Acute brief heat stress in late gestation alters neonatal calf innate immune functions¹

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ABSTRACT

Heat stress, as one of the environmental stressors affecting the dairy industry, compromises the cow milk production, immune function, and reproductive system. However, few studies have looked at how prenatal heat stress (HS) affects the offspring. The objective of this study was to evaluate the effect of HS during late gestation on calf immunity. Calves were born to cows exposed to evaporative cooling (CT) or HS (cyclic 23–35°C) for 1 wk at 3 wk before calving. Both bull and heifer calves (CT, $n = 10$; HS, $n = 10$) were housed in similar environmental temperatures after birth. Both CT and HS calves received 3.78 L of pooled colostrum within 12 h after birth and were fed the same diet throughout the study. In addition to tumor necrosis factor α , IL-1 β , IL-1 receptor antagonist (IL-1RA), and toll-like receptor (TLR)2, and TLR4 mRNA expression, the expression of CD14⁺ and CD18⁺ cells, and DEC205⁺ dendritic cells were determined in whole blood samples at d 0, 3, 7, 14, 21, and 28. The neutrophil to lymphocyte ratio, differential cell counts, and the hematocrit were also determined. During late gestation, the HS cows had greater respiration rates, rectal temperatures, and tended to spend more time standing compared with the CT cows. The HS calves had less expression of tumor necrosis factor- α and TLR2 and greater levels of IL-1 β , IL-1RA, and TLR4 compared with CT calves. The HS calves also had a greater percentage of CD18⁺ cells compared with the CT calves. Additionally, a greater percentage of neutrophils and lesser percentage of lymphocytes were in the HS calves compared with the CT calves. The results indicate that biomarkers of calves' immunity are affected in the first several weeks after birth by HS in the dam during late gestation.

Key words: dairy cattle, calf, heat stress, innate immunity

INTRODUCTION

Heat stress (HS) is a major environmental concern in the dairy industry. In dairy cattle, HS occurs when ambient temperatures are above 25°C (Armstrong, 1994). Heat stress causes greater rectal temperatures (RT) and elevated respiration rates (RR; Ominski et al., 2002; do Amaral et al., 2011). Dairy cattle also spend less time lying when exposed to hot temperatures (Overton et al., 2002; Legrand et al., 2011). In addition to behavioral responses, HS in dairy cattle reduces feed intake (Adin et al., 2009), milk production (Collier et al., 2006), and reproductive performance (Hansen, 2009). Heat exposure before parturition caused decreased neutrophil phagocytosis and oxidative burst (do Amaral et al., 2011) after animals were returned to a thermoneutral environment following calving. Reduction of lymphocyte proliferation (Lacetera et al., 2006) and reduced IgG concentrations (do Amaral et al., 2011) occurred in cows under HS during the dry period and decreased (do Amaral et al., 2010) tumor necrosis factor α (TNF- α) responses of lymphocytes from HS cows. The immune impairment due to HS can cause increased susceptibility of dairy cattle to many diseases (Lacetera et al., 2006; do Amaral et al., 2009).

Maternal HS during late gestation also inhibits the immune response of the offspring, by modifying T and B cell function (Merlot et al., 2008), decreasing IgG concentration in the calf (Donovan et al., 1986; Tao et al., 2012), and compromising the proliferation rate of mononuclear cells (Tao et al., 2012). Moreover, sows under HS during the last 2 wk of gestation causes lower circulating IgG in piglets compared with piglets from sows under a thermoneutral environment (Machado-Neto et al., 1987). The immune system in the neonate is fully developed at birth but it is unprimed (Tizard, 1992). The fetus is protected primarily by the innate immune system, but the phagocytic activity of its immune cells is not fully developed until late in gestation

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(Barrington, 2001). B cells in the fetus only make up 1% of total lymphocytes compared with the 4% at 1 wk after birth and with the 10% in mature calves (Kampen et al., 2006). This results in the lack of any endogenous antibody response until 2 to 4 wk of age making the ingestion of colostrum extremely important in providing an immunologic defense to the calf during the first 2 to 4 wk of life (Chase et al., 2008). Adequate colostrum transfer has been recognized to have beneficial effects on the calf's immune response earlier in life (Furman-Fratczak et al., 2011), by the uptake of cytokines (Nguyen et al., 2007; Chase et al., 2008) and the absorption of immunoglobulins during the first 48 h (Sangild, 2003). Recent work has isolated the effects of colostrum from heat-stressed cows and the absorptive ability of the neonate from heat-stressed cows (Monteiro et al., 2014), determining that calves had reduced passive transfer regardless of colostrum source. Previous research in our laboratory (unpublished data), conducted during the summer with a short heat event during the last month of gestation, resulted in calves that were more prone to disease and grew slower even when born after the HS event, prompting the hypothesis of this research.

Currently, few data are available on the effects of maternal HS during late gestation on the immune function in dairy cattle. Therefore, the objective of the present study was to evaluate the effect of a brief maternal HS during the late gestation period on the postnatal immune function of dairy calves. Our hypothesis was that a brief late gestation heat stress would alter neonatal calf innate immune measures.

