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PRODUCTION AND MANAGEMENT: *Original Research*

Providing heat-stress abatement to late-lactation Holstein cows affects hormones, metabolite blood profiles, and hepatic gene expression but not productive responses

Rocío Soledad Martínez,[1]* Rafael Alejandro Palladino,[2,3] Georgget Banchero,[1] Rafael Fernández-Martín,[2,4] Mariana Nanni,[5] Nicolás Juliano,[4] Jesica Iorio,[4] and Alejandro La Manna[1]
[1]Instituto Nacional de Investigación Agropecuaria (INIA), Ruta 50 km 11.5, Colonia, Uruguay, 70000; [2]Instituto de Producción Animal (INPA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina C1417DSE; [3]Facultad de Ciencias Agrarias, Universidad Nacional de Lomas de Zamora, Buenos Aires, Argentina B1828; [4]Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina C1417DSE; and [5]Instituto de Investigación Tecnología de los Alimentos–Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina B1686

ABSTRACT

Objective: Our objective was to evaluate the effects of providing shade and shade combined with evaporative cooling on production, cow activity, metabolism, and hepatic gene expression of late-lactation Holstein dairy cows under moderate heat-stress conditions.

Materials and Methods: Forty-eight multiparous Holstein cows were used in a completely randomized block-design trial and randomly assigned to 1 of 3 treatments: control (CTL), without access to shade; access to artificial shade (SH); and shade combined with evaporative cooling (SHplus). Results were analyzed using a mixed procedure with repeated measures.

Results and Discussion: No differences were observed in DMI. Milk yield was not different among treatments, but lactose concentration was greater in SHplus. Treatments CTL and SH had greater BW losses than SHplus. Control cows spent less time grazing. The CTL and SH had higher p.m. rectal temperatures than SHplus, whereas CTL had the highest p.m. respiration rate. Control cows had greater serum insulin levels. Control and SH had greater BHB and urea concentrations and lower glucose concentration compared with SHplus. The hepatic expression of *PCK1*, *PDK4*, and *HP* genes was downregulated in SH and SHplus relative to control. Hepatic expression of *NFKB1* was downregulated, whereas *SOCS2* was upregulated, for SHplus compared with CTL.

Implications and Applications: Despite the absence of treatment effects on productive variables, changes in blood profiles and hepatic expression of target genes were observed among treatments. These results suggest that the provision of shade combined with evaporative cooling was effective in alleviating the negative effects of heat stress.

Key words: heat stress, late lactation, energy metabolism, hepatic gene expression

INTRODUCTION

Heat stress (**HS**) causes significant economic losses to the dairy industry (Scharf et al., 2014) that are estimated to be about $1,500 million per year only for United States (St-Pierre et al., 2003). It is expected that the detrimental effects of HS on animal production will be intensified as a consequence of global warming (Beniston et al., 2007). Additionally, the dairy industry has been selecting for more productive animals, which are also more sensitive to HS (Ravagnolo et al., 2000; Kadzere et al., 2002).

Heat stress occurs when environmental conditions prevent the animal from maintaining its physiological body temperature (Bligh, 1973). Under HS, mechanisms to maintain homeothermy are prioritized, such as reduce DMI (West, 2003), reduce gastrointestinal absorption (Beede and Collier, 1986), and increase maintenance requirements (Beede and Collier, 1986; Baumgard and Rhoads, 2013). Moreover, the metabolism and utilization of carbohydrates, lipids, and proteins are altered (Baumgard and Rhoads, 2013). All these changes have negative effects on milk yield (West, 2003), animal welfare (Polsky and von Keyserlingk, 2017), reproduction (Jordan, 2003),

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*Corresponding author: rmartine@agro.uba.ar

and health (Silanikove, 2000; Kadzere et al., 2002). The reduction in milk yield is partially explained by the decrease in feed intake (Wheelock et al., 2010) but also by changes in postabsorptive metabolism and nutrient partitioning (Baumgard and Rhoads, 2013). In fact, during HS, glucose that would normally be directed to the mammary gland is diverted into muscle to obtain energy as its oxidation is more efficient and produces less cellular heat than fatty acids (O'Brien et al., 2010). Additionally, because ruminants obtain little to no glucose directly from dietary digestion, gluconeogenesis is vital to supply glucose to extrahepatic tissues. In this context, a healthy liver plays a central role during HS (Collins et al., 1980; Febbraio, 2001).

