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Dietary supplementation of vitamin D_3 and calcium partially recover the compromised time budget and circadian rhythm of lying behavior in lactating cows under heat stress

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ABSTRACT

Heat stress (HS) impedes cattle behavior and performance and is an animal comfort and welfare issue. The objective of this study was to characterize the time budget and circadian rhythm of lying behavior in dairy cows during HS and to assess the effect of dietary supplementation of vitamin D_3 and Ca. Twelve multiparous Holstein cows (42.2 ± 5.6 kg milk/d; 83 ± 27 d in milk) housed in tiestalls were used in a split-plot design with the concentration of dietary vitamin E and Se as main plots (LESe: 11.1 IU/kg and 0.55 mg/kg, and HESe: 223 IU/kg and 1.8 mg/kg, respectively). Within each plot cows were randomly assigned to (1) HS with low concentrations of vitamin D_3 and Ca (HS, 1,012 IU/kg and 0.73%, respectively), (2) HS with high concentrations of vitamin D_3 and Ca (HS+D_3/Ca; 3,764 IU/kg and 0.97%, respectively), or (3) thermoneutral pair-fed (TNPF) with low concentrations of vitamin D_3 and Ca (1,012 IU/kg and 0.73%, respectively) in a Latin square design with 14-d periods and 7-d washouts. Lying behavior was measured with HOBO Loggers in 15-min intervals. Overall, cows in HS spent less time lying per day relative to TNPF from d 7 to 14. Daily lying time was positively correlated with milk yield, energy-corrected milk yield, and feed efficiency, and was negatively correlated with rectal temperature, respiratory rate, fecal calprotectin, tumor necrosis factor-α, and C-reactive protein. A treatment by time interaction was observed for lying behavior: the time spent lying was lesser for cows in HS than in TNPF in the early morning (0000–0600 h) and in the night (1800–2400 h). The circadian rhythm of lying behavior was characterized by fitting a cosine function of time into linear mixed model. Daily rhythmicity of lying was detected for cows in TNPF and HS+D_3/Ca, whereas only a tendency in HS cows was observed. Cows in TNPF had the highest mesor (the average level of diurnal fluctuations; 34.2 min/h) and amplitude (the distance between the peak and mesor; 17.9 min/h). Both the mesor and amplitude were higher in HS+D_3/Ca relative to HS (26.6 vs. 25.2 min/h and 3.91 min/h vs. 2.18 min/h, respectively). The acrophase (time of the peak) of lying time in TNPF, HS, and HS+D_3/Ca were 0028, 0152, and 0054 h, respectively. Lastly, a continuous increase in daily lying time in TNPF was observed during the first 4 d of the experimental period in which DMI was gradually restricted, suggesting that intake restrictions may shift feeding behavior and introduce biases in the behavior of animals. In conclusion, lying behavior was compromised in dairy cows under HS, characterizing reduced daily lying time and disrupted circadian rhythms, and the compromised lying behavior can be partially restored by supplementation of vitamin D_3 and Ca. Further research may be required for a more suitable model to study behavior of cows under HS.

Key words: circadian rhythm, dairy cow, heat stress, lying behavior

INTRODUCTION

Heat stress (**HS**) is a growing concern for animal production and has attracted considerable research attention in dairy cattle over the past few decades. With a cascade of adverse effects on metabolic process (Baumgard and Rhoads, 2013), endocrine status (Wise et al., 1988), and immune response (Bagath et al., 2019), HS detrimentally affects dairy production and animal welfare. It is well-known that HS can induce inflammation, indicated by increased proinflammatory cytokines such as tumor necrosis factor-α (**TNF-α**; Chen et al., 2018). The inflammatory state might be attributed to the impaired intestinal barrier, allowing the passage of LPS into bloodstream, consequently trigger-

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ing an immune response (Lambert et al., 2002; Pearce et al., 2013; Fontoura et al., 2022). Another potential mechanism involves oxidative stress, where heat shock proteins could activate toll-like receptors in antigen-presenting cells, prompting a proinflammatory reaction (Archana et al., 2017; Dubrez et al., 2020).

In addition to its role in Ca homeostasis, vitamin D_3 also modulates immune responses by regulating the proliferation, differentiation, and function of immune cells (Guillot et al., 2010; Kamen and Tangpricha, 2010; Yin and Agrawal, 2014). Concurrent vitamin D_3 and Ca supplementation have been demonstrated to alleviate the severity of inflammatory bowel disease in mice (Zhu et al., 2005) and reduce both inflammatory makers and rectal temperatures in heat-stressed cows (Ruiz-González et al., 2023). Acting as antioxidants, dietary supplementation of vitamin E and Se above recommendations (NRC, 2012) has shown the ability to alleviate oxidative stress and intestinal permeability in heat-stressed pigs (Liu et al., 2016).

Lying behavior is an important component of time budget for cows in the context of animal welfare and production efficiency (Tucker et al., 2021). It has been reported in cross-farm research that dairy cows spend 8 to 13 h/d lying down, among which cows at pasture have relatively lower lying time (9 h/d), whereas cows kept in tiestalls use more of their daily time budget lying down (12.5 h/d; Tucker et al., 2021). If cows are deprived of an adequate time of lying, problems in animal health might occur. For instance, a reduced lying time is associated with an increased risk of health issues, such as hoof disease (Leonard et al., 1996; Thomsen et al., 2012; Cook, 2020). Numerous studies have reported the effects of HS on lying behavior. When given the opportunity, cows are more likely to stand and seek cooler microenvironments as the ambient temperature rises in freestalls (Tucker et al., 2008). The percentage of cows standing in freestalls during daytime increases as the weather becomes hot (Shultz, 1984; Allen et al., 2015), and cows spend less time lying down with an increasing temperature-humidity index (**THI**) during summer (Cook et al., 2007; Zähner et al., 2016).

Whether dietary supplementation of vitamin D_3 and E, as well as Ca and Se above recommendations can alleviate the HS-induced disturbance on the time budget and circadian rhythm of lying behavior in dairy cows has not been specifically investigated. However, given that vitamin D_3 reduced rectal temperatures of dairy cows (Ruiz-González et al., 2023), it may also alter behavioral changes related to such alleviation of HS. Insights into the effects of dietary supplementation of vitamins and minerals on lying behavior of cows under HS could contribute to the development of nutritional strategies that can help to alleviate the negative consequences of HS in dairy cows. We hypothesized that cows under HS have a decreased daily lying time and a disrupted diurnal pattern of lying behavior, and diet supplementation of vitamins and minerals may recover the compromised lying behavior in heat-stressed cows. Therefore, the objective of the present study was to investigate the time budget and circadian rhythm of lying behavior in lactating dairy cows under HS and to investigate if diet supplementation of above-mentioned vitamins and minerals can help cows to alleviate HS consequences.