MATERIALS AND METHODS

Animals and Experimental Design

This study was conducted at the Purdue Animal Sciences Research and Education Dairy Unit from May to September 2014. The experimental protocol was approved by the Animal Care and Use Committee at Purdue University. Twenty multiparous Holstein cows were randomly assigned to 1 of 2 environmental treatments, control (CT) or HS, and moved into a metabolism room approximately 3 wk before calving. All cows were managed in a metabolism barn with tie stalls. A single treatment was in the room at one time, using 3 replications per treatment. Cows were moved into the metabolism room for an acclimation period of 7 d and then were on the treatment for the following 7 d. The metabolism barn for the CT cows was equipped with evaporative cooling equipment. For the HS cows, the temperature was increased to $35^{\circ}\text{C} \pm 6$ over 12 h each

Table 1. Guaranteed analysis of milk replacer and starter for calves

Nutrient	Milk replacer ¹	Calf starter ²
CP, minimum, %	22	20
Fat minimum, %	20	4.5
Crude fiber minimum, %	0.15	6.0
ADF minimum, %		8.0
Ca minimum, %	1.0	1.0
P minimum, %	0.7	0.3
Salt minimum, %		0.525
Selenium minimum, %		0.3
Vitamin A, IU/kg	44,000	19,800
Vitamin D ₃ , IU/kg	11,000	4,400
Vitamin E, IU/kg	220	110
Monensin, g/t		40 g/ton

¹Land O'Lakes, Amplifier Max MOS, Shoreview, MN.

²Momenta, Vita Plus Corporation, Madison, WI.

day and was reduced to $23^{\circ}\text{C} \pm 4$ over 12 h each night. Following the treatment conditions, cows were moved to box stalls under geothermal, thermal neutral ($22^{\circ}\text{C} \pm 2$), environmental conditions for approximately 1 wk before their expected calving date.

Both bull and heifer calves born to those cows were used in the current study (CT: bulls = 6, heifers = 4; HS: bulls = 4, heifers = 6). Both CT (n = 10) and HS (n = 10) calves received 3.78 L of pooled superior (defined as greater than 50 mg/mL of IgG) colostrum (from multiparous cows in geothermally controlled environments) within 12 h after birth and were fed the same diet throughout the study. Calves from both treatments were removed from their dam immediately after birth and housed in individual hutches exposed to ambient temperatures. Calves were fed commercial milk replacer of all milk components (Table 1) and offered dry feed (Table 1) from d 3. Water was replaced twice daily and always available.

Measurements and Sample Collection

The respiration rates (RR), rectal temperatures (RT), and posture of cows in both treatments were monitored hourly for 12 h during 7 d of HS or CT treatment from 0800 to 1900 h. Two consecutive RR were measured in breaths per minute using a stopwatch. The average of those 2 consecutive RR per cow was recorded hourly. The posture of the cow, and whether they were lying or standing at the moment before RR, were counted (an hourly instantaneous scan sample) and calculated as the percentage of observations. The RT were measured using a digital thermometer hourly and disinfected between individual cows with 70% isopropyl alcohol. The room temperature and relative humidity were recorded hourly by taking the average of

Table 2. Effects of heat stress for 1 wk during late gestation on cow respiration rate, rectal temperature, and posture¹ during that week²

Item	Treatment		P-value
	CT	HS	
Parity			
2	2	2	—
3	7	5	—
4	1	3	—
Min THI	<64	75	—
Mean THI	69	83	—
Max THI	79	85	—
Min RR, breaths/min	34.77 ± 1.02	46.90 ± 1.02	<0.01
Mean RR, breaths/min	47.86 ± 1.29	64.36 ± 1.29	<0.01
Max RR, breaths/min	62.56 ± 1.72	82.64 ± 1.72	<0.01
Min RT, °C	37.73 ± 0.19	38.63 ± 0.19	<0.01
Mean RT, °C	38.46 ± 0.03	38.96 ± 0.03	<0.01
Max RT, °C	39.02 ± 0.04	39.45 ± 0.04	<0.01
Standing, ³ %	53.35 ± 3.71	62.80 ± 3.71	0.09

¹All means reported are least squares means ± SE (n = 10 cows/treatment) created by mixed model analysis.

²CT = control cows; HS = heat-stressed cows; min THI = temperature and humidity index for the minimum room temperature; mean THI = temperature and humidity index for the mean room temperature; max THI = temperature and humidity index for the maximum room temperature; Min RR = mean of the minimum respiration rates; mean RR = mean of the average respiration rates; max RR = mean of the maximum respiration rates; min RT = mean of the minimum rectal temperatures; mean RT = mean of the average rectal temperatures; max RT = mean of the maximum rectal temperatures.

³The percentage of observations based on whether the cow was lying or standing before the respiration rates were counted (12 observations/day per cow).

2 readings and was also digitally recorded every 15 min by a data logger (Onset HOBO, UX100-003; 10526645) and the THI values were calculated (Thom, 1959). Each hourly observation was conducted in the same recording sequence: room temperature, humidity, RT, posture, RR. Cows were multiparous in lactations 2, 3, and 4 (Table 2).

