Seasonal heat load is more potent than the degree of body weight loss in dysregulating immune function by reducing white blood cell populations and increasing inflammation in Holstein dairy cows

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ABSTRACT

We investigated the effects of seasonal heat stress and the degree of body weight (BW) loss postpartum (PP) on immune and inflammatory markers in transition dairy cows. Blood sampled twice weekly during the transition period (3 wk prepartum to 3 wk PP) from 12 peripartum Holstein cows in summer and 12 in winter was analyzed for plasma nonesterified fatty acids, the oxidative stress marker malondialdehyde (MDA), haptoglobin, and tumor necrosis factor- α (TNF- α). Complete blood counts and white blood cell (WBC) subpopulations (CD4, CD8, CD25, WC1, and CD335) were examined weekly during the transition period. Adipose tissue biopsies were taken at 7 d PP from summer-calving cows. In cows calving during seasonal heat stress, plasma TNF- α concentrations were 4.9-fold higher prepartum and 5.1-fold higher PP, and MDA concentrations were higher prepartum and PP than in winter. Hematocrit was lower prepartum and tended to be lower PP in summer compared with winter, and after adjusting to hematocrit values fewer WBC prepartum (–17%) and PP (–22%), lower monocytes prepartum (–37%) and PP (–49%), and fewer neutrophils (–25%) PP were found in summer compared with winter-calving cows. The percentages of cytotoxic T cells (CD8) and natural killer cells (CD335) were lower, as well as prepartum gamma delta-T cells (WC1) in summer compared with winter-calving cows. Moreover, lower red blood cell counts prepartum and mean platelet volume and red blood cell distribution width prepartum and PP, as well as higher hemoglobin prepartum and PP, were found in summer than

winter-calving cows. In summer, cows that lost more BW PP had higher plasma MDA and TNF- α concentrations, fewer basophil numbers prepartum, a decrease in the percentage of cells expressing the IL-2 receptor (CD25), increased ratio of T helper cells (CD4):CD8, and a tendency for increased WC1 percentages in blood prepartum, as well as a higher percentage of WC1 PP and higher abundance of TNF- α and lower lipopolysaccharide-binding protein in adipose tissue compared with cows that lost less BW PP. In conclusion, seasonal heat stress during the peripartum period was associated with lower WBC counts coupled with increased levels of subacute inflammation, indicating dysregulation of immune function, whereas the degree of BW loss had minor effects. This suggests that heat load is a more potent stressor associated with immune function than the variation in the degree of BW loss PP in high-yielding peripartum dairy cows.

Key words: heat stress, metabolic stress, immune, inflammation, dairy cow

INTRODUCTION

The main stressors affecting high-yielding dairy cows are peripartum metabolic stress and environmental heat load due to elevated ambient temperature and humidity. During the peripartum period, a reduction in immune competence coupled with a state of systemic subacute inflammation has been well characterized and includes lymphocytes' decreased ability to respond to mitogens, as well as changes in neutrophil function (Sordillo and Mavangira, 2014; Trevisi and Minuti, 2018). This is linked with a mild release of proinflammatory cytokines, without signs of infection or pathology (Bertoni and Trevisi, 2013; Bradford et al., 2015). Chronic heat load during the summer can contribute to systemic inflammation during the peripartum period by increasing the expression of proinflammatory

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cytokines in the blood cells of dairy cattle (Tao et al., 2013; Zhang et al., 2014). Moreover, immune dysfunction has been demonstrated in vitro by a lower ability of blood neutrophils to phagocytize pathogens postpartum (**PP**) in heat-stressed cows compared with those cooled prepartum (do Amaral et al., 2011). Indeed, according to data from ~430,000 calvings recorded in the Israeli Herd Book between the years 2005–2016, cows calving during the summer months (July–September) had higher incidences of ketosis and retained placenta compared with those calving in the winter (January–March; $P < 0.0001$; our unpublished data). This may suggest that cows calving during heat stress are in a greater risk of developing PP disease. Heat stress may also contribute indirectly to inflammation by promoting gut leakiness; the combination of an altered rumen environment and a leakier gut under heat stress might contribute to LPS-induced inflammation PP (Pearce et al., 2013; Bradford et al., 2015). However, only limited information is available on the effects of chronic seasonal heat stress on in vivo immune and inflammatory markers [blood count, plasma cytokines concentrations, and subpopulations of white blood cells (**WBC**) by flow cytometry] in peripartum high-yielding dairy cows.

In our previous work, we demonstrated that during the summer season, with an average thermal heat index (**THI**) of 72.4, cows in late pregnancy had increased systemic concentrations of oxidative stress marker compared with cows calving in winter (average THI = 57.8; Zachut et al., 2017). Moreover, enrichment of their adipose tissues (**AT**) with proteins related to the nuclear factor erythroid 2-related factor 2 (*Nrf2*)-mediated oxidative stress response was found in summer-calving cows, as part of their regulatory adaptations to chronic heat load in summer (Zachut et al., 2017). During the peripartum period, cows suffer from elevated oxidative stress, related to an excess of nonesterified fatty acids (**NEFA**) in the blood as a result of AT lipolysis (Abuelo et al., 2019). Disturbances in lipid metabolism are related to immune function because excess NEFA can downregulate the immune response in dairy cows (Wood et al., 2009; Zarrin et al., 2014). As heat stress also affects immune function by contributing to oxidative stress (Bradford et al., 2015), we postulate that cows that calve in the summer, under chronic heat stress, will experience a higher extent of immune dysfunction and subacute inflammation than cows that calve in winter.

Moreover, as the magnitude and duration of the inflammatory state is widely variable among cows (Bradford et al., 2015; Trevisi and Minuti, 2018), we assume that cows that experience a high degree of metabolic stress (represented by a high degree of BW loss) during the peripartum period will have an increased level of

subacute inflammation, in accordance with the premise of Bradford et al. (2015) regarding the effects of multiple stressors on immune function. We therefore examined the effects of degree of BW loss PP, using our previous definition of this criterion based on the extent of BW loss during the first 5 wk PP, classifying cows as experiencing either high weight loss (**HWL**) or low weight loss (**LWL**) PP (Zachut et al., 2013; Zachut and Moallem, 2017). The degree of BW loss PP is repeated within cows between lactations and has long-term implications on their reproductive performance (Zachut and Moallem, 2017). Because immune activation involves large and rapid increases in leukocyte populations and activity that requires energy (Wolowczuk et al., 2008), we hypothesized that HWL cows, which exhibit a severe negative energy balance PP (Zachut and Moallem, 2017), will experience dysregulated immune function and an increased inflammatory response compared with LWL cows, which experience less metabolic stress. Moreover, we hypothesized that cows that calve under chronic heat stress in the summer and lose a high degree of BW (HWL) will suffer from both heat stress and metabolic stress, which will affect immune function and subacute inflammation compared with LWL cows.

Because heat stress and peripartum metabolic stress contribute to immune dysfunction and inflammation, we investigated the effects of chronic heat stress during the summer and the degree of BW loss PP, and their interactions, on in vivo measures of immune function and inflammation in peripartum high-yielding dairy cows.