MATERIALS AND METHODS

Experimental Design, Treatments, and Management

Experimental procedures were approved by the Centre de recherche en sciences animales de Deschambault (CRSAD) animal care committee (2019-BL-386), according to the Canadian Council on Animal Care Guidelines for the Use of Farm Animals (1993). The current study was part of a larger experiment to evaluate the effects of dietary supplementation of vitamins and minerals in cows under HS. In the present study, the HS and treatment effects on lying behavior and its circadian pattern are presented, while the lactation performance, physiological indicators, and inflammation symptoms of HS were reported recently by Ruiz-González et al. (2023).

The experiment was conducted between October and December 2020 at the CRSAD research farm in Deschambault, QC, Canada. Twelve multiparous Holstein cows averaging (mean ± SD) 42.2 ± 5.6 kg milk/d and 83 ± 27 DIM were housed in a tiestall barn and used in a split-plot design, with the main plot being the dietary concentrations of vitamin E and Se, including low (**LESe**; 11.1 IU/kg vitamin E and 0.55 mg/kg Se, respectively; n = 6) and high concentration (**HESe**; 223 IU/kg vitamin E and 1.8 mg/kg Se, i.e., 20- and 3.2-fold higher, respectively; n = 6). The 2 main plots were balanced for parity, DIM, and milk yield at the start of the experiment. Treatment subplots were arranged in a replicated 3 × 3 Latin square design within main plots with three 14-d experimental periods. Treatments were (1) HS and low vitamin D_3 and Ca (1,012 IU/kg vitamin D_3 and 0.73% Ca), (2) HS and high concentrations of vitamin D_3 and Ca supplementation (**HS+D$_3$/Ca**; 3,764 IU/kg vitamin D_3 and 0.97% Ca, i.e., 3.7- and 1.3-fold higher, respectively), and (3) cows were kept in thermoneutrality and pair-fed (**TNPF**) to their HS counterparts and received the same concentrations of vitamin D_3 and Ca as the HS group.

Thermal conditions were controlled via climate-controlled chambers (Edgetech Instrument Inc.). Cows

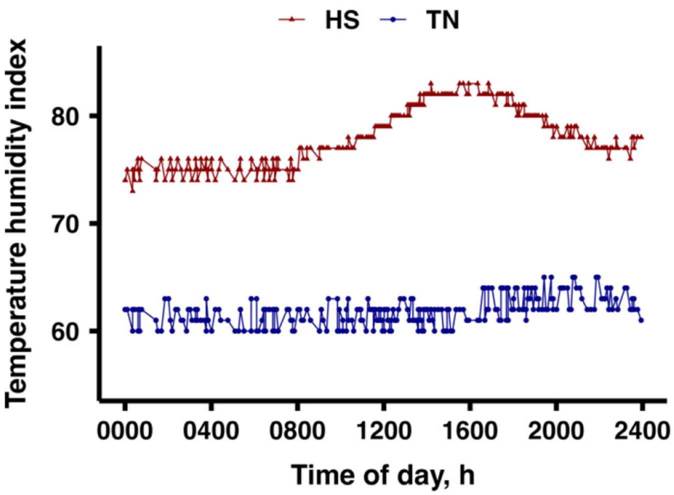

Figure 1. Diurnal patterns of temperature-humidity index in heat stress (HS) or thermoneutral (TN) chambers during the experimental period (Ruiz-González et al., 2023).

under HS experienced a cyclical daily THI (calculated according to NRC, 1971) varying between 72.0 and 82.0 (Figure 1). Temperature in the HS chambers was automatically controlled during the 14-d experimental periods as follows: temperature increase from 29 to 39°C at a rate of 1.6°C/h from 0800 to 1400 h, constant temperature from 1400 to 1700 h, temperature reduction to 29°C at a rate of 1.6°C/h from 1700 to 2300 h, and a constant temperature of 29°C from 2300 to 0800 h. The relative humidity in HS chambers was not controlled and ranged from 20 to 50%. Temperature in the thermoneutral chamber was maintained at 20°C and relative humidity ranged from 55 to 64% (THI = 61.0 to 64.0, Figure 1). Following each experimental period, cows were kept in thermoneutral conditions (THI = 61.0 to 64.0) for 7 d to allow recovery from the previous experimental period. Removal from HS chambers and exclusion of cows from the experiment was considered if DMI reduction was greater than 50% for a period of 48 h. No animals were removed from the experiment.

All cows were individually fed a TMR twice daily at 0900 and 1400 h and refusals were recorded daily before morning feeding. Cows under HS had ad libitum access to feed, while cows in the TNPF group were restricted to the same level of feed intake as their heat-stressed counterparts (Ruiz-González et al., 2023). Four diets that had similar chemical compositions: 15.7% CP, 30.2% starch, and 26.3% NDF, were formulated to meet predicted energy and nutrient requirements (NRC, 2001). The concentrations of vitamins and minerals under investigation are shown in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.25047950.v1, Wang, 2024). Drinking water was always available.

Data and Sample Collections and Measurements

Lying behavior was recorded by a data logging accelerometer (HOBO Onset Pendant G, 64k; Onset Computer Corporation) and determined by an algorithm developed in IGOR (WaveMetrics Inc.) according to validated parameters (Ledgerwood et al., 2010). Raw data of time lying down was recorded with a frequency of 15 min from d 0 to 14 of each period and summed over 1 h and 24-h intervals to obtain hourly lying time (min/h) and daily lying time (min/d) respectively.

Data for milk production, milk components, feed intake, hyperthermia indicators, oxidative stress, and intestinal inflammation markers were used to test their association with lying time. Details of the sample collection and processing procedure are described in Ruiz-González et al. (2023). Briefly, milk samples were collected twice daily (0700 and 1700 h) on d 0, 3, 5, 7, 10, 12, and 14 of each experimental period, and analyzed for fat, protein, and lactose concentrations by infrared absorption spectroscopy with a Foss MilkoScan FT 6000 instrument (Foss). Milk yield was recorded by an integrated milk meter (Flomaster Pro; DeLaval). Yield of ECM was calculated according to Madsen et al. (2008), and feed efficiency (**FE**) was determined as milk yield (kg/d) per DMI (kg/d).