Calf jugular blood samples were collected at d 1, 3, 7, 14, 21, and 28 into 8.5-mL tubes containing 1.50 mL of ACD (acid-citrate dextrose) for flow cytometry and PCR and 8.5-mL tubes containing 0.10 mL of EDTA (BD, Franklin Lakes, NJ) for hematology analysis. Immediately following blood collection, blood was stored on ice, transported to the laboratory, and then placed in a water bath at 37°C for 30 min or brought to room temperature for hematology analysis.

Hematology and Passive Antibody Transfer

White blood cell (WBC) counts (K/ μ L), the neutrophil to lymphocyte (N/L) ratio, the hematocrit percentage, and the percentage and cell counts of neutrophils (PMNL), lymphocytes (LYM), monocytes (MO), eosinophils (EO), and basophils (BA) were measured using a hematology analyzer (Genesis, Oxford Science Inc., Oxford, CT) using the calf setting and standards. Plasma protein concentrations (g/dL) were measured using a refractometer (A 300 CL Clinical, Jenco In-

ternational Inc., Portland, OR) to determine passive transfer of maternal antibodies.

PCR

Cytokines TNF- α , IL-1 β , IL-1 receptor antagonist (IL-1RA), and toll-like receptor 2 (TLR2), and 4 (TLR4) mRNA expression in whole blood were detected by real-time PCR using primers and probes diluted to 10 μ M solutions (Table 3) and developed with Primer Express (Applied Biosystems). The RNA was extracted per instructions using the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA; catalog no. 52304; Felver-Gant et al., 2012). Reverse transcription was conducted in the Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO) and amplified using cycling conditions (Felver-Gant et al., 2012). All samples were measured in duplicates and standards in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%. Data are expressed as relative abundance of the gene of interest using a relative standard curve and normalized to the stable (CV <2% across all samples) reference gene 18S.

Flow Cytometry

Cellular surface expression of CD14, CD18, and DEC205 were measured using flow cytometry. Five mi-

Table 3. Bovine specific primers and probes used for real-time PCR

Gene ¹	Primer	Amplification efficiency, %	Product length, bp	Reference/accession no.
TNF- α	(f ²) TGGGAAGCTTACCTTTTCCTTTC (r ³) CTTCTTCATGACCCAGATACATCCT (p ⁴) CCTCAAGTAACAAGCCG (VIC/MGB)	98	61	Bienhoff and Allen, 1995
IL-1 β	(f) TTCCTGTGGCCTTGGGTATC (r) TGGGCGTATCACCTTTTTTCA (p) CAAGAATCTATACCTGTCTTGT (VIC/MGB)	78	69	Ito and Kodama, 1996
IL1-RA	(f) CCTCCTTCTCACCCAGATC (r) AGAAAATGGAAGCCGCTTAGG (p) CAG GCGCTCACTTC (VIC/MGB)	96	64	Kirisawa et al., 1998
TLR2	(f) CCACGGAAGGAGCCTCTGA (r) GCCATCGCAGACACCAGTT (p) CAGGCTTCTTCTCTGTCTT (VIC/MGB)	86	65	AF368419
TLR4	(f) CCGGATCCTAGACTGCAGCTT (r) TCCTTGGCAAATTCTGTAGTTCTTG (p) CCGTATCATGGCCTCT (VIC/MGB)	104	71	AAG32061

¹TNF = tumor necrosis factor α ; IL1-Ra = IL-1 receptor antagonist; TLR = toll-like receptor.

²Forward primer.

³Reverse primer.

⁴Probe.

croliters each of labeled monoclonal mouse anti-human phycoerythrin-labeled CD14 (Serotec; Raleigh, NC; catalog no. MCA1568PE) and fluorescein isothiocyanate labeled monoclonal mouse anti-human CD18 (BD, San Diego, CA; catalog no. 555923) were added to 500 μ L of blood to measure CD14 and CD18. Additionally, 5 μ L of fluorescein isothiocyanate labeled monoclonal mouse anti-bovine DEC205 (catalog no. MCA1651, Serotec, Raleigh, NC) was added to a second tube to measure dendritic cell presence. Each of those antibodies have been validated in our laboratory. The control for CD14, CD18, and DEC205 was 500 μ L of cells only. All tubes were incubated for another 30 min at 37°C in a water bath. After incubation, 900 μ L of sterile cold water was added to each tube of blood. After 45 s of contact, 100 μ L of 10 $\times$ Hanks' balanced salt solution was added. The tubes were centrifuged for 3 min at 1,800 $\times g$ and supernatant was then discarded. One milliliter of 1 $\times$ HBSS was added to each tube, centrifuged for 3 min at 1,800 $\times g$, and then the supernatant was discarded. Lastly, cell pellets were resuspended in 350 μ L of 2% paraformaldehyde in HBSS and these samples were kept at 20°C until analysis. Expression of CD14, CD18, and DEC205 were then investigated using flow cytometry (Accuri C6, Accuri, Ann Arbor, MI). For each sample, a total population of 10,000 cells was analyzed. The percentage of cells that were positive and the average relative fluorescence intensity of cells expressing CD14, CD18, and DEC205 above the control cells was calculated. Analysis was conducted on all cells, low to medium forward scatter (FS)/high side scatter (SS; neutrophils), high FS/low SS (monocytes), and low FS/low SS (lymphocytes).