MATERIALS AND METHODS

Animals and Experimental Procedures

The experimental protocol for the study was approved by the Volcani Center Animal Care Committee (approval numbers 679/16 IL and 697/17 IL), and the experiment was performed in accordance with the relevant guidelines and regulations. The experiment was conducted at the Volcani Center experimental farm in Rishon Lezion, Israel. The study design was comparative (summer vs. winter) and retrospective (HWL vs. LWL). Twelve high-yielding, 255 ± 5 d pregnant dry multiparous Holstein dairy cows with an average parity of 3.5 (range 2–7) that were randomly available at the dairy farm during the summer season (July–September 2017) participated in this study. A formal sample size calculation was not performed due to absence of prior data, and a sample of 12 cows was enrolled for each season based on our previous studies in transition cows (Zachut et al., 2017, 2018). The cows calving in summer were not cooled during late pregnancy, but they

were exposed to 5 cooling sessions per day PP according to routine cooling management on the dairy farm, as previously described (Honig et al., 2012). Ambient temperature and relative humidity were recorded every 3 h by the Israel Meteorological Service (Bet Dagan, Israel), from a station that is located 2 km from the dairy farm, and THI was calculated as described previously (Bohmanova et al., 2007). All cows were group-housed in a covered pen with an adjacent outside yard, and fed ad libitum once a day at 1100 h with a standard Israeli diet that contained 16.5% CP and 1.78 Mcal of NE_L per kg of DM. The cows were group-housed in a shaded loose pen from calving to 21 d PP that was equipped with a real-time electronic individual feeding system. Each feeding station included an individual identification system (ID tag; SAE Afikim, Kibbutz Afikim, Israel) that allowed each cow to enter a specific feeding station and automatically recorded each meal. After calving, milk production and BW were recorded electronically 3 times daily in the milking parlor (SAE Afikim). In addition, milk samples were collected once a month from 3 consecutive milking sessions and analyzed for milk fat, protein, lactose, and urea by infrared analysis (standard IDF 141C:2000) at the laboratories of the Israeli Cattle Breeders' Association (Caesarea, Israel). According to routine management in Israel, all cows were examined by a veterinarian 5 to 10 d after calving, treated according to the farm's routine management, and clinical events and treatments were recorded.

To compare some of the seasonal effects between summer and winter, another group of 12 late pregnant cows with an average parity of 3.3 (range 2–5) that were randomly selected from the farm during the following winter season (December 2017–January 2018) were subjected to blood sampling twice a week during the peripartum period as described above, and BW and milk production and composition data were collected as described. The nutritional values of diets in winter and summer were identical, and the rations were composed of almost the same ingredients. In both seasons, the blood samples were used for the following analyses: blood sampled twice a week was analyzed for plasma NEFA, malondialdehyde (MDA), haptoglobin (HP), and tumor necrosis factor α (TNF- α). Blood sampled once a week was analyzed for full blood count and flow cytometry of subpopulations of WBC.

During the experiment in the summer season, the average calculated THI was 77.5, compared with an average THI of 58.3 during the winter. We previously demonstrated that cows in the same barn under similar cooling management during the summer are heat-stressed based on rectal temperature and respiratory rate (Honig et al., 2012).

Defining Cow Subgroups According to Their Degree of BW Loss PP

To examine differential BW loss in the first month PP as an indicator of metabolic stress in PP cows, we divided the cows from both seasons retrospectively into LWL and HWL, as described previously (Zachut et al., 2013, 2018; Zachut and Moallem, 2017). Briefly, cows losing more than the mean BW loss of all cows during the first month PP (6% BW loss) were regarded as HWL, and those losing less than this mean were defined as LWL. Accordingly, in the summer season, 6 cows were defined as **S-HWL** (on average, $7.9 \pm 1.1\%$ BW loss, range 6.3–9.5%), and 6 cows were **S-LWL** (on average, $3.2 \pm 2.6\%$ BW loss, range –1.4–5.8%). In the winter season, 6 cows were defined as **W-HWL** (on average, $8.6 \pm 2.1\%$ BW loss, range 6.1–11.0%), and 6 cows were **W-LWL** (on average, $4.1 \pm 0.8\%$ BW loss, range 2.9–5.4%). As shown in Figure 1, S-HWL and W-HWL cows lost significantly more BW (as percentage of BW at wk 1 PP) than did S-LWL and W-LWL cows between wk 2 and 6 PP.

Analysis of NEFA, Oxidative Stress, and Inflammatory Markers in Blood Plasma

Plasma samples obtained from heparin-coated tubes from both seasons were analyzed for NEFA, MDA, HP, and TNF- α . Blood samples were collected twice weekly from 3 wk before expected calving until 3 wk

Figure 1. Percentage of BW loss relative to wk 1 postpartum (PP) in cows that differ in degree of metabolic stress. Peripartum dairy cows that calved under chronic heat stress in the summer were divided retrospectively into those with high weight loss during the first 5 wk PP (S-HWL, $n = 6$), and those with low weight loss (S-LWL, $n = 6$). Another group of peripartum cows that calved in the winter was similarly divided into W-HWL ($n = 6$) and W-LWL ($n = 6$). Data presented as mean $\pm$ SEM.

PP from the jugular vein into vacuum tubes containing lithium heparin (Becton Dickinson Systems, Cowley, England). The blood samples were collected after the morning milking at 0700 h, and plasma was separated after centrifugation at $4,000 \times g$ for 15 min at 4°C and then stored at -80°C pending analyses. Plasma NEFA concentration was determined as previously described (Zachut et al., 2017), with an intraassay coefficient of variation of 6.7%. Plasma MDA concentration was measured by the fluorometric thiobarbituric acid reactive substances assay (Feldman, 2004); the intra- and interassay coefficients of variation were 9.5% and 2.6%, respectively. Plasma HP concentrations were determined by ELISA (E-10HPT, ICL, Portland, OR), and TNF- α concentration was determined with a Bovine TNF- α DuoSet ELISA Kit (R&D Systems Inc., Minneapolis, MN); the intra- and interassay coefficients of variation were 9.4 and 5.7%, respectively.

In addition, we examined the concentrations of the acute-phase protein and marker of endotoxemia LPS-binding protein (**LBP**) and of complement C3 in the plasma during the first 2 wk PP. Plasma LBP was determined by a commercial bovine LBP ELISA Kit (OKCA02041, Aviva System Biology, San Diego, CA), and plasma complement C3 by a commercial Bovine C3 ELISA Kit (LS-F13127, LSBio, Seattle, WA). Plasma iron concentrations were determined from 14 d prepartum to 14 d PP using a Cobus C111 auto-analyzer (Roche Holding GmbH, Grenzach-Wyhlen, Germany) with the reagent set IRON2 that first liberates Fe³⁺ ions from the transferrin complex using pH <2, then ascorbate reduces Fe³⁺ to Fe²⁺ ions, and the reaction of the Fe²⁺ ions with FerroZine gives a colored complex that is directly proportional to the iron concentration and was measured photometrically.

Blood Counts

Whole blood samples from EDTA-coated tubes obtained in both seasons were sent once a week during the experiments in both seasons to the laboratory of the Veterinary Hospital of Hebrew University (Rishon Lezion, Israel), and a complete blood count was performed using a hematology analyzer (Advia 2120i, Siemens Medical Solutions, Erfurt, Germany).