Rectal temperature was measured daily from d 0 to 14 of each period at 0800, 1400, and 1700 h using a hand-held cattle rectal thermometer (AG-102 angled; AG-Medix) inserted 30 mm into the rectum of the animal for ~60 s until a stable measurement was obtained. Respiratory rate was measured on d 0, 3, 5, 7, 10, 12, and 14 of each period, at 0800, and 1700 h by visually counting flank movements of uninterrupted breathing during 30 s and then converting to the number of breaths. Rectal temperature and respiratory rate were averaged across 3 times and 2 times repeated measurements in a day, respectively. As described by Ruiz-González et al. (2023), fecal samples were collected at 0700 h on d 0, 7, and 14 of each period to determine the concentration of fecal calprotectin (the intra- and interassay CV were 4.21% and 4.98%, respectively), and blood samples were collected from the coccygeal vein at 0 and 4 h relative to morning feeding, on d 0, 7, and 14 to test lipopolysaccharide-binding protein (**LBP**; the intra- and interassay CV were 3.78% and 3.45%, respectively), TNF-α (the intra- and interassay CV were 4.23% and 4.78%, respectively), and C-reactive protein (**C-RP**; the intra- and interassay CV were 4.01% and

4.44%, respectively) by ELISA using commercial kits (Mybiosource).

Statistical Analysis

Lying data from the first experimental period were excluded from analysis because of large amounts of missing values. All data analysis were performed in R statistical language (R Core Team 2022; version 4.2.2). Daily lying time was analyzed using mixed model with repeated measures using *lmer* procedure (Bates et al., 2015), and denominator degrees of freedom were adjusted by the Kenward-Rogers method. The model was as follows:

$$Y_{ijklm} = \mu + B_i + P_j + T_k + C_l + D_m + B_i \times T_k + T_k \times D_m + B_i \times D_m + e_{ijklm},$$

where Y_{ijklm} is daily lying time, μ is the overall mean, B_i is the fixed effect of main plot ($i = 1$ to 2), P_j is the fixed effect of period ($j = 1$ to 3), T_k is the fixed effect of treatment ($k = 1$ to 3), C_l is the random effect of cow ($l = 1$ to 12), D_m is the fixed effect of day of period ($m = 0$ to 14), $B_i \times T_k$ is the interaction of main plot and treatment, $T_k \times D_m$ is the interaction of treatment and day of period, $B_i \times D_m$ is the interaction of main plot and day of period, and e_{ijklm} is the random error. A reduced model without the fixed effect of day was used to test the effect of HS and diet treatments on daily lying time using data from d 8 to 14.

Pearson correlations were calculated to characterize the relationship between daily lying time and milk production, rectal temperature, respiratory rate, and inflammation using data from d 7, 10, and 14 of each period.

Similarly, hourly lying time over 24-h from d 8 to 14 was analyzed using mixed model with repeated measures using *lmer* procedure (Bates et al., 2015). The model was as follows:

$$Y_{ijklmn} = \mu + B_i + P_j + T_k + C_l + H_n + T_k \times H_n + e_{ijklmn},$$

where Y_{ijklmn} is hourly lying time, H_n is the fixed effect of time of day ($n = 0$ to 24), and other terms in the model are the same as described above.

To characterize daily patterns of lying behavior, a cosine function of time modeling a repeated 24-h rhythm was fitted in mixed model as a linear component. Lying time within 1 h after milking (0700 and 1700 h) and feeding (0900 and 1400 h) were excluded from data. The model as follows:

$$Y_{ijklmn} = M + B_i + P_j + T_k + C_l + \alpha\cos\left(\frac{2\pi H_n}{24}\right) + \beta\sin\left(\frac{2\pi H_n}{24}\right) + T_k \times \alpha_k\cos\left(\frac{2\pi H_n}{24}\right) + T_k \times \beta_k\sin\left(\frac{2\pi H_n}{24}\right) + e_{ijklmn},$$

where Y_{ijklmn} is hourly lying time, M is the mesor of cosine function (the average level of diurnal fluctuations), α and β are the linear coefficients of cosine function. Amplitude was calculated as $\sqrt{\alpha^2 + \beta^2}$ and phase was calculated as $[\phi arctan(\beta/\alpha) \times 12/\pi]$ and reported as acrophase (Niu et al., 2014). Determination of these parameters provides a quantitative description of the rhythms of complex data. Zero-amplitude test comparing cosine fit to null model without time term was performed to determine the significance of the cosinor pattern in each treatment. The 95% confidence intervals were calculated for mesor, amplitude, and phase according to Bourdon et al. (1995), and significance of pre-planned contrasts were defined as a difference between treatments greater than 1.96 times the square root of the standard errors (Knezevic, 2008).

To investigate the change in lying behavior at different times of the day over the experimental periods, hours were binned into 6-h time frames, i.e., early morning (0000–0600 h), morning (0600–1200 h), afternoon (1200–1800 h), and night (1800–2400 h). Hourly lying time in each time frame was analyzed using following mixed model:

$$Y_{ijklmn} = \mu + B_i + P_j + T_k + C_l + D_m + T_k \times D_m + e_{ijklmn},$$

where Y_{ijklmn} is hourly lying time and other terms in the model are the same as described above.

In all analyses, data points with studentized residuals outside of ± 3 were considered outliers and were removed from analysis, no more than 3 entries were removed in all analysis. Significance and tendency of the main effect was declared at $P < 0.05$ and $0.05 < P < 0.10$, respectively. Significance of the interactions was declared at $P < 0.10$. The pre-planned contrasts were TNPF versus HS and HS+D_3/Ca versus HS.

RESULTS

Effects of HS and Dietary Treatments on Daily Lying Time

The differences in daily lying time between treatments are presented in Figure 2. Figure 2A shows the