Statistical Analysis

Cow and calf data were checked for normal distribution and non-normal data were transformed as noted below and analyzed as a randomized complete block design with repeated measures using the MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). Flow data were transformed to log₁₀ before statistical analysis. No transformations were necessary for cow or calf PCR and hematology data. Sex and replications were tested in the model for interactions with the treatments. No interactions with treatment were found, and they were removed from the analysis where appropriate. The calf model included treatment, day, and treatment $\times$ day interaction. The cow model included treatment. Statistical inferences of significance were based on $P < 0.05$ and trends on $P < 0.10$.

RESULTS

No refusals of milk replacer occurred, and starter consumptions were not different ($P > 0.10$) between treatments (data not shown). Water intake was not measured. Plasma protein, indicative of maternal antibody transfer, was 6.47 ± 0.23 for calves from CT cows and 6.08 ± 0.23 for calves from HS cows ($P = 0.23$). In the present study, HS cows had greater minimum, mean, and maximum RR and RT than the CT cows ($P < 0.05$, Table 2). The HS cows also tended ($P = 0.09$) to spend more time standing compared with the CT cows (Table 2).

No difference was found among treatments in hematocrit ($P > 0.05$, Table 4). The plasma protein concen-

Table 4. Hematology measurements¹ of calves born to cows under heat stress for 1 wk during late gestation²

Measure	Treatment		Main effects and interactions (<i>P</i> -value)		
	CT	HS	Treatment	Day	Treatment × day
PP, g/dL (d 0)	6.47 ± 0.23	6.08 ± 0.23	0.25	—	—
WBC, K/μL	7.65 ± 0.47	8.17 ± 0.45	0.43	0.01	0.08
N/L ratio	2.89 ± 0.38	3.36 ± 0.37	0.38	0.03	0.87
PMNL, K/μL	5.07 ± 0.51	5.33 ± 0.50	0.16	0.55	0.55
LYM, K/μL	2.31 ± 0.17	2.22 ± 0.16	0.70	0.20	0.51
MO, K/μL	1.22 ± 0.33	0.49 ± 0.33	0.12	0.31	0.46
EO, K/μL	0.44 ± 0.11	0.12 ± 0.10	0.03	0.66	0.60
BA, K/μL	0.11 ± 0.05	0.01 ± 0.05	0.21	0.32	0.34
PMNL, %	54.57 ± 1.89	62.90 ± 1.86	<0.01	0.01	0.93
LYM, %	32.85 ± 1.89	27.92 ± 1.86	0.07	0.01	0.59
MO, %	8.01 ± 0.54	6.75 ± 0.53	0.10	0.01	0.74
EO, %	3.98 ± 0.78	2.27 ± 0.77	0.12	0.07	0.74
BA, %	0.60 ± 0.25	0.17 ± 0.24	0.22	0.29	0.31
HCT, %	24.34 ± 1.25	25.64 ± 1.20	0.45	0.61	0.83

¹All means reported are least squares means ± SE created by mixed model analysis (n = 10).

²CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress; PP = plasma protein concentration; WBC = white blood cell count; N/L = neutrophil to lymphocyte ratio; PMNL = neutrophil count or percentage; LYM = lymphocyte count or percentage; MO = monocyte count or percentage; EO = eosinophil count or percentage; BA = basophil count or percentage; HCT = percentage hematocrit.

trations were less ($P < 0.05$) in HS calves, and plasma protein concentration peaked at d 3 and decreased after d 7 ($P < 0.05$, data not shown).

No treatment effects were found on WBC counts ($P > 0.05$), but a trend ($P = 0.08$) was found for a treatment by day interaction such that CT calves had a peak of WBC numbers on d 7, whereas the HS calves did not increase WBC numbers until d 28 ($P = 0.0004$; Figure 1).

Compared with controls, EO counts were less ($P < 0.05$) in HS calves, but none of BA counts differed ($P > 0.05$). The percentage of PMNL were greater ($P < 0.05$) in HS calves compared with CT calves (Table 4) and tended to be less for LYM ($P = 0.07$) and for MO ($P = 0.10$).

No treatment effect was found for N/L ratio ($P > 0.05$, Table 4). However, the N/L ratio was greatest on d 7 ($P = 0.03$). The day main effect (data not shown) of PMNL percent was greatest at d 0 and 7 ($P = 0.006$) and of LYM percent was greatest on d 3 compared with d 0 and 7 and increased to another peak at d 28 ($P = 0.01$). The percentage of MO was greatest on d 3 and increased after d 7 ($P = 0.01$), but the percentage of EO increased after d 3 and decreased after d 21 ($P = 0.07$).