Peripheral Blood Leukocyte Populations by Flow Cytometry

Peripheral blood leukocyte populations in both seasons were analyzed from an additional heparin-coated tube once a week. Blood samples were put immediately on ice and were analyzed within 1 h of blood sampling using flow cytometry as described previously (Lange et

al., 2016), with some modifications; the following antibodies (Bio-Rad, Hercules, CA) were used: T helper cells were identified using anti-bovine CD4 (MCA 1653 PE); cytotoxic T lymphocytes were identified using anti-bovine CD8 (MCA837 A647); cells expressing the IL-2 receptor (CD25) were identified using anti-bovine CD25 (MCA2430F); gamma delta-T cells were identified using anti-bovine WC1 (MCA1655F); and natural killer cells were identified using anti-bovine CD335 (MCA2365PE). For the analysis, 7 μ L each of the various antibodies was transferred into FACS glass tubes (DWK Life Sciences LLC, Milville, NJ), and 50 μ L of whole blood was added and incubated for 30 min at 4°C in the dark. The red blood cells (**RBC**) were lysed by adding 9 parts distilled water for no more than 1 min. Subsequently, one part of $10 \times$ PBS was added (02-023-5A, Biological Industries, Beit HaEmek, Israel) with mixing. Samples were then centrifuged for 10 min at $400 \times g$ and 4°C. The samples were then washed with 2 mL of PBS and centrifuged at $400 \times g$ for 7 min at 4°C. Finally, cell pellets were suspended in 300 μ L of PBS and analyzed using the Accuri C6 Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ). A gate for leukocytes was drawn containing lymphocytes and monocytes based on their forward and side scatter characteristics. For each cell type, the percentage of cells within this gate expressing the surface marker was determined. An example of the gating strategy is shown in Supplemental File S1 (<https://doi.org/10.3168/jds.2020-18547>).

AT Biopsies and Immunoblot Analyses

Subcutaneous AT biopsies were obtained on d 7 PP from cows that calved during the summer season. The AT samples were taken from the subcutaneous fat pad around the pin bones as previously described (Zachut et al., 2013), immediately frozen in liquid nitrogen, and stored at -80°C. For immunoblots of AT, the protein concentration of the sample homogenate was measured by bicinchoninic acid assay. Then, a 20- μ g sample in Laemmli loading buffer was resolved by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane with antibodies to TNF- α (1:1,000, OCAO4183, Aviva System Biology, San Diego, CA), LBP (1 μ g/mL, ARP41546_P050, Aviva System Biology), and β -actin (1:1,000, ab46805, Abcam Biotech). Goat anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch 111-035-003, West Grove, PA) at a concentration of 1:10,000 was used in an electrochemiluminescence reaction for protein detection. Data were processed and analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). To ensure that quantitative data were

obtained, chemiluminescence signals were measured during at least 5 consecutive exposure times to determine each antibody's linear range of signal intensity. Specific band signals were normalized to β -actin.

Statistical Analysis

Plasma concentrations of NEFA, MDA, HP, TNF- α , LBP, C3, iron, as well as milk yields, hematology parameters, and subpopulations of WBC, were analyzed as repeated measurements with the MIXED procedure (SAS, version 9.2, SAS Institute Inc., Cary, NC), using the following model:

$$Y_{ijkl} = \mu + S_i + SG_j + C(S \times SG)_{ijk} + SSG_{ijkl} \\ + DIM_{ijklmn} + E_{ijklmn},$$

where μ = the overall mean; S_i = the season effect (i = summer or winter); SG_j = the subgroup effect (j = HWL or LWL); $C(S \times SG)_{ijk}$ = cow k nested in season i and subgroup j ; SSG_{ijkl} = season $\times$ subgroup; DIM_{ijklmn} = day in lactation as a continuous variable; and E_{ijklmn} = the random residual.

For all hematology parameters, we have included the effect of hematocrit (**HCT**) and the interaction of HCT $\times$ season in the statistical model above. When the effect of HCT $\times$ season was not significant, it was excluded from the model.

Protein abundance in AT from immunoblots was analyzed with the GLM procedure of SAS. The effect of disease events was examined and was not significant for any of the tested variables; therefore, it was excluded from the model. Significance was set at $P \leq 0.05$ and tendencies were set at $P \leq 0.10$.

RESULTS

Cow Performance and Circulating Concentrations of Metabolic and Inflammatory Markers

In cows calving during the summer, milk yields during the first month PP were 40.3 kg/d in S-HWL and 37.8 kg/d in S-LWL cows (SEM = 1.1, $P = 0.2$). In the winter, milk yields were higher for W-HWL than for W-LWL cows (47.9 vs. 43.5 kg/d, respectively, SEM = 0.8, $P = 0.004$). The PTA values of the cows were not different between summer- and winter-calving cows ($P = 0.76$), or between HWL and LWL cows ($P = 0.20$). Dry matter intake was similar between S-HWL and S-LWL cows during the first 30 d PP (19.4 and 19.5 kg/d, respectively, SEM = 0.9, $P = 0.9$), and the average calculated energy balance PP was -6.7 Mcal/d in S-HWL compared with -2.8 Mcal/d in S-LWL cows

(SEM = 0.9, $P = 0.02$). All cows were examined by a veterinarian at 5 to 10 d PP according to the farm's routine; 1 S-HWL, 3 S-LWL, and 1 W-LWL cows had metritis, were treated once, and recovered; 2 W-HWL cows had ketosis, were treated once i.v. with a dextrose infusion, and were rechecked 4 d later and found healthy. All cows were monitored regularly during the study for any signs of clinical disease.

Prepartum, average plasma NEFA concentrations were higher in winter than in summer late pregnant cows ($P = 0.01$; Table 1), whereas concentrations of the oxidative stress marker MDA were higher in summer versus winter cows ($P = 0.008$; Table 1 and Figure 2), and tended to be higher in S-HWL than in S-LWL cows ($P = 0.06$). No differences were observed in HP concentrations between subgroups or seasons (Table 1). Plasma TNF- α concentrations were 4.9-fold higher prepartum in summer versus winter (1,504.9 vs. 305.0 pg/mL, respectively, SEM = 262.3, $P = 0.0002$; Table 1 and Figure 2), and tended to be higher in S-HWL than in S-LWL cows ($P = 0.06$).

In a similar manner, PP plasma NEFA concentrations were higher in winter-calving than in summer-calving cows ($P < 0.0001$; Table 1); they tended to be higher in W-HWL than in W-LWL cows ($P = 0.07$), and the interaction subgroup $\times$ season tended to be significant ($P = 0.07$; Table 1). Plasma MDA concentrations were higher in summer than in winter ($P = 0.002$), were higher in S-HWL than in S-LWL cows ($P = 0.03$), and the effect of subgroup tended to be significant ($P = 0.10$; Table 1 and Figure 2). When analyzing both periods (prepartum and PP) together, the overall effect of subgroup (HWL vs. LWL) on MDA concentrations was significant ($P = 0.04$). No differences were observed in HP concentrations PP (Table 1). Plasma TNF- α concentrations were 5.1-fold higher in summer-calving versus winter-calving cows PP (1,185.7 vs. 233.9 pg/mL, respectively, SEM = 189.7, $P < 0.0001$), and were higher in S-HWL than in S-LWL cows ($P = 0.03$; Table 1).