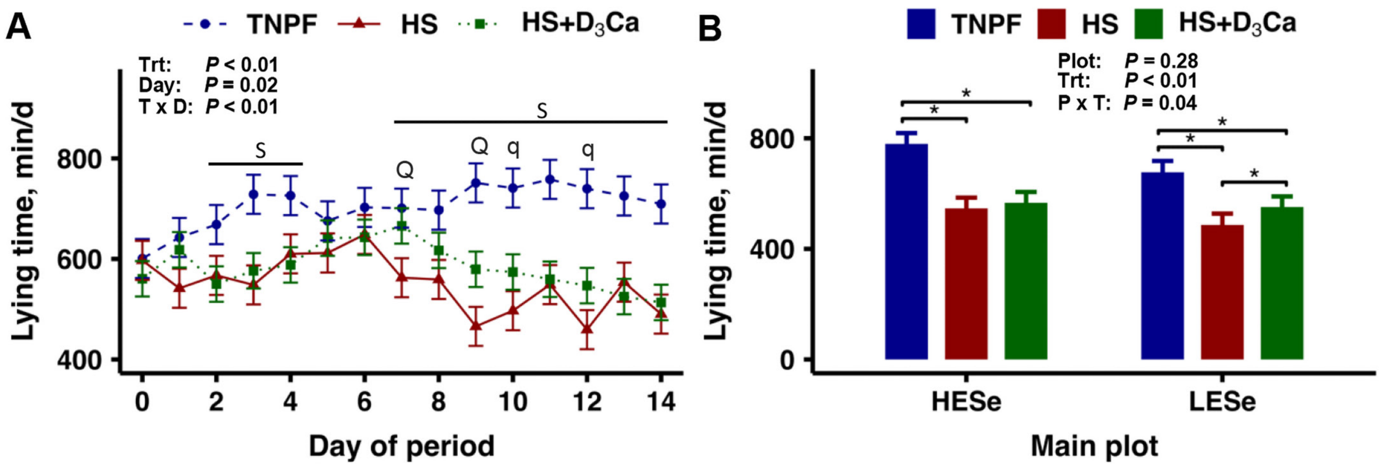

Figure 2. Effects of heat stress (HS) and diet treatments on daily lying time. Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (high, HESe, n = 6; and low, LESe, n = 6). Within each plot, cows were randomly allocated to HS induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF). (A) Daily LSM of daily lying time in each treatment during the 14-d experimental period. Main effects of treatment (Trt) and day (Day) and their interaction (T × D) are shown on the plot. Statistical differences ($P < 0.05$) of pre-planned contrasts on each day are indicated by uppercase S (HS vs. TNPF) and Q (HS vs. HS+D_3/Ca); tendencies ($0.05 < P < 0.10$) are indicated by lowercase s (HS vs. TNPF) and q (HS vs. HS+D_3/Ca). (B) Overall LSM of daily lying time from d 8 to d 14. Main effects of main plot (Plot) and treatment (Trt) and their interaction (P × T) are shown on the plot. Statistical inference of pairwise contrasts in each plot are indicated by * ($P < 0.05$). Error bars represent SE.

daily LSM of daily lying time in each treatment (means are reported in Supplemental Table S2; https://doi.org/10.6084/m9.figshare.25047950.v1, Wang, 2024). There was a treatment by day interaction ($P < 0.01$) where daily lying time was greater ($P < 0.01$) for TNPF versus HS from d 7 to 14, and greater ($P < 0.05$) for HS+D_3/Ca versus HS on d 7 and d 9. Figure 2B shows the overall LSM of daily lying time in each group across d 8 to 14 (means are reported in Supplemental Table S3; https://doi.org/10.6084/m9.figshare.25047950.v1, Wang, 2024). There was a plot by treatment interaction ($P = 0.04$): cows in HS spent less ($P < 0.01$) time lying than TNPF group in both HESe (−233.5 min/d) and LESe (−187.8 min/d) plots, whereas the daily lying time was lower ($P = 0.03$) for HS versus HS+D_3/Ca in the LESe plot (−63.4 min/d), but no significant difference was observed for HS versus HS+D_3/Ca in the HESe plot. The main effect of plot was not significant for daily lying time.

Relationships Between Daily Lying Time, Production, and HS Indicators

Correlations between daily lying time and production traits in each treatment and across all treatments are shown in Figure 3. Overall, daily lying time was positively correlated ($P < 0.01$) with milk yield, ECM, and FE. A positive correlation ($P = 0.03$) between daily lying time and ECM was also present in HS and HS+D_3/Ca. Daily lying time was positively correlated ($P = 0.01$) with milk fat concentration in HS and was positively correlated ($P < 0.01$ and $P = 0.03$, respectively) with milk lactose concentration in HS and TNPF. No overall correlation was observed between daily lying time and milk composition.

Correlations between daily lying time, rectal temperature, respiratory rate, and inflammation indicators are shown in Figure 4. Overall, daily lying time was negatively correlated with rectal temperature and respiratory rate ($P < 0.01$). Similarly, daily lying time was negatively correlated ($P < 0.05$) with fecal calprotectin, TNF-α, and C-RP across all treatments.

Diurnal Pattern of Lying Behavior

Figure 5 shows lying time over a 24-h period from d 8 to 14 (means are reported in Supplemental Table S4; https://doi.org/10.6084/m9.figshare.25047950.v1, Wang, 2024). A treatment by time interaction ($P < 0.01$) was observed (Figure 5A): the time spent lying (min/h) was reduced ($P < 0.01$) for HS versus TNPF during early morning (0000–0600 h) and night (1800 to 2400 h). There was no difference between treatments during daytime (0600–1800 h) except for 0800, 1400, and 1500 h: the hourly lying time was greater ($P < 0.01$) for TNPF versus HS at 0800 h and was lower for TNPF versus HS at 1400 and 1500 h ($P < 0.01$). Cows in HS+D_3/Ca had greater ($P < 0.05$) hourly lying time at 0200, 0800, and 2300 h compared with HS, and there were tendencies ($0.05 < P < 0.10$) for greater lying time for HS+D_3/Ca versus HS at 0300, 0900, and 2100 h.