Tumor necrosis factor- α and TLR2 expression were less in the HS calves than the CT calves ($P < 0.05$, Table 4). The HS calves tended ($P = 0.10$) to have greater IL-1 β , and had greater IL-1RA and TLR4 RNA expressions than the CT calves ($P < 0.05$; Table 4). As a day effect, TNF- α decreased after d 0, whereas IL-1 β , IL-1RA, and TLR4 expressions decreased after d 7 ($P < 0.05$). Day had no effect on TLR2 ($P > 0.05$; Table

5). No treatment by day effect was observed on TNF- α , IL-1 β , IL-1RA, TLR2, or TR-4 ($P < 0.05$, Table 5).

Considering all leukocytes without gating, the HS calves had greater percentages of CD18⁺ ($P = 0.0438$; Table 6) compared with the CT calves. No differences were observed in the percentages of CD14⁺ ($P = 0.25$; Table 6) and DEC205⁺ ($P = 0.26$; Table 6), or the mean fluorescence of CD18⁺ ($P = 0.14$; Table 7), CD14⁺ ($P = 0.86$; Table 7), and DEC205⁺ ($P = 0.18$; Table 7) among treatments in all leukocytes. Additionally, total leukocytes of the HS calves tended to have a greater mean fluorescence of CD18 on leukocytes on d 7 compared with the CT calves ($P = 0.08$, Figure 2).

No treatment ($P > 0.05$) and day ($P > 0.05$) effects were found on the percentage or mean fluorescence for CD18⁺, CD14⁺, or DEC205⁺ in lymphocytes (Tables 6 and 7, respectively, for percentage and mean fluorescence). However, the HS calves also tended to have a greater mean fluorescence of CD14⁺ on lymphocytes on d 0 and 14 compared with the CT calves ($P = 0.06$; Figure 3).

Heat-stressed calves had a greater percentage and mean fluorescence of CD18 on monocytes compared with the CT calves ($P < 0.05$; Tables 6 and 7 for percentage and mean fluorescence, respectively). No differences in the percentage of CD14⁺ or DEC205⁺ in monocytes ($P > 0.05$; Table 6) were found. However, DEC205⁺ tended to have greater mean fluorescence on monocytes in HS calves ($P = 0.07$; Table 7).

More monocytes were CD14⁺ in HS calves on d 3 and fewer were CD14⁺ on d 28 compared with the CT calves ($P = 0.03$; Figure 4, data shown as percentage of all cells). Percentage of CD18⁺ expression on mono-

Table 5. mRNA expression of cytokines and toll-like receptors in leukocytes of calves born to cows under heat stress for 1 wk during late gestation^{1,2}

Item	TNF-α	IL-1β	IL-1RA	TLR2	TLR4
Treatment					
CT	2.21 ± 0.26	0.16 ± 0.02	0.34 ± 0.05	0.41 ± 0.07	0.29 ± 0.03
HS	1.42 ± 0.26	0.21 ± 0.02	0.55 ± 0.05	0.20 ± 0.07	0.42 ± 0.03
Day					
d 0	4.16 ^a ± 0.48	0.26 ^a ± 0.04	0.61 ^a ± 0.09	0.39 ± 0.12	0.52 ^a ± 0.06
d 3	2.26 ^b ± 0.44	0.26 ^a ± 0.03	0.55 ^a ± 0.08	0.34 ± 0.12	0.44 ^a ± 0.05
d 7	1.72 ^{bc} ± 0.46	0.26 ^a ± 0.04	0.61 ^a ± 0.09	0.32 ± 0.12	0.50 ^a ± 0.05
d 14	0.85 ^c ± 0.44	0.11 ^b ± 0.03	0.29 ^b ± 0.08	0.23 ± 0.12	0.25 ^b ± 0.05
d 21	1.06 ^c ± 0.44	0.13 ^b ± 0.03	0.32 ^b ± 0.08	0.35 ± 0.11	0.24 ^b ± 0.05
d 28	0.81 ^c ± 0.45	0.10 ^b ± 0.04	0.27 ^b ± 0.09	0.21 ± 0.12	0.18 ^b ± 0.05
P-value					
Treatment	0.03	0.10	<0.01	0.04	<0.01
Day	<0.01	<0.01	<0.01	0.89	<0.01
Treatment × day	0.55	0.13	0.37	0.49	0.13

^{a-c}Means within a column with differing superscripts differ ($P \leq 0.05$).
¹All means reported are least squares means ± SE in relative abundance to 18S created by mixed model analysis (n = 10).
²CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress. TNF-α = tumor necrosis factor α; IL-1RA IL-1 receptor antagonist; TLR2 = toll-like receptor 2; TLR4 = toll-like receptor 4.

cytes, regardless of treatment (day effect), increased after d 7 ($P = 0.05$, data not shown). The mean fluorescence of CD18 on monocytes was less in CT calves on d 7 compared with the HS calves ($P < 0.05$; Figure 4). Monocytes also increased percentage of cells that

were DEC205⁺ after d 7 ($P < 0.05$, day effect data not shown).
No treatment differences were present in the percentage of CD14⁺ or DEC205⁺ (Table 6) in PMNL cells ($P > 0.05$). However, the percentage of cells positive for

WBC

Figure 1. The treatment by day effect on white blood cell counts (WBC) of calves born to cows under heat stress for 1 wk during late gestation. Data are shown as mean values ± SE (n = 10). * indicates differences between treatments on that day ($0.05 < P < 0.01$).