As mentioned, concentrations of the acute-phase protein and marker of endo-toxemia LBP and of complement C3 were examined during 0–14 d PP; the average concentration of LBP was higher on d 7 PP in HWL than in LWL cows across seasons (7.3 vs. 4.3 ng/mL, respectively, SEM = 1.0, $P = 0.05$), and the average concentrations of plasma complement C3 tended to be higher in summer-calving than winter-calving cows on d 7 PP (816.5 vs. 620.3 pg/mL, SEM = 84.1, $P = 0.10$). Plasma iron was examined from 14 d prepartum to 14 d PP, and across seasons the average iron concentrations were decreased by 27% at 0 to 2 d PP compared with 1 to 3 d prepartum, and the lower plasma iron concentrations were maintained until 10 d PP (-18%

Table 1. Plasma nonesterified fatty acids (NEFA), oxidative stress, and inflammatory markers in cows with high (HWL) or low (LWL) weight loss during the transition period in summer and winter seasons

Item	Season ¹				SEM	P-value		
	Summer		Winter			Subgroup (HWL vs. LWL)	Season	Subgroup × season
	S-HWL ²	S-LWL	W-HWL	W-LWL				
Prepartum								
NEFA, μEq/L	143.1 ^b	202.0 ^b	463.6 ^a	367.4 ^{ab}	84.8	0.82	0.01	0.37
MDA, μM	300.5 ^a	162.0 ^a	93.9 ^b	68.2 ^b	50.6	0.12	0.008	0.28
HP, pg/mL	62.5	22.5	72.4	49.3	39.5	0.44	0.65	0.83
TNF-α, pg/mL	1,855.0 ^a	1,154.7 ^a	339.6 ^b	270.4 ^b	262.3	0.16	0.0002	0.24
Postpartum								
NEFA, μEq/L	180.3 ^b	242.9 ^b	774.9 ^a	542.3 ^a	76.5	0.28	<0.0001	0.07
MDA, μM	431.0 ^a	216.1 ^b	93.2 ^c	73.7 ^c	51.7	0.10	0.002	0.16
HP, pg/mL	245.3	235.3	147.9	257.3	86.1	0.58	0.67	0.50
TNF-α, pg/mL	1,470.9 ^a	900.5 ^b	266.0 ^c	201.9 ^c	189.7	0.11	<0.0001	0.20

^{a-c}Values with different superscript letters in a row are significantly different at $P \leq 0.05$.

¹Peripartum dairy cows (from 3 wk prepartum to 3 wk postpartum) under chronic heat stress in summer ($n = 12$) or in winter ($n = 12$) were compared for plasma NEFA, oxidative stress, and inflammatory markers. HP = haptoglobin; MDA = malondialdehyde; TNF = tumor necrosis factor.

²Cows were divided retrospectively into those with high weight loss during the first 5 wk postpartum in summer (S-HWL, $n = 6$) and those with low weight loss in summer (S-LWL, $n = 6$), and similarly in winter into W-HWL ($n = 6$) and W-LWL ($n = 6$) cows.

compared with prepartum). However, when analyzing the effects of season and subgroups, no differences were found prepartum (23.4, 22.6, 23.9, and 24.5 $\mu\text{mol/L}$ for W-HWL, W-LWL, S-HWL, and S-LWL, respectively, SEM = 2.3, $P > 0.6$) or PP (21.5, 20.7, 20.6, and 22.0 $\mu\text{mol/L}$ for W-HWL, W-LWL, S-HWL, and S-LWL, respectively, SEM = 1.5, $P > 0.5$) between seasons, subgroups, or their interaction.

Blood Counts

Prepartum HCT was lower in summer-calving than in winter-calving cows ($P = 0.002$; Table 2), therefore the blood counts were adjusted to HCT in the statistical model. As shown in Table 2, prepartum WBC counts were 17% lower (7.77 vs. 9.38 $10^3/\mu\text{L}$ in summer and winter, respectively; $P = 0.05$; Figure 3), and the number of monocytes (−37%; $P = 0.03$; Figure 3), and large uncharacterized cells (−61%; $P = 0.02$) were lower in the summer versus winter prepartum cows. For the number of monocytes, the interaction of HCT × season was significant ($P = 0.01$). The number of lymphocytes prepartum tended to be lower in summer than in winter (−20%; $P = 0.10$; Figure 3). The number of basophils prepartum was lower in S-HWL than in S-LWL cows ($P = 0.02$), and the effect of the subgroup tended to be significant ($P = 0.08$; Table 2). In addition, average percentages of monocytes tended to be lower (−28%, $P = 0.07$), and lower basophils (−46%, $P < 0.0001$) and large uncharacterized cells (−63%, $P = 0.008$) were found prepartum in summer compared with winter.

The interaction of HCT × season was significant ($P = 0.04$) for percentage of monocytes.

The average RBC counts were lower in summer versus winter prepartum cows (5.91 vs. 6.07 $10^6/\mu\text{L}$, respectively, $P = 0.009$). The effect of HCT ($P < 0.0001$) and the of interaction HCT × season ($P = 0.008$) were significant for RBC counts prepartum. The average hemoglobin counts during the prepartum period were higher in the summer than in winter ($P = 0.0009$; Table 2), and the effect of HCT on hemoglobin was significant ($P < 0.0001$). Reductions in RBC distribution width (RDW; $P = 0.01$), hemoglobin distribution width (HDW; $P < 0.0001$), and a tendency for lower mean platelet volume (MPV; $P = 0.06$; Table 2) were also observed in summer compared with winter prepartum cows. For RDW, the effect of HCT ($P = 0.02$) and the interaction of HCT × season ($P = 0.005$) were significant. For MPV, the interaction of HCT × season was significant ($P = 0.04$). The percentages of hyperchromic RBC ($P < 0.0001$) were lower in summer versus winter prepartum cows (Table 2). The effect of subgroup on the number of platelets (PLT) tended to be significant ($P = 0.08$), and S-HWL tended to have lower PLT than S-LWL ($P = 0.10$).

During the PP period the HCT tended to be lower in summer than in winter cows ($P = 0.09$; Table 3). Postpartum, WBC counts were 22% lower in summer versus winter-calving cows (8.0 vs. 10.3 $10^3/\mu\text{L}$, respectively, $P = 0.01$; Figure 3 and Table 3), and the effect of subgroup was significant ($P = 0.04$; Table 3), as W-HWL had higher WBC counts than W-LWL cows ($P =$

0.02). The effect of HCT was significant ($P = 0.01$) for WBC PP. The average number of lymphocytes tended to be lower in summer than in winter (-14% ; $P = 0.10$), and the effect of subgroup was significant ($P = 0.04$), as W-HWL had lower numbers of lymphocytes than W-LWL cows ($P = 0.05$). The average numbers of monocytes (-49% , $P < 0.0001$; Figure 3) and large uncharacterized cells (-62% ; $P = 0.002$) were lower in the summer versus winter PP cows (Table 3). The number of neutrophils was lower in summer than in winter cows (-25% ; $P = 0.05$; Table 3). The number of large uncharacterized cells was lower in summer versus

winter ($P = 0.01$). In addition, the number of basophils was lower in W-HWL than in W-LWL cows ($P = 0.002$), and the effect of the subgroup was significant ($P = 0.003$; Table 3). For the number of basophils, the effect of HCT was significant ($P = 0.02$). Lower average percentages of monocytes (-37% , $P < 0.0001$), basophils (-26% , $P = 0.005$), and large uncharacterized cells (-57% , $P = 0.01$; Table 3) in summer versus winter-calving cows were found PP. For the percentages of monocytes, the effect of HCT was significant ($P = 0.05$). The percentage of eosinophils tended to be higher in summer than in winter cows ($P = 0.07$; Table 3), and the effect of HCT was significant ($P = 0.003$).