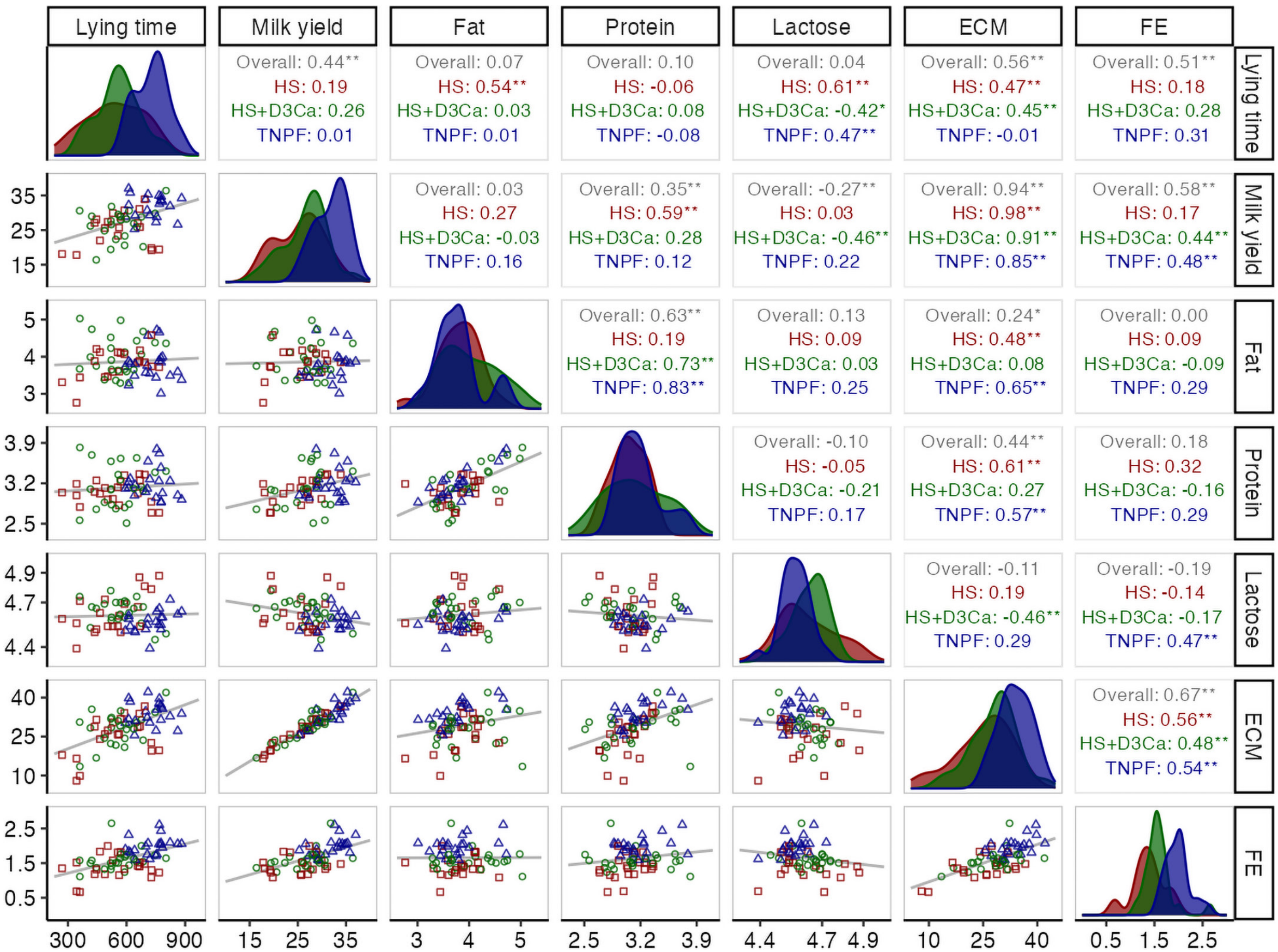

Figure 3. Correlations between daily lying time (min/d) and production traits. Data from d 7, d 10, and d 14 were used for the analysis. Production traits include milk yield (kg/d), fat (%), protein (%), lactose (%), ECM, and feed efficiency (FE, kg milk/DMI kg). The overall correlations and correlations in each treatment group are shown on the upper triangle. Distribution of each variable are shown by treatments on the diagonal. Lower triangle shows pairwise scatter plots with linear regression lines. The legend of colors coding treatments is indicated on the upper triangle. Significance was indicated using ** ($P < 0.05$), and tendency was indicated using * ($0.05 < P < 0.10$). Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (high, HESe, n = 6; and low, LESe, n = 6). Within each plot, cows were randomly allocated to heat stress (HS) induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF).

Circadian rhythm curves of lying behavior in each treatment are presented in Figure 5B, and rhythmicity parameters of cosine function are summarized in Table 1. Significant diurnal rhythmicity (zero-amplitude test, $P < 0.01$) of lying behavior was detected in TNPF, characterizing the highest mesor (34.2 min/h) and amplitude (17.9 min/h). The acrophase of TNPF occurred at 0028 h. A disrupted circadian curve ($P = 0.06$) with the lower ($P < 0.01$) mesor (25.2 min/h) and amplitude (2.18 min/h) was observed for cows in HS compared with cows in TNPF, whereas HS+D_3/Ca restored rhythmicity ($P < 0.01$) and upregulated ($P < 0.01$) the mesor and amplitude by 1.4 min/h and 1.73 min/h, respectively, compared with HS. The peak of hourly lying time in HS and HS+D_3/Ca was at 0152 and 0054 h, respectively.

Lying Behavior at Different Times of Day

Figure 6 shows average hourly lying time in 6-h timeframes of day over the experimental period (d 0 to 14). There was a treatment by day interaction ($P < 0.01$) for lying time during early morning (0000–0600 h; Figure 6A): lying duration in this timeframe was greater ($P < 0.05$) for TNPF versus HS from d 4 to 6 and d 8 to 14, and there was a tendency ($P = 0.08$) for greater hourly