Table 6. Percentage of cells positive for cell surface expression of CD14, CD18, and DEC205 of calves born to cows under heat stress for 1 wk during late gestation^{1,2}

Item	Treatment		P-value		
	CT	HS	Treatment	Day	Treatment × day
No gate					
CD18, %	40.63 ± 3.95	51.96 ± 3.92	0.04	0.80	0.90
CD14, %	24.23 ± 2.19	27.78 ± 2.17	0.25	0.47	0.41
DEC205, %	3.70 ± 1.84	6.60 ± 1.83	0.26	0.18	0.47
Lymphocytes					
CD18, %	0.08 ± 0.08	0.11 ± 0.08	0.78	0.92	0.92
CD14, %	0.60 ± 0.07	0.66 ± 0.07	0.50	0.17	0.88
DEC205, %	0.69 ± 0.01	0.71 ± 0.01	0.19	0.45	0.26
Monocytes					
CD18, %	23.80 ± 2.97	32.12 ± 2.94	0.05	0.05	0.22
CD14, %	2.22 ± 0.13	2.41 ± 0.12	0.28	0.05	0.03
DEC205, %	1.78 ± 0.15	1.91 ± 0.14	0.55	<0.01	0.11
PMNL					
CD18, %	40.69 ± 4.06	51.96 ± 4.02	0.05	0.80	0.90
CD14, %	3.11 ± 0.11	3.19 ± 0.11	0.61	0.87	0.77
DEC205, %	1.99 ± 0.20	1.93 ± 0.19	0.81	0.75	0.95

¹All means reported are least squares means ± SE created by mixed model analysis (n = 10).

²CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress. CD18 = cluster of differentiation CD18; CD14 = cluster of differentiation CD14; DEC205 = cluster of differentiation DEC205.

CD18 on PMNL cells was less in the CT calves ($P = 0.05$; Table 6) on d 7 but not any other day. No day effect was present on the mean fluorescence expression of CD14⁺ and DEC205⁺ in PMNL cells ($P > 0.05$). The HS calves had greater day effects on the mean fluorescence of CD18 on PMNL cells compared with the CT calves ($P < 0.05$, Figure 5).

None of the growth measures were different between HS and CT calves (Table 8), only a trend ($P = 0.10$) for initial heart girth. We detected differences in initial and final BW, hip height, and heart girth for sex, but

changes (between initial and final measures) in those measures did not differ between sexes.

DISCUSSION

Increasing RR and RT are 2 major indicators of HS in dairy cattle (Ominski et al., 2002; do Amaral et al., 2011). Lower RR and RT in CT cows suggest that less heat load is carried compared with HS cows (Tao et al., 2012). Dairy cattle also spend less time lying when exposed to high temperatures (Overton et al., 2002; Le-

Table 7. Mean fluorescence of leukocytes from calves born to cows under heat stress for 1 wk during late gestation^{1,2}

Item	Treatment		P-value		
	CT	HS	Treatment	Day	Treatment × day
No gate					
CD18	8.19 ± 0.16	8.51 ± 0.16	0.14	0.07	0.08
CD14	7.62 ± 0.19	7.57 ± 0.20	0.87	0.17	0.77
DEC205	6.10 ± 0.32	6.10 ± 0.40	0.18	0.42	0.42
Lymphocytes					
CD18	4.16 ± 0.75	5.64 ± 0.52	0.11	0.23	0.49
CD14	4.71 ± 0.62	5.79 ± 0.54	0.20	0.95	0.06
DEC205	5.76 ± 0.61	4.96 ± 0.62	0.35	0.60	0.11
Monocytes					
CD18	7.76 ± 0.17	8.41 ± 0.16	0.06	0.01	0.03
CD14	8.25 ± 0.18	8.43 ± 0.17	0.49	0.21	0.80
DEC205	6.60 ± 0.18	7.06 ± 0.17	0.07	0.36	0.11
PMNL					
CD18	8.19 ± 0.16	8.51 ± 0.16	0.14	0.07	0.08
CD14	7.62 ± 0.19	7.57 ± 0.20	0.86	0.17	0.77
DEC205	6.10 ± 0.32	6.80 ± 0.40	0.18	0.42	0.24

¹All means reported are least squares means ± SE created by mixed model analysis (n = 10).

²CT = calves born to thermal neutral dams; HS = calves exposed to prenatal heat stress. CD18 = cluster of differentiation CD18; CD14 = cluster of differentiation CD14; DEC205 = cluster of differentiation DEC205.