No differences were found in the average RBC counts in summer compared with winter cows PP (Table 3), and the effect of HCT on RBC was significant ($P < 0.0001$). Average hemoglobin was higher in summer than in winter ($P = 0.04$), and the effect of HCT on hemoglobin was significant ($P < 0.0001$). Average RDW was lower PP in summer versus winter PP cows ($P = 0.0001$; Table 3). The average MPV was lower in summer than in winter cows PP ($P = 0.0008$). For MPV, the effect of HCT ($P = 0.01$) and the interaction of HCT $\times$ season ($P < 0.0001$) were significant.

The average percentages of both hypochromic ($P = 0.004$) and hyperchromic RBC ($P = 0.05$) were lower in summer versus winter PP cows (Table 3), and the interaction subgroup $\times$ season tended to be significant both for hypochromic and hyperchromic RBC ($P = 0.09$, Table 3). For hypochromic RBC, the interaction of HCT $\times$ season was significant ($P = 0.01$), and for hyperchromic RBC the effect of HCT ($P = 0.04$) and the interaction of HCT $\times$ season ($P = 0.01$) were significant.

When analyzing both time periods (prepartum and PP) across seasons, the WBC counts were lower in HWL than in LWL cows ($P = 0.03$), and PLT tended to be lower in HWL than in LWL cows ($P = 0.08$). In addition, the number of lymphocytes tended to be lower in HWL than in LWL cows ($P = 0.09$) and the number of basophils was lower in HWL than in LWL cows ($P = 0.009$). We also analyzed the blood count data between prepartum and PP periods, and except for monocyte counts ($P = 0.02$) and percentages ($P = 0.009$), which were higher PP than prepartum, all other parameters and the interactions of period $\times$ season were not significant.

Subpopulations of WBC in the Blood

As shown in Table 4, during the prepartum period the average percentage of CD4 cells did not differ between subgroups or seasons; however, the S-HWL tended to have a higher percentage of CD4 than the

Figure 2. Plasma concentrations of tumor necrosis factor- α (TNF- α ; A) and the oxidative marker malondialdehyde (MDA; B) during the peripartum period. Peripartum dairy cows that calved under chronic heat stress in the summer were divided retrospectively into those with high weight loss during the first 5 wk postpartum (S-HWL, $n = 6$), and those with low weight loss (S-LWL, $n = 6$). Another group of peripartum cows that calved in the winter was similarly divided into W-HWL ($n = 6$) and W-LWL ($n = 6$). Data presented as mean $\pm$ SEM.

Table 2. Hematology during the prepartum period in cows with high (HWL) or low (LWL) weight loss in summer and winter

Parameter ^{1,2}	Season ³				SEM	<i>P</i> -value		
	Summer		Winter			Subgroup (HWL vs. LWL)	Season	Subgroup × season
	S-HWL ⁴	S-LWL	W-HWL	W-LWL				
WBC parameter								
WBC, 10 ³ /μL	7.43 ^b	8.10 ^{ab}	8.93 ^{ab}	9.83 ^a	0.74	0.30	0.05	0.87
Lymphocytes, 10 ³ /μL	2.93 ^b	3.67 ^{ab}	3.86 ^{ab}	4.42 ^a	0.46	0.17	0.10	0.84
Monocytes, 10 ³ /μL	0.34 ^b	0.33 ^b	0.46 ^{ab}	0.61 ^a	0.06	0.27	0.03	0.18
Neutrophils, 10 ³ /μL	3.68	3.66	3.76	4.08	0.33	0.66	0.52	0.63
Eosinophils, 10 ³ /μL	0.41	0.32	0.48	0.43	0.13	0.59	0.52	0.87
Basophils, 10 ³ /μL	0.04 ^b	0.14 ^a	0.13 ^{ab}	0.14 ^a	0.03	0.07	0.29	0.17
Large uncharacterized cells, 10 ³ /μL	0.03 ^b	0.04 ^b	0.10 ^a	0.07 ^{ab}	0.02	0.53	0.02	0.23
Lymphocytes, %	39.69	45.94	42.49	44.90	2.88	0.14	0.78	0.50
Monocytes, %	4.76 ^{ab}	3.99 ^b	5.94 ^{ab}	6.27 ^a	0.64	0.71	0.07	0.37
Neutrophils, %	48.96	44.82	43.80	42.12	2.89	0.31	0.22	0.67
Eosinophils, %	7.22	3.86	30.05	35.00	12.63	0.95	0.07	0.73
Basophils, %	0.71 ^b	0.78 ^b	1.30 ^a	1.46 ^a	0.08	0.20	<0.0001	0.58
Large uncharacterized cells, %	0.35 ^b	0.37 ^b	1.21 ^a	0.76 ^{ab}	0.18	0.25	0.008	0.20
RBC parameter								
RBC, 10 ⁶ /μL	5.85	5.97	6.12	6.02	0.15	0.94	0.009	0.50
HGB, g/dL	10.72 ^a	10.71 ^a	10.49 ^b	10.51 ^b	0.04	0.87	0.0009	0.67
HCT, %	27.13 ^b	27.12 ^b	30.19 ^a	28.86 ^{ab}	0.69	0.34	0.002	0.35
RDW, %	19.53 ^{ab}	18.89 ^b	18.83 ^b	20.25 ^a	0.42	0.37	0.01	0.02
HDW, g/dL	2.22 ^b	2.16 ^b	2.77 ^a	2.93 ^a	0.06	0.43	<0.0001	0.08
PLT, 10 ³ /μL	358.77 ^{ab}	442.07 ^a	327.12 ^b	377.48 ^{ab}	36.8	0.08	0.25	0.65
MPV, fL	7.64	7.75	7.80	9.15	0.76	0.31	0.06	0.39
HYPO, %	0.25	0.29	0.28	0.17	0.09	0.73	0.69	0.40
HYPER, %	1.72 ^b	1.02 ^b	10.39 ^a	13.19 ^a	1.66	0.53	<0.0001	0.29

^{a,b}Values in a row with different superscripts are significantly different at $P \leq 0.05$.

¹WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; HCT = hematocrit; RDW = RBC distribution width; HDW = hemoglobin distribution width; PLT = platelets; MPV = mean platelet volume; HYPO = hypochromic RBC; HYPER = hyperchromic RBC.

²Reference intervals for bovine hematology (Wood and Quiroz-Rocha, 2010): RBC 4.9–7.5 10⁶/μL; HGB: 8.4–12.0 g/dL; HCT: 21–30%; RDW: 16–20%; PLT: 160–650 10³/μL; MPV: 4.6–7.4 fL; neutrophils: 1.7–6.2 10³/μL; lymphocytes: 1.8–8.1 10³/μL; monocytes: 0.1–0.7 10³/μL; eosinophils: 0.1–1.2 10³/μL; basophils: 0–0.2 10³/μL.

³Peripartum dairy cows (from 3 wk prepartum to 3 wk postpartum) under chronic heat stress in summer ($n = 12$) or in winter ($n = 12$) were compared for weekly analysis hematological parameters.

⁴Cows were divided retrospectively into those with high weight loss during the first 5 wk postpartum in summer (S-HWL, $n = 6$) and those with low weight loss in summer (S-LWL, $n = 6$), and similarly in winter into W-HWL ($n = 6$) and W-LWL ($n = 6$).