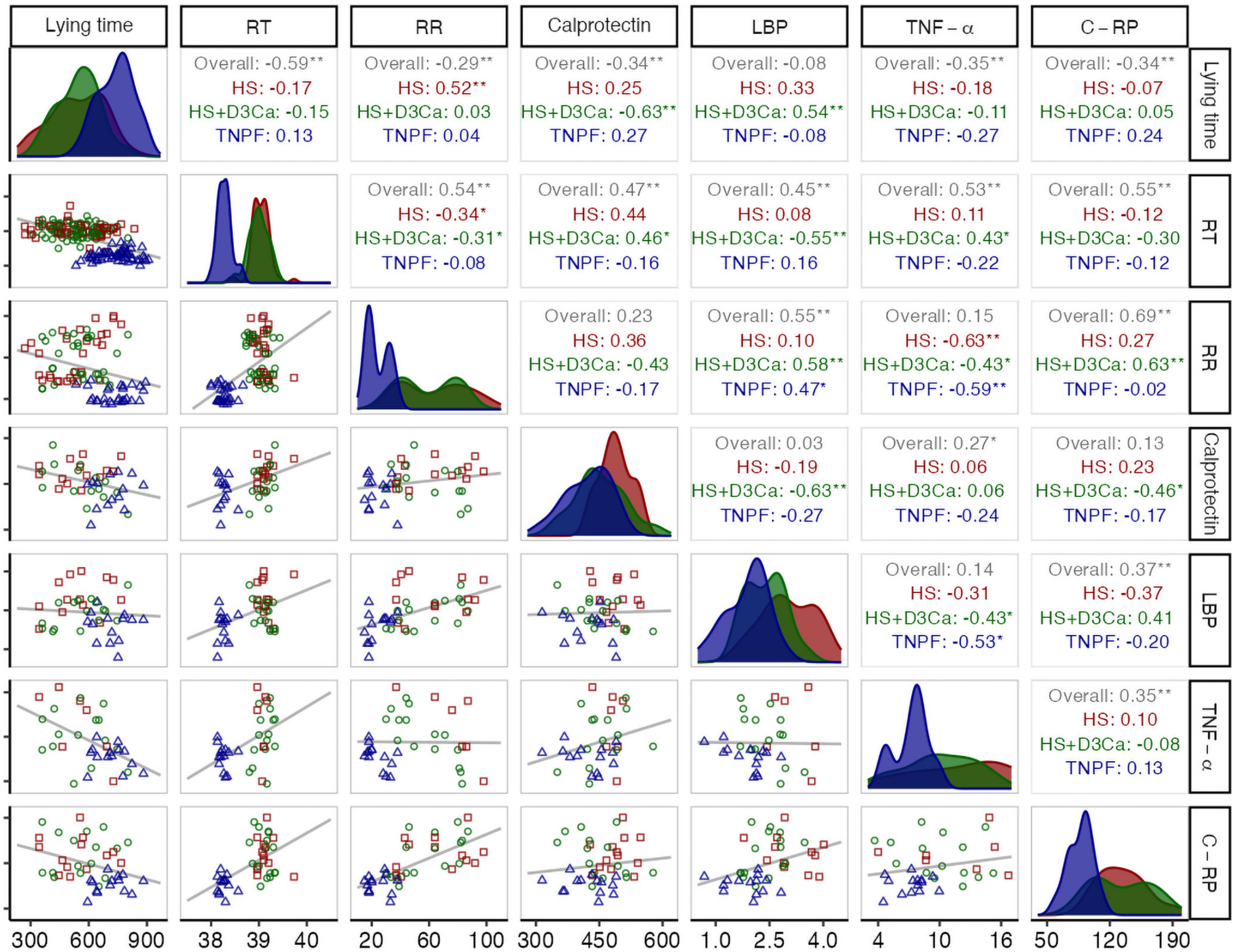

Figure 4. Correlations between daily lying time (min/d), rectal temperature (RT, °C), respiratory rate (RR, breaths/min), and inflammation markers. Inflammation markers include fecal calprotectin (ng/mL), lipopolysaccharide-binding protein (LBP, ng/mL), tumor necrosis factor-α (TNF-α; ng/mL), and C-reactive protein (C-RP, ng/mL). Data from d 7 and d 14 were used for the analysis. The overall correlations and correlations in each treatment group are shown on the upper triangle. Distribution of each variable are shown by treatments on the diagonal. Lower triangle shows pairwise scatter plots with linear regression lines. The legend of colors coding treatments is indicated on the upper triangle. Significance was indicated using ** ($P < 0.05$), and tendency was indicated using * ($0.05 < P < 0.10$). Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (high, HESe, n = 6; and low, LESe, n = 6). Within each plot, cows were randomly allocated to heat stress (HS) induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF).

lying time for TNPF versus HS at d 7. No main effect of treatment and treatment by day interaction were observed during morning (0600–1200 h; Figure 6B). Treatment by time interactions ($P < 0.01$) were also found for afternoon (1200–1800 h; Figure 6C) and night (1800–2400 h; Figure 6D). The time spent lying was greater ($P < 0.05$) for TNPF versus HS during night from d 3 to 14 (Figure 6D) but was lower ($P < 0.05$) for TNPF versus HS during afternoon on d 6 to 8, d 13 and 14 (Figure 6C). Tendencies ($0.05 < P < 0.10$) of reduced lying time during afternoon for TNPF versus HS were observed on d 4, 10, and 11 (Figure 6C).

DISCUSSION

Numerous studies have consistently demonstrated that lying time is compromised in cows under heat challenge (Shultz, 1984; Cook et al., 2007; Zähner et al., 2016), which was also evident by the decreased lying time of heat-stressed cows in the current experiment. Cook et al. (2007) reported that lying time reduced from 10.9 h/d in the coolest period (THI from 51.3 to 58.9) to 7.9 h/d in the hottest period (THI from 68.1 to 80) between June and September in a free stall setting. This finding is in line with the present study: heat-

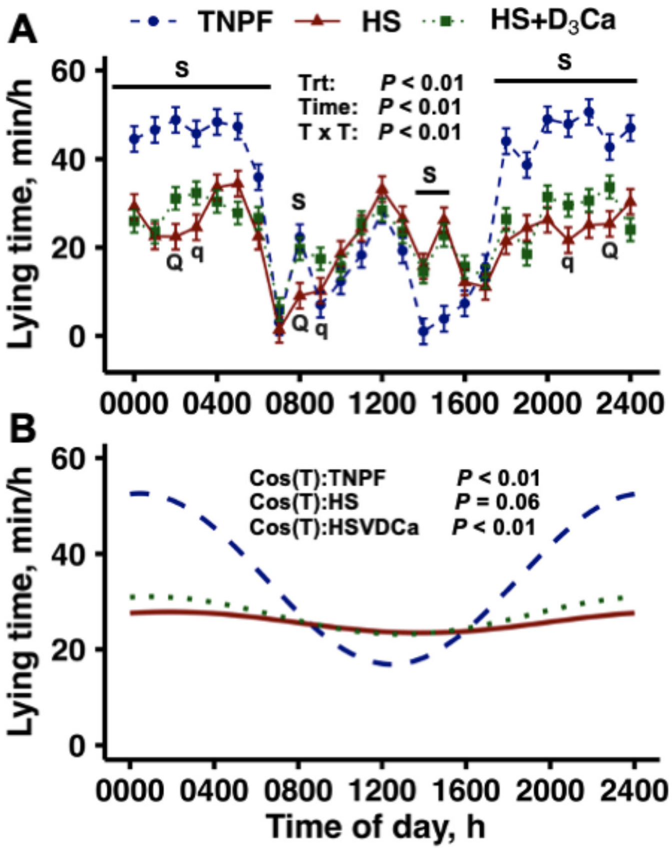

Figure 5. Effect of treatments on diurnal patterns of lying time. Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (high, HESe, n = 6; and low, LESe, n = 6). Within each plot, cows were randomly allocated to heat stress (HS) induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF). Data from d 8 to d 14 were used in analysis. (A) LSM of hourly lying time in each treatment across time of day. Main effects of treatment (Trt) and time of day (Time) and their interaction (T × T) are shown on the plot. Statistical differences ($P < 0.05$) of pre-planned contrasts in each hour are indicated by uppercase S (HS vs. TNPF) and Q (HS vs. HS+D_3/Ca); tendencies ($0.05 < P < 0.10$) are indicated by lowercase s (HS vs. TNPF) and q (HS vs. HS+D_3/Ca). Error bars represent SE. (B) Fitted cosine function curves in each treatment. Statistical significance of zero-amplitude test for each treatment is shown on the plot. Data during milking (0700 and 1700 h) and feeding (0900 and 1400 h) were excluded for cosine function fit; only spontaneous lying behavior was used.

stressed cows (THI from 72 to 82) spent 3 to 4 h/d less lying than their thermoneutral counterparts (THI from 61 to 64). However, cows under the thermoneutral condition in our study had greater lying time (12.1 vs. 10.9 h/d) compared with Cook et al. (2007), which might be explained by different housing conditions (tiestalls vs. freestalls; Tucker et al., 2021) and different studying methods, i.e., restricted pair-feeding in our study and ad libitum feeding in Cook et al. (2007).