Figure 2. The treatment by day effect on the fluorescence intensity of cluster of differentiation (CD) 18 on all leukocytes of calves born to cows under heat stress for 1 wk during late gestation. Data are shown as mean values $\pm$ SE ($n = 10$). Fluorescence intensity was log-transformed before statistical analysis. * ($0.05 < P < 0.01$) and ** ($P < 0.05$) indicate significant differences between treatments on that day.

grand et al., 2011). Like previous studies, in the current study cows exposed to HS had greater minimum, mean, and maximum RR and RT than CT cows. The HS cows also tended to spend more time standing than the CT cows. This measure was reflective of the cows under each of the treatments and did not show changes within the cow from the acclimation phase to the treatment phase. It also demonstrates the difficulties encountered when collecting both behavioral and physiological data. Getting the rectal temperatures was a process that disturbed the cows' postures, and was the reason for the hourly measurements. The changes in RR, RT, and posture indicate a greater core body temperature. Additionally, as previously reported by Tao et al. (2012), the elevated RR and RT during the dry period of HS cows compared with CT cows provided evidence that the HS model used in the present study was successful.

As part of the recognition of gram-positive bacteria and bacterial carbohydrate moiety, TLR2 plays a role in mediating the innate immune response by releasing cytokines. In the present study, the expression of TLR2 and TNF- α were both less in the HS calves compared

with the CT calves. Supporting the current results, Couret et al. (2009) found that maternal stress during late gestation decreased TNF- α production in piglets. In cows, TNF- α concentration was also decreased after exposure to HS (do Amaral et al., 2010). Eicher et al. (2004) also found that calves treated with growth hormone and dexamethasone had lower TNF- α expression in blood leukocytes at 2 wk of age. In corroborating the previous findings, we hypothesize that the lower TNF- α concentration in the current study could be caused by an increase in circulating stress hormones in the calves exposed to maternal HS. However, Tao et al. (2012) found no differences among treatments in circulating cortisol levels of the calves from cows that were exposed to longer heat stress than in our study, and in the current study, the stress hormones of the calves were not measured.

A complex on the cell surface made up of TLR4 and CD14 recognizes the lipopolysaccharide of gram-negative bacteria and activates macrophages to secrete inflammatory cytokines (Gioannini and Weiss, 2007), which establishes the first line of defense against injury

or disease (Uematsu and Akira, 2007). The expression of CD14 was higher on monocytes in the HS calves on d 3 and tended to be higher on d 14. However, the number of monocytes and the percentage of cells expressing CD14 did not change. This shows that the CD14 per cell was upregulated rather than a change in the number of cells with CD14 expression. Similarly, TLR4 mRNA expression was increased in the HS calves in our study, thus supporting an increased ability to recognize gram-negative bacteria. In contrast, the expression of TLR4 mRNA in human blood monocytes was decreased after strenuous exercise in the heat (34°C) from an increase in circulating cortisol (Lancaster et al., 2005). Du et al. (2012) also suggested that major stress hormones suppress the response of murine macrophages through downregulation of TLR4. We did not measure cortisol in the calves, but the dams had greater cortisol concentrations at d 3 following the initiation of the 7-d heat stress (unpublished data). However, those studies are of mature subjects exposed to heat stress, rather than offspring exposed to prenatal heat stress.

The adhesion cell surface molecule CD18 is responsible for the recruitment of leukocytes to the site of infection and had greater expression in the total leukocyte population, and more specifically on monocytes and PMNL cells. Additionally, the upregulation of CD18 expression along with the recognition of lipopolysaccharide of gram-negative bacteria by the TLR4/CD14 complex has been shown to benefit the immune defense system, leading to phagocytosis of bacteria (Nunes et al., 2010). The expression of CD18 was greater in monocytes and neutrophils of our HS calves, mostly caused by a drop in CD18 fluorescence intensity of control calves on d 7.

The DEC205 is a receptor expressed on dendritic cells and monocytes, which plays a role in antigen presentation to T cells as part of regulating the adaptive immune response (Jiang et al., 1995). Jiang et al. (1995) showed that the antigen-presentation function of lymphocytes is associated with high levels of DEC205 expression on antigen-presenting cells. We were not expecting any treatment differences in DEC205 expression because of the association of DEC205 with mature

Lymphocyte CD14

Figure 3. The treatment by day effect on the fluorescence intensity of cluster of differentiation (CD) 14 on lymphocytes of calves born to cows under heat stress for 1 wk during late gestation. Data are shown as mean values $\pm$ SE ($n = 10$). Percent positive data and the fluorescence intensity were log-transformed before statistical analysis. * ($0.05 < P < 0.01$) and ** ($P < 0.05$) indicate significant differences between treatments on that day.