S-LWL cows ($P = 0.07$). The average percentage of CD8 cells prepartum was lower in summer-calving versus winter-calving cows (12.0 vs. 20.7%, respectively, SEM = 3.0, $P = 0.009$), and the interaction subgroup × season tended to be significant ($P = 0.06$; Table 4). The CD4:CD8 ratio was higher in S-HWL versus S-LWL cows prepartum ($P = 0.02$), and the interaction subgroup × season was significant ($P = 0.03$; Table 4). The percentage of WC1 cells was lower in summer versus winter prepartum cows (23.3 vs. 30.6%, SEM = 3.6, $P = 0.05$; Table 4). The effect of subgroup (HWL vs. LWL) on the percentage of WC1 cells tended to be significant ($P = 0.06$; Table 4), and the percentage of WC1 cells tended to be higher in S-HWL cows than in S-LWL cows prepartum ($P = 0.10$). The percentage of CD25 cells was lower in summer versus winter prepartum cows (26.4 vs. 38.4%, SEM = 4.4, $P = 0.01$; Table 4). The average percentage of CD335 cells pre-

partum was lower in summer than in winter cows (2.7 vs. 6.9%, SEM = 0.4, $P < 0.0001$; Table 4), the effect of subgroup on CD335 cells was significant ($P = 0.05$; Table 4) and it tended to be higher in W-HWL than in W-LWL prepartum ($P = 0.08$).

Postpartum, no differences were found in the percentages of CD4 cells, but the average percentages of CD8 (11.2 vs. 22.6%, respectively, SEM = 2.1, $P = 0.009$) was lower in summer-calving than in winter-calving cows. No differences in the CD4:CD8 ratio were observed PP (Table 4). Regarding the percentage of WC1 cells PP, it should be noted that in the winter, WC1 was not monitored PP due to a technical problem. Therefore, the effect of subgroup was significant ($P = 0.02$) due to the higher percentage of WC1 in S-HWL than in S-LWL PP ($P = 0.02$; Table 4). The average percentage of CD25 was not different PP, but the average percentage of CD335 cells (3.1 vs. 5.2%,

Figure 3. Blood counts adjusted to hematocrit of white blood cells (WBC; A), number of lymphocytes (B), and number of monocytes (C) in periparturient dairy cows that calved under chronic heat stress in the summer ($n = 12$) and cows that calved in winter ($n = 12$). * $P \leq 0.05$; *** $P \leq 0.001$; † $P \leq 0.1$. Data presented as mean $\pm$ SEM.

respectively, SEM = 0.7, $P = 0.005$; Table 4) was lower in summer-calving than in winter-calving cows. The interaction subgroup $\times$ season for CD335 tended to be significant ($P = 0.06$), as CD335 tended to be lower in W-LWL than in W-HWL ($P = 0.08$).

Protein Abundance in the AT

During the summer season, the abundance of TNF- α in the AT of cows 7 d PP was higher for the S-HWL versus S-LWL group (1.7 vs. 0.9 arbitrary units in HWL and LWL AT, respectively, SEM = 0.1, $P = 0.001$), whereas the abundance of LBP was lower in S-HWL than in S-LWL AT (0.2 vs. 0.5 arbitrary units, respectively, SEM = 0.08, $P = 0.05$).

DISCUSSION

The findings of the present study demonstrate that environmental heat stress resulting from continuous high ambient temperature and humidity during the summer is more potent than the degree of BW loss PP in altering immune and inflammatory markers in vivo in high-yielding periparturient dairy cows. Heat stress during the transition period was associated with increased markers of subacute inflammation and oxidative stress and reduced WBC and some RBC-related counts. Although other seasonal factors such as photoperiodic and circadian rhythm may contribute to these differences, we presume that heat stress was the main effector, as previously stated (Bernabucci et al., 2002; Zachut et al., 2017). Generally, most of the effects on immune and inflammatory markers between seasons were similar during the prepartum and PP period. Therefore, in the following sections, we discuss the effects of seasonal heat stress (summer vs. winter), the effects of degree of BW loss PP (HWL vs. LWL), and their interaction on the examined immune and inflammatory parameters during the transition period.

Association Between Seasonal Heat Load, Blood Counts, and WBC Populations in Periparturient Cows

In this study, we found lower HCT prepartum and a tendency for lower HCT PP in summer- versus winter-calving cows, and after adjusting the hematological parameters to HCT, several WBC-related counts were lower in summer- versus winter-calving cows. Very limited information is available on hematological values during summer and winter seasons in healthy Holstein cows. In the present study, during summer heat load lower WBC counts prepartum (−17%) and PP (−22%) were found in the cows compared with winter. This was accompanied by lower percentages of CD8 and CD335

cells, and lower WC1 cells in blood prepartum. Overall, the blood counts (Wood and Quiroz-Rocha, 2010) and flow cytometry findings (Banos et al., 2013; Lange et al., 2016) in the present study are within the range normally observed in peripartum dairy cows. Lacetera et al. (2005a) reported that cows calving in summer (vs. spring) had lower DNA synthesis in peripheral blood mononuclear cells in response to a mitogen in vitro, suggesting lower lymphocyte proliferation in heat-stressed peripartum cows. A decrease in lymphocyte proliferation during heat stress could partly explain the lower WBC count in the present study. In addition, we found that cows calving during summer heat stress had 37 to 49% fewer monocyte numbers and lower percentages of monocytes PP compared with winter-calving cows. Interestingly, monocytopenia is associated with endotoxemia (Tornquist and Rigas, 2010) and inflammation

(Jones and Allison, 2007), which could be related to the increase in gut permeability in heat-stressed cows (Pearce et al., 2013; Bradford et al., 2015). Moreover, we found lower percentages of basophils prepartum and PP, as well as reduced numbers of neutrophils PP in cows calving during summer versus winter, which is in accordance with the decreased basophil counts reported for Holstein Friesian-crossbred cows in the summer (Narayan et al., 2007).

The number of blood lymphocytes observed in our study tended to be lower prepartum and PP in summer- versus winter-calving cows. Lymphocytopenia can be caused by acute stress or immune suppression (Roland et al., 2014). Recently, inconclusive findings were reported on the effect of chronic heat stress on blood leukocytes in several cattle breeds (Bagath et al., 2019). Taken together, our findings confirm in

Table 3. Hematology during the postpartum period in cows with high (HWL) or low (LWL) weight loss in summer and winter

