone for 4 h. Accumulation of (A) IL-8 (CXCL8), (B) IP-10 (CXCL10), (C) MCP-1 (CCL2), (D) MIP-1α (CCL3), and (E) MIP-1β (CCL4) were evaluated using a multiplex assay. Bars represent the mean ± SEM, and each dot represents an individual sample. Data were analyzed with models that included the fixed effects of cow treatment, LPS challenge, and the interaction between cow treatment and LPS challenge. $^*P \leq 0.05$.

and chemokines (MIP-1α) compared with the blood of CL cows. In a previous study of *Streptococcus uberis* mastitis challenge, we observed an increase in IL-10 expression in blood following prepartum HS (Thompson et al., 2014), along with HS-induced reduction in *TLR2* expression. Thus, the current data are consistent with a persistent effect of prepartum HS on immune function postpartum, even after cooling is provided.

Exposure of dairy cows to temperatures above their thermoneutral zone induces a stress response, which includes activation of the hypothalamic-pituitary-adrenal axis and secretion of glucocorticoids, mainly cortisol, into the blood (Wise et al., 1988; Ouellet et al., 2020). Basal plasma concentrations of inflammatory cytokines IL-1β, IL-6, IFNγ, and TNFα are greater in cows experiencing HS (THI = 80.5) relative to cows under thermoneutral (THI = 66.0) conditions (Chen et al., 2018). In the present experiment, no differences in basal concentrations of cytokines between CL and HS cows were observed; however, cytokine concentrations were analyzed in blood samples collected from cows on d 21 postpartum, when all cows were receiving CL and, thus, were better able to regulate their body temperature.

The innate immune response is the first line of defense against pathogens associated with uterine disease (Sheldon et al., 2019). Pathogenic bacteria such as *E. coli*, *T. pyogenes*, *F. necrophorum*, and *P. melaninogenica* trigger an inflammatory response in the uterus characterized by secretion of pro-inflammatory cytokines and chemokines. Although production of inflammatory cytokines is key to innate immunity toward pathogens and prevention of disease, an even greater production of inflammatory cytokines in response to pathogens likely exacerbates or prolongs inflammatory responses and impairs tissue repair and resolution of disease (Barth et al., 2013). Resistance is the collective ability of an animal to avoid disease by limiting

pathogen proliferation, primarily using inflammation and innate immunity. Exposure of animals to stressors affects the host immune system; however, the resultant immune effect depends on the species, health status of the animal, and the type and length of the stressor (Dhabhar, 2009). For example, dairy cows exposed to elevated environmental temperature during the dry period upregulate molecular pathways related to immunity in the mammary gland (Dado-Senn et al., 2018). Moreover, long-term exposure to elevated temperatures induces an inflammatory state in lactating dairy cows (Min et al., 2016). Moreover, Marins et al. (2021) reported that mononuclear cells collected from dairy cows during HS accumulated greater concentrations of cytokines after in vitro challenge with LPS compared with cooled cows. However, data here describe a carry-over effect of prepartum HS on postpartum immune function in blood collected 21 d postpartum when all cows were receiving CL to alleviate HS. Importantly, we found no difference in the clinical disease incidence in our study population that could have influenced the observed changes to peripheral immune function. Collectively, the data herein suggest that exposure to prepartum HS has carry-over effects on the postpartum immune response of a cow, which is not overcome by CL during the first weeks of lactation. Moreover, these data highlight the importance of providing proper heat abatement to pregnant heifers that are typically housed on pasture with minimal access to shade, especially in southeastern regions such as Florida that experience 70% of the year under risk of HS (Ferreira et al., 2016).

It is important to consider the manner in which uterine disease was categorized in the current population of cows. Here, we used the robust model of vaginal mucus grade that is associated with pathogen density in the uterus and subsequent fertility (Williams et al., 2005; Sheldon et al., 2006, 2009); however, others have suggested that vaginal mucus grade or purulent vaginal discharge score is not closely associated with endometrial inflammation based on endometrial cytology (McDougall et al., 2011, 2020). Future studies should consider evaluating uterine pathogens and endometrial inflammation using cytology.

The present findings suggest that the increased incidence and persistence of uterine disease we have observed during summer months when environmental temperatures are increased is not associated with increased pathogen load in the vagina, confirming our earlier work (Molinari et al., 2022). The increased incidence and persistence of uterine disease during periods of elevated environmental temperature may be due to HS-induced perturbations of the systemic inflammatory response, reducing the immune resistance of cows to uterine disease. It is important to note that it is unclear whether the effects observed here on whole blood cells could be directly translated to the uterine environment. Further investigation is necessary to evaluate the effects of HS on the uterine response to pathogens and to determine the mechanisms responsible for HS-induced chronic inflammation in the dairy cow.

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