The physiological mechanisms underlying the reduced lying time in response to HS is not well understood.

Table 1. Effects of heat stress and diet treatments[1] on parameters of diurnal patterns

Treatment	Mesor,[2] min/h	Amplitude,[3] min/h	Acrophase,[4] h	P-value[5]
TNPF	34.2*	17.9*	0028*	<0.01
HS	25.2	2.18	0152	0.06
HS+D_3/Ca	26.6*	3.91*	0054*	<0.01

[1]Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (Adequate, n = 6; and High, n = 6). Within each plot, cows were randomly allocated to heat stress (HS) induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF).

[2]Mesor: the average level of diurnal fluctuations.

[3]Amplitude: the distance between the peak and mesor.

[4]Acrophase: the time of the peak of fitted curve.

[5]Significance of the zero-amplitude tests for the cosine fit.

*Significantly ($P < 0.05$) different from HS; pre-planned contrasts tested TNPF versus HS and HS+D_3/Ca versus HS.

Standing up may increase the body surface area of cows to improve cooling through convection, evaporation, and radiation (Allen et al., 2015) and may make the respiration of cows more effective (Tucker et al., 2021). Allen et al. (2015) found core body temperature decreased after a standing bout during summer seasons. Nordlund et al. (2019) also observed a decrease in core body temperature at a rate of 0.25°C/h during standing in freestall pens. As reported by Ruiz-González et al. (2023), heat-stressed cows in the current experiment, had a greater rectal temperature compared with cows under thermoneutral conditions, which indicates a need for heat dissipation. The negative association between respiratory rate and lying time in the current study suggested that more standing and panting under HS would mitigate heat load in cows. Importantly, although standing seems to be an important coping mechanism during HS, reduced lying time has also critical implications for animal welfare, health, and production (Dawkins, 2004).

The positive correlations between lying time, milk yield, ECM, and FE indicated negative implications of reduced lying time on milk production under HS. Reduced production performance is a well-established consequence of HS, which is partially accounted for by decreased DMI under HS (Rhoads et al., 2009). However, correlations between daily lying time and production traits in the current study were not confounded by DMI, because the data used in correlation analysis were from days when DMI was stabilized (d 7 to 14), during which there was no difference in DMI between treatments (Ruiz-González et al., 2023). Correlations between lying time and production efficiency might be explained by the limited blood flow to the udder (Rulquin and Caudal, 1992) and increased maintenance

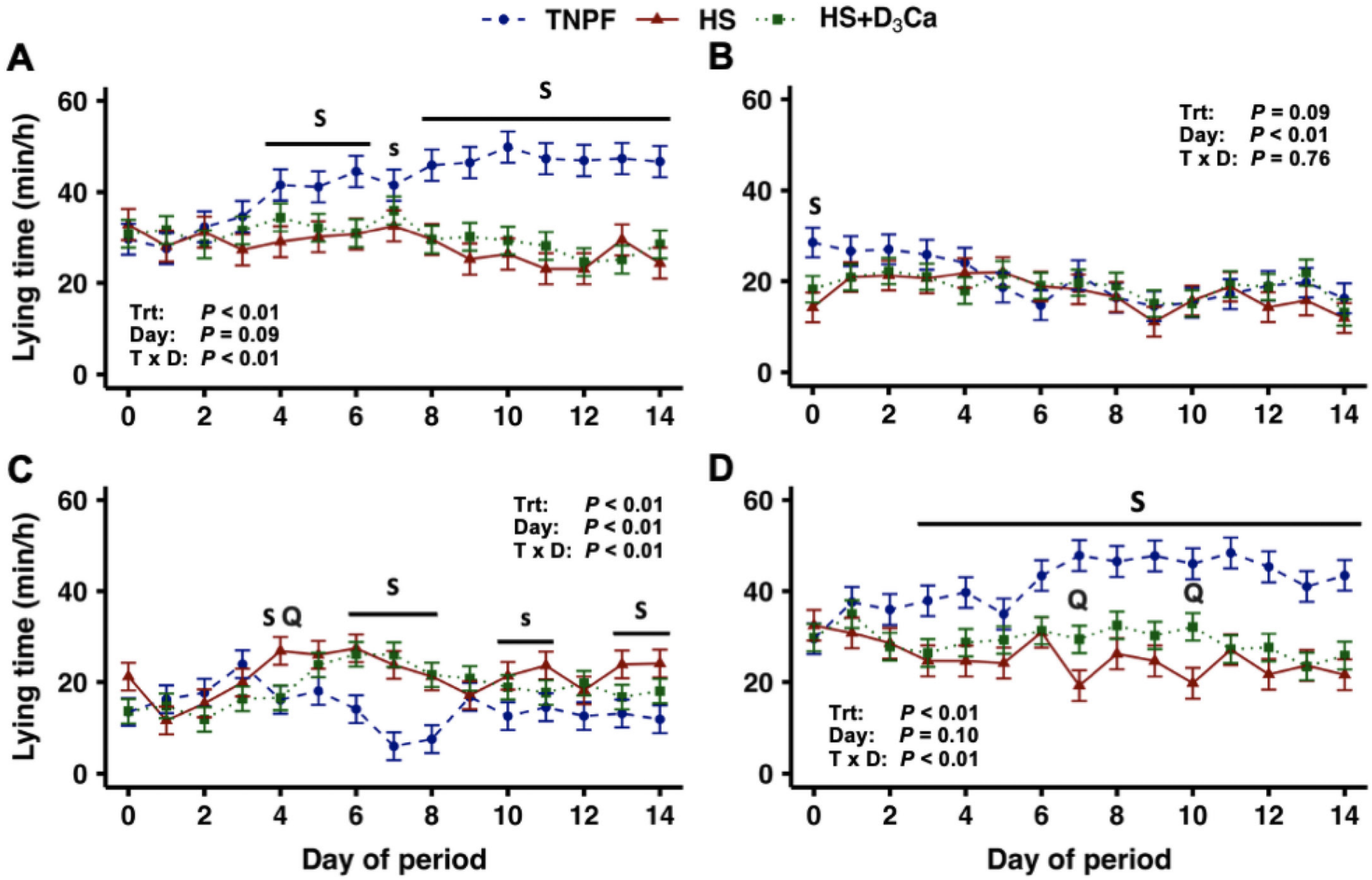

Figure 6. Effects of heat stress (HS) and diet treatments on hourly lying time during different periods of day. (A) early morning (0000–0600 h), (B) morning (0600–1200 h), (C) afternoon (1200–1800 h), and (D) night (1800–2400 h). Treatments were arranged in a split-plot design where the main plot was the concentrations of dietary vitamin E and Se (high, HESe, n = 6; and low, LESe, n = 6). Within each plot, cows were randomly allocated to HS induction; HS supplemented with higher vitamin D_3 and Ca (HS+D_3/Ca); thermoneutral pair-feeding to the HS group (TNPF). Main effects of treatment (Trt) and day (Day) and their interaction (T × D) are shown on the plots. Statistical differences ($P < 0.05$) of pre-planned contrasts on each day are indicated by uppercase S (HS vs. TNPF) and Q (HS vs. HS+D_3/Ca); tendencies ($0.05 < P < 0.10$) are indicated by lowercase s (HS vs. TNPF) and q (HS vs. HS+D_3/Ca). Error bars represent SE.