Parameter ^{1,2}	Season ³				SEM	P-value		
	Summer		Winter			Subgroup (HWL vs. LWL)	Season	Subgroup × season
	S-HWL ⁴	S-LWL	W-HWL	W-LWL				
WBC parameter								
WBC, 10 ³ /μL	7.75 ^b	8.23 ^b	8.71 ^b	11.80 ^a	0.79	0.04	0.01	0.12
Lymphocytes, 10 ³ /μL	2.89 ^b	3.26 ^{ab}	3.13 ^b	4.03 ^a	0.29	0.04	0.10	0.39
Monocytes, 10 ³ /μL	0.51 ^b	0.36 ^b	0.81 ^a	0.89 ^a	0.07	0.69	<0.0001	0.14
Neutrophils, 10 ³ /μL	4.17 ^b	4.18 ^b	4.60 ^{ab}	6.48 ^a	0.62	0.15	0.05	0.15
Eosinophils, 10 ³ /μL	0.18	0.20	0.18	0.17	0.07	0.96	0.78	0.80
Basophils, 10 ³ /μL	0.06 ^b	0.07 ^b	0.07 ^b	0.13 ^a	0.01	0.003	0.15	0.05
Large uncharacterized cells, 10 ³ /μL	0.04 ^b	0.05 ^b	0.13 ^a	0.11 ^a	0.02	0.81	0.002	0.54
Lymphocytes, %	39.65	42.77	37.84	36.20	3.07	0.81	0.19	0.45
Monocytes, %	5.79 ^b	4.71 ^b	8.90 ^a	7.84 ^a	0.63	0.11	<0.0001	0.99
Neutrophils, %	50.70	48.49	50.00	50.20	3.42	0.99	0.67	0.53
Eosinophils, %	2.48	2.68	0.91	1.66	0.68	0.49	0.07	0.69
Basophils, %	0.77 ^b	0.74 ^b	0.92 ^{ab}	1.12 ^a	0.08	0.36	0.005	0.18
Large uncharacterized cells, %	0.55 ^b	0.51 ^b	1.44 ^a	1.02 ^{ab}	0.25	0.39	0.01	0.48
RBC parameter								
RBC, 10 ⁶ /μL	5.40	5.49	5.68	5.53	0.13	0.81	0.26	0.39
HGB, g/dL	9.90 ^a	9.87 ^{ab}	9.76 ^b	9.81 ^{ab}	0.05	0.83	0.04	0.43
HCT, %	25.57 ^b	25.61 ^b	27.51 ^a	25.84 ^{ab}	0.60	0.20	0.09	0.18
RDW, %	19.66 ^b	18.91 ^b	21.29 ^a	21.23 ^a	0.43	0.30	0.0001	0.36
HDW, g/dL	2.26 ^{ab}	2.15 ^{ab}	2.04 ^b	2.46 ^a	0.13	0.27	0.74	0.06
PLT, 10 ³ /μL	458.36	566.37	480.05	514.63	50.67	0.17	0.77	0.48
MPV, fL	7.46 ^{bc}	7.25 ^b	7.85 ^{ac}	7.98 ^a	0.17	0.78	0.0008	0.29
HYPO, %	0.18 ^c	0.16 ^c	0.84 ^a	0.49 ^b	0.09	0.08	0.004	0.09
HYPER, %	1.63 ^b	1.34 ^b	2.62 ^b	8.39 ^a	1.75	0.13	0.05	0.09

^{a-c}Values in a row with different superscripts are significantly different at $P \leq 0.05$.

¹WBC = white blood cells; RBC = red blood cells; HGB = hemoglobin; HCT = hematocrit; RDW = RBC distribution width; HDW = hemoglobin distribution width; PLT = platelets; MPV = mean platelet volume; HYPO = hypochromic RBC; HYPER = hyperchromic RBC.

²Reference intervals for bovine hematology (Wood and Quiroz-Rocha, 2010): RBC 4.9–7.5 10⁶/μL; HGB: 8.4–12.0 g/dL; HCT: 21–30%; RDW: 16–20%; PLT: 160–650 10³/μL; MPV: 4.6–7.4 fL; neutrophils: 1.7–6.2 10³/μL; lymphocytes: 1.8–8.1 10³/μL; monocytes: 0.1–0.7 10³/μL; eosinophils: 0.1–1.2 10³/μL; basophils: 0–0.2 10³/μL.

³Peripartum dairy cows (from 3 wk prepartum to 3 wk postpartum) under chronic heat stress in summer (n = 12) or in winter (n = 12) were compared for weekly analysis hematological parameters.

⁴Cows were divided retrospectively into those with high weight loss during the first 5 wk postpartum in summer (S-HWL, n = 6) and those with low weight loss in summer (S-LWL, n = 6), and similarly in winter into W-HWL (n = 6) and W-LWL (n = 6).

Table 4. Subpopulations of white blood cells (WBC) during the transition period in cows with high (HWL) or low (LWL) weight loss during the first month postpartum in summer and winter

Item	Season				SEM	<i>P</i> -value		
	Summer ¹		Winter			Subgroup (HWL vs. LWL)	Season	Subgroup × Season
	S-HWL ²	S-LWL	W-HWL	W-LWL				
Prepartum								
CD4, %	19.2	13.7	16.2	16.3	2.1	0.22	0.92	0.21
CD8, %	9.6 ^b	14.3 ^b	24.5 ^a	16.8 ^{ab}	3.0	0.63	0.009	0.06
CD4:CD8	2.1 ^a	1.1 ^b	1.1 ^b	1.5 ^{ab}	0.3	0.36	0.25	0.03
WC1, %	27.4 ^{ab}	19.1 ^b	33.6 ^a	27.6 ^{ab}	3.6	0.06	0.05	0.76
CD25, %	19.3 ^b	33.4 ^a	38.8 ^a	38.0 ^a	4.4	0.14	0.01	0.11
CD335, %	2.9 ^b	2.4 ^b	7.4 ^a	6.3 ^a	0.4	0.05	<0.0001	0.53
Postpartum								
CD4, %	14.7	13.6	16.0	16.7	2.7	0.94	0.41	0.75
CD8, %	10.7 ^b	11.6 ^b	20.0 ^a	25.1 ^a	2.1	0.17	<0.0001	0.32
CD4:CD8	1.5	1.2	1.3	1.3	0.2	0.54	0.80	0.55
WC1, ³ %	25.0 ^a	9.5 ^b	—	—	3.8	0.02	—	—
CD25, %	27.2	20.9	23.6	31.1	5.4	0.90	0.55	0.21
CD335, %	3.4 ^b	2.7 ^b	4.2 ^{ab}	6.2 ^a	0.7	0.36	0.005	0.06

^{a,b}Values in a row with different superscripts are significantly different at $P \leq 0.05$.

¹Peripartum dairy cows (from 3 wk prepartum to 3 wk postpartum) under chronic heat stress in summer ($n = 12$) or in winter ($n = 12$) were compared for weekly analysis of subpopulations of WBC by flow cytometry.

²Cows were divided retrospectively into those with high weight loss during the first 5 wk postpartum in summer (S-HWL, $n = 6$) and those with low weight loss in summer (S-LWL, $n = 6$), and similarly in winter into W-HWL ($n = 6$) and W-LWL ($n = 6$).

³WC1 percentage in the winter season was only examined prepartum due to a technical problem.

an in vivo comprehensive study that in high-yielding Holstein dairy cows, chronic heat stress reduces blood leukocytes, in a manner that is not merely explained by lower HCT in summer, during the peripartum period. This is in accordance with the hypothesis that multiple external insults (i.e., heat stress + metabolic stress) induce a systemic inflammatory response, and that the number and severity of these challenging events may progressively reduce immunocompetence and increase susceptibility to inflammation (Bradford et al., 2015; Trevisi and Minuti, 2018). Generally, the comparable magnitude of change in the WBC parameters between the prepartum and PP periods in the present study indicates that heat stress affects the transition period in a comparable extent already during late gestation, before the metabolic challenge for milk production begins.

In the present study, we found moderate reductions in RBC-related blood counts (lower prepartum RBC, a tendency for lower MPV prepartum and lower MPV PP, and lower RDW prepartum and PP) in summer-calving compared with winter-calving cows. Shaffer et al. (1981) examined 169 Holstein cows and found a decline in hemoglobin, oxyhemoglobin, and packed cell volume with increasing environmental temperature. They suggested that the depression of these measures during heat stress are related to the reduction in cellu-

lar oxygen requirements to reduce metabolic heat load (Lee et al., 1976); therefore, the oxygen binding capacity of the blood is decreased under heat stress (Shaffer et al., 1981). Lee et al. (1976) found that during the hot season packed cell volume depression was partially due to a decrease in erythrocytes, and postulated a possible destruction of erythrocytes during heat stress. However, in the present study, although some RBC-related counts were lower in summer, the hemoglobin was higher and plasma iron concentrations were not different between seasons, implying that iron availability was sufficient in cows calving during heat stress.