Figure 4. The treatment by day effect on the percentage of monocytes expressing cluster of differentiation (CD) 14 (top panel) and on mean fluorescence of CD18 (bottom panel) on the surface of monocytes of calves born to cows under heat stress for 1 wk during late gestation. Data are shown as mean values $\pm$ SE ($n = 10$). Percent positive data and the fluorescence intensity was log-transformed before statistical analysis. $^*(0.05 < P < 0.01)$, $^{**}(P < 0.05)$, $^{***}(P < 0.001)$ indicate significant differences between treatments on that day.

dendritic cells (Butler, 2007). However, the HS calves tended to have a higher fluorescence intensity of DEC205 on monocytes compared with CT calves. The higher fluorescence intensity in DEC205 was also seen in calves challenged to *Salmonella* Dublin compared with control calves (Eicher et al., 2011). These findings indicate that maturation of dendritic cells and monocytes was already being modulated in the HS calves compared with CT calves, suggesting prenatal HS may cause the neonate to have an altered adaptive response sooner than if they were not pre-exposed to maternal HS. A measure of responses to a vaccine or disease challenge would be necessary to determine if these changes are beneficial or detrimental.

In the present study, the mRNA expressions of IL-1 β and IL-1RA were both upregulated in the HS calves compared with the CT calves. Along with TNF- α , IL-1 β is a pro-inflammatory cytokine mediated by recognition molecules responsible for a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Interleukin-1 expression is rapidly upregulated following immune stimulation (Ito and Kodama, 1994). Interleukin-1RA acts as an inhibitor to IL-1 by competing with the IL-1 receptors. The levels of IL-1 β and IL-1RA were also both upregulated in rats exposed to HS (Lin et al., 1995). Additionally, previous studies reported that the pretreatment of IL-1RA reduced damage caused by HS in rats (Lin et al., 1995).

Table 8. Probability of fixed variables (temperature, sex, week) and their interactions for growth and feed intake of calves born to cows under heat stress for 1 wk during late gestation

Variable ¹	Temperature (T)	Sex (G)	Week	T × G	T × week
IBW	0.14	<0.01	—	0.92	—
FBW	0.46	<0.01	—	0.68	—
ΔBW	0.20	0.19	—	0.47	—
IHH	0.70	<0.01	—	0.84	—
FHH	0.33	0.01	—	0.65	—
ΔHH	0.39	0.82	—	0.73	—
IHG	0.10	<0.01	—	0.45	—
FHG	0.54	0.06	—	0.46	—
ΔHG	0.27	0.19	—	0.86	—
F:G	0.41	0.12	<0.01	0.70	0.79

¹IBW = initial BW; FBW = final BW; ΔBW = change in BW; IHH = initial hip height; FHH = final hip height; ΔHH = change in hip height; IHG = initial heart girth; FHG = final heart girth; ΔHG = change in heart girth; and F:G = feed to gain ratio for the entire 4 wk.

and rabbits (Lin et al., 1994). Thus, based on previous HS studies in other species, on d 0 and 7 the HS calves may have greater IL-1RA expression to counter the increase in IL-1β expressed during the prenatal HS. Furthermore, IL-1RA, IL-1β, and TLR4 all decreased after d 7, whereas TNF-α decreased earlier, in the first few days after the initiation of heat stress on d 0 to 3.

It has been established that due to the increase in the number of neutrophils and the decrease in lym-

phocytes, the N/L is a suitable measure often used to assess the stress response in cattle (Friend et al., 1987; Stull and McDonough, 1994). Increased neutrophil and decreased lymphocyte and eosinophil percentages were also reported to occur after various types of environmental stressors including bacterial infection in cattle (Radostist et al., 1994).

Tao et al. (2012) observed decreased hematocrit, total plasma protein, and IgG concentrations in heifer

Figure 5. The treatment by day effect on the mean fluorescence of cluster of differentiation (CD) 18 on the surface of in polymorph nuclear cells (PMNL) of calves born to cows under heat stress for 1 wk during late gestation. Data are shown as mean values ± SE (n = 10). Percent positive data and the fluorescence intensity was log-transformed before statistical analysis. *(0.05 < P < 0.01), ** (P < 0.05), *** (P < 0.001) indicate significant differences between treatments on that day.

calves born to cows exposed to late-gestational HS without heat abatement. Similarly, late-gestation HS in neonate calves (Stott, 1980) and piglets (Machado-Neto et al., 1987) caused a decrease in the concentration of IgG. In previous studies, the duration and intensity of heat stress exceeded that imposed in this experiment. In the current study, no difference in the hematocrit was found, but a lower plasma protein concentration was observed in the HS calves compared with the CT calves; however, that concentration is still considered adequate passive transfer. Monteiro et al. (2014) demonstrated that it is the calf lack of passive transfer that is responsible for less IgG concentrations rather than the colostrum quality. This suggests that our calves had adequate passive transfer, regardless of the heat stress. Growth measures of our calves were not different between HS and CT calves. This also demonstrates a milder heat stress (duration and temperature).

CONCLUSIONS

The current data provide immunological evidence of altered innate immunity during the first few weeks after birth in neonates due to maternal HS during late gestation in the first few weeks after birth. We conclude from the current study that a brief late gestation prenatal HS alters the calf's innate immune function by changes in cellular interactions with pathogens (CD18 and CD14) and changes in acute phase cytokines and pathogen recognition molecules that would alter immune responses.

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