requirements (West, 2003) when cows spend more time standing. Interestingly, daily lying time was correlated with ECM in both HS and HS+D_3/Ca groups, but not in TNPF, indicating the varying lying time within the normal range under thermoneutral conditions may not have implications on production performance, whereas greater lying time under HS may reflect higher heat resistance.

Although the cows in our experiment showed no clinical symptoms of illness, the observed negative associations between lying time and inflammatory markers (TNF-α and C-RP) suggest that cows were experiencing discomfort as a result of activated immune response due to HS (Zhu et al., 2005). As reported by Ruiz-González et al. (2023), an increase in fecal calprotectin and plasma LBP concentrations was observed in heat-stressed cows, suggesting increased gut permeability (Grootjans et al., 2010), which was partially recovered in the HS+D_3/Ca group (Ruiz-González et al., 2023).

Cows′ lying behavior follows a distinct circadian rhythm, with 59.4% lying happening at night (Winckler et al., 2015). This pattern was evident by the peak of lying time occurring at night for all treatments in our study. However, only a tendency for rhythmicity was observed for heat-stressed cows in our study, and the lying time curve was flattened over the day, indicating a disruption of the diurnal pattern of the cows experiencing HS. As light-dark cycle is the dominant factor that entrain circadian rhythms in animals (Asher and Schibler, 2011), an important biological function of lying down during night is rest. For example, dairy cows sleep ~4 h/d, including 3 h REM and 45 min NREM sleep (Ternman et al., 2012) and must lie down to have REM sleep (Ruckebusch, 1974). Reduced lying time at night suggested a lack of sufficient rest for cows under HS, which may interrupt sleep duration and quality in cows. However, we did not measure sleep in this study, and very little is known about how changes in lying time affect the duration and state of sleep, an area that warrants further research.

Given its ability to modulate immune responses, increased vitamin and mineral supplementation (e.g., vitamin D_3 and Ca) could be easily implemented to alleviate the negative effects of hyperthermia (Liu et al., 2016; Ruiz-González et al., 2023). The use of vitamin E/Se as a blocking factor in our study was because of the bioaccumulation of Se in tissues such as liver (Mehdi and Dufrasne, 2016), which could have had residual effects across periods. No effect of vitamin E/Se on lying behavior was observed in the current study, indicating increased concentrations of vitamin E/Se has a limited effect. However, the increased daily lying time and restored rhythmicity of lying behavior in HS+D_3/Ca suggested that vitamin D_3 and Ca may be used as a dietary approach to alleviate the effect of HS, as was also evident from the improved production and inflammation status independently of basal concentrations of vitamin E and Se, as reported in Ruiz-González et al. (2023). The partially restored lying behavior suggest a gradual alleviation of discomfort in cows, potentially attributed to reduced inflammation as stated by Ruiz-González et al. (2023). Meanwhile, the additional heat generation resulting from the inflammation-related pyretic response (Blatteis, 2006; Waldron et al., 2006) could be modulated by supplying vitamin D_3. This idea is supported by (Ruiz-González et al., 2023), where a reduced rectal temperature was observed in cows during HS with higher vitamin D_3 supplementation.

Interestingly, differences in lying time between the treatments were observed within 2 h after PM feeding. It is reported that cows eat over 27% of the daily intake during the first 2 h after PM feeding when fed 2x/d (Niu et al., 2014). In the current study, cows under HS spent more time lying down, ranging from 13 to 19 min/h, during the first 2 h following PM feeding. The greater lying time during this period was most likely related to the reduced feed intake of the heat-stressed cows, i.e., the time budgeting of the cows shifted from eating behavior to lying behavior. Increased lying time after feeding indicate that cows under HS may have less motivation to eat after fresh feed delivery compared with cows in thermoneutral conditions.

It is worth noting that daily lying time in TNPF increased during the first 4 d (adaptation) of the experimental periods, which might be explained by trade-offs between lying and feed availability, as well as the respective eating behavior of cows. We used a pair-feeding design to eliminate the confounding effect of nutritional conditions on treatment comparisons in the current study (Ruiz-González et al., 2023). The feed was restricted at the same rate for the TNPF group as DMI dropped by 28.6% in the first 4 d of HS induction (Ruiz-González et al., 2023). The restricted feeding in TNPF may shift feeding behavior of the cows and consequently affect lying behavior. As a result, the cows in TNPF increased their daily lying time by 20.9% during the first 4 d, mainly due to the increased lying time during early morning and night. On the contrary, cows in TNPF had lower lying time during afternoon, especially within 2 h after PM feed delivery. As mentioned above, cows in TNPF perhaps used most of the time eating after fresh feed delivery and spent more time lying during the overnight period, while cows in HS may shift their eating time budget at night. Importantly, although pair-feeding is demonstrated a good model to investigate the effect of hyperthermia separate from feed intake reductions, it may introduce biases when used to determine animal behavior as shown by the behavioral changes in pair-feeding over time.

CONCLUSIONS

Dairy cows experiencing a heat challenge exhibited a reduced time lying and a disrupted circadian rhythm of lying behavior. The effect of HS on lying behavior was most noticeable during night when heat-stressed cows spent less time lying. Milk yield, milk ECM and feed efficiency were positively correlated with lying time, indicating the importance of sufficient lying time for the productive performance of lactating dairy cows. Supplementation of vitamin D_3 and Ca partially restored lying time and its rhythmicity by increasing lying time during the overnight period. Overall, based on the data from the current experiment, reduced lying time under HS has implications for productivity and welfare of lactating dairy cows, and increasing dietary vitamin D_3 and Ca concentrations may be a viable dietary approach for alleviation of the negative effects of HS. Further research may be required for a more suitable model to study behavior of cows under HS as pair-feeding might introduce biases when used for animal behavior study.

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