There are several possibilities regarding the cows' water balance during chronic heat stress in summer, which could affect their WBC and RBC counts. First, lower values of blood measures could be caused by a hemodilution effect, as more water may be transported in the circulatory system for evaporative cooling (Shaffer et al., 1981). The reduced HCT prepartum and the tendency for lower HCT PP in summer- versus winter-calving cows support this notion, although adjusting the hematological parameters to HCT did not diminish the differences in numerous WBC and RBC counts between summer and winter, suggesting that lower HCT alone, which may imply hemodilution, cannot explain the observed reductions in blood counts in the summer-calving cows. In another study, the HCT values were

not different between cooled and heat-stressed peripartum dairy cows (Fabris et al., 2017), which is not in accordance with our findings.

Second, it is also plausible that there is no difference in the volume of water in blood between chronically heat-stressed and nonstressed cows as they adjust to the environment (i.e., balance water consumption and evaporation). Nevertheless, in our study, we found that the HCT was lower prepartum and tended to be lower PP in summer, which might be because of higher blood volume or lower blood cell proliferation during heat stress, and this issue warrants additional investigation.

Increased Subacute Inflammation and Oxidative Stress in Summer-Calving Cows

We found a significant increase in plasma TNF- α concentrations, a tendency for increased plasma complement C3 concentrations at d 7 PP, and an increased oxidative stress marker (MDA) in the summer-calving cows, suggesting that chronic heat stress increased the degree of subacute inflammation and oxidative stress in the PP cows. Heat stress has been shown to increase TNF- α expression in peripheral blood mononuclear cells during the peripartum period (Tao et al., 2013), and increased expression of TNF- α and NF- κ B has been reported in the muscle of acute heat-stressed ewes (Chauhan et al., 2014). Thus, the higher concentrations of TNF- α in heat-stressed cows may reflect an adaptive response to the environmental heat stress (Tao et al., 2019).

Moreover, we found higher concentrations of the oxidative stress marker MDA in S-HWL than in S-LWL cows, and numerically higher concentrations in W-HWL versus W-LWL cows (effect of subgroup $\times$ season; $P = 0.12$). This could imply that the increased AT lipolysis in HWL cows causes an increase in oxidative stress. In this study, the average plasma NEFA was lower in summer-calving than in winter-calving cows, which is in accordance with the finding that heat-stressed cows do not display the increased NEFA concentrations that are typical of a lower nutritional level (Shwartz et al., 2009). Because the cows in this study were defined as HWL or LWL based on their degree of BW loss PP, and the higher oxidative stress in the HWL cows supports the premise of increased lipolysis in those cows, a difference in plasma NEFA between HWL and LWL cows was expected. The lack of response in plasma NEFA could possibly be explained by elevated fatty acid oxidation in the liver, which occurs in states of limited energy supply; however, this issue remains unclear. We presume that the increased NEFA in winter- versus summer-calving cows did not have a detrimental effect on immune parameters in winter-calving cows because

NEFA concentrations PP were not very high (~ 660 μ Eq/L), and hence may avoid the negative effects on immune regulation.

Associations Between Immune Function, Inflammation, and Heat Stress in Cows with a High Degree of BW Loss (S-HWL)

Although the effect of season was much more pronounced, we found some specific indications of immune dysregulation in cows that calved under the summer heat load and lost more BW PP (S-HWL) compared with S-LWL cows. These included higher plasma MDA and TNF- α concentrations, fewer basophil numbers prepartum, a decrease in the percentage of CD25, increased CD4:CD8, and a tendency for increased WC1 percentages in blood prepartum, as well as a higher percentage of WC1 PP in S-HWL compared with S-LWL. Finally, TNF- α in the AT of S-HWL cows PP was more abundant, whereas LBP was less abundant, as compared with S-LWL AT. Throughout the transition period, we found a lower percentage of gamma delta-T (WC1) cells in the blood of S-LWL versus S-HWL cows. In ruminants, gamma delta-T cells form a major regulatory and suppressive T-cell compartment, and they can produce cytokines and have cytolytic activity (Guzman et al., 2014). Dairy cows with a high cell-mediated immune response have been reported to have more gamma delta-T cells than those with a low immune response (Hine et al., 2012), which is in accordance with the elevated inflammatory response in our S-HWL cows. Based on the suppressive role of gamma delta-T cells in ruminants, we suggest that their proportion was higher in S-HWL cows as a mechanism to reduce the inflammatory tone in these cows. Interestingly, most of the effects of degree of metabolic stress PP on inflammatory and immune function in the present study were found in summer and not during the winter season. This suggests that the winter-calving cows were able to maintain homeostasis and limit immune dysregulation and inflammation, whereas the summer-calving cows could not, due to the additional load of heat stress.

Association Between Degree of BW Loss During Summer and Inflammation in AT

In the present study, the AT of S-HWL cows had a higher abundance of TNF- α than that of S-LWL cows, indicating a stronger inflammatory response in the AT of cows with a higher degree of metabolic stress. Severe body fat mobilization is known to alter immunocompetence (Lacetera et al., 2005b), and the production of reactive oxygen species induces inflammation (Sordillo

and Aitken, 2009; Sordillo and Mavangira, 2014); this suggests that the higher AT lipolysis in S-HWL cows had detrimental effects on their immune function. Indeed, it has been shown that cows with higher lipolysis PP have more macrophages in their AT (De Koster et al., 2018; Newman et al., 2019). Taken together, we propose that the S-HWL cows had increased inflammation in their AT compared with S-LWL cows, which may have contributed to the increased systemic inflammation (via TNF- α secretion). The AT of S-HWL cows also had a lower abundance of LBP compared with S-LWL cows. Inflamed AT could be the result of LPS exposure to the resident macrophages (Newman et al., 2019). According to Hersoug et al. (2016), adipocytes can serve as an intermediate storage site for LPS, which might imply that during dramatic AT mobilization, LPS is released in large quantities from the AT into the circulation (Khiaosa-Ard and Zebeli, 2018). The decreased abundance of LBP in the AT of S-HWL cows, coupled with increased LBP in the plasma on d 7 PP, suggests that LPS release from the AT of HWL cows is part of their inflammatory response. This issue warrants further investigation.

We would like to mention that the limitations of our study are the lack of concurrent comparison between cows calving in both seasons, which is inevitable in this design, and the relatively limited number of cows due to the comprehensive simultaneous analyses of numerous online and offline immune and inflammatory blood markers.

CONCLUSIONS

In this study, we reported on in vivo immune (blood counts, subpopulations of WBC) and inflammatory markers in blood of peripartum high-yielding cows calving during summer heat stress or the winter season. We showed that seasonal heat stress during the peripartum period is associated with lower WBC counts coupled with increased levels of subacute inflammation, indicating dysregulation of immune function under heat stress. In addition, increased oxidative stress was found in summer-calving cows. Moreover, the subgroup of cows that calved during the summer and lost more BW PP had some specific changes in immune parameters in the blood and AT, indicating that these cows suffer from both metabolic and heat stresses that affect their immune function. Based on the vast differences in immune and inflammatory markers in cows calving during summer compared with winter, we conclude that heat stress is a more potent effector on immune function than the variation in the degree of BW loss PP between cows.

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