In the southern hemisphere, late lactation normally coincides with the end of spring and early summer, when temperature–humidity indexes (**THI**) are high (Saravia, 2009) and pasture growth and quality are low (Fariña and Chilibroste, 2019). These scenarios might negatively affect body condition at a time when cows should start gaining BW and BCS (reach 5 on a 1-to-10-point scale or 3 points on a 1-to-5-point scale; Roche et al., 2017) to face the transition period with a healthy immune system (Hoedemaker et al., 2009; Sordillo and Mavangira, 2014; Roche et al., 2015). Therefore, although the late lactation stage is not usually considered as relevant as others stages of lactation, improved management including HS mitigation strategies would potentially enhance future cow performance and reduce health issues.

Environmental modifications through the use of shade and evaporative cooling (**EC**) are the most effective strategies to reduce the negative effects of HS on dairy cows (Beede and Collier, 1986). Overall, the predicted economic benefit could be up to 40% with HS abatement strategies (St-Pierre et al., 2003). In confinement systems, the provision of shade improved rectal temperature (**RT**), respiration rate (**RR**), DMI, and milk yield (17 vs. 19.4 kg/d; Schneider et al., 1984). In addition, when EC (sprinklers and fans) was added to shade, the beneficial effects increased. Chen et al. (1993), working with mid-lactation cows fed TMR, found that when shade was combined with EC, solid-corrected milk increased. Tarazón-Herrera et al. (1999) found that EC augmented 3.5% FCM (~10%) and diminished RR and BW losses in late-lactation cows compared with cows that only had access to shade. In grazing systems, Kendall et al. (2006) found in mid-lactation cows that the provision of shade in the grazing paddock improved milk yield (+0.5 kg). These authors also reported that grazing activity patterns were modified with the provision of shade, but unexpectedly, shade did not affect the total grazing time. Finally, in mixed systems, Valtorta et al. (1997) reported greater milk yield in cows provided with shade during the day (daily confinement) with the grazing session allocated at night. In the same way and more recently, Román et al. (2017) reported that cows in early lactation that had access to shade and shade combined with fans and sprinklers produced more solid-corrected milk (+15% compared with control cows) and had greater DMI. The objective of this study was to determine the effects of shade and shade combined with EC on cow performance and physiology traits, including milk yield; DMI; BCS; cow activity; metabolism; and hepatic expression of genes involved in carbohydrate metabolism, heat-shock response, chronic stress, and inflammatory processes of late-lactation Holstein dairy cows in a mixed grazing system with mild HS conditions (THI below 72). We hypothesized that cows with access to shade or shade combined with EC would produce more milk and have lower body condition losses, which would be reflected in better physiological parameters, compared with control cows.

MATERIALS AND METHODS

The experiment was conducted at La Estanzuela Experimental Station of the Instituto Nacional de Investigación Agropecuaria (Route 50, km 11, Colonia, Uruguay; 34°20′23.72″S, 57°41′39.48″W) during the summer of 2015 (from January 9, 2015, to March 9, 2015). All experimental procedures were approved by the Instituto Nacional de Investigación Agropecuaria Animal Welfare Committee (No. 0009/11).

Colonia's summer (December to March) average temperature range was 21.2 to 23.7°C; maximum temperature range was 26.4 to 27.3°C; minimum temperature range was 17.2 to 19.2°C; relative humidity range was 69 to 74%, and accumulated precipitation range was 87 to 125 mm during the period 1961 to 1990 (https://www.inumet.gub.uy).

Experimental Design, Animals, and Management

The experimental design was a completely randomized block design. Forty-eight multiparous Holstein dairy cows (calving period from March 15, 2014, till July 15, 2014) were assigned to 1 of 16 blocks by DIM (197 ± 48), lactation number (2.4 ± 1.3), BCS (2.8 ± 0.23), and BW (436 ± 41 kg; see Appendix Table A1). Within each block, cows were randomly assigned to 1 of 3 treatments: control (**CTL**): cows stayed from 0700 to 2000 h in an unshaded yard; shade (**SH**): cows stayed in a yard where they had access to artificial shade from 0700 to 2000 h; and shade combined with EC (**SHplus**): cows stayed in a yard where they had access to artificial shade from 0700 to 2000 h combined with 2 EC sessions. The first cooling session was after the milking at 0500 h and the second before the milking at 1500 h. Each session consisted of 2 series of 2 min of sprinkling (300 L/h) followed by 15 min of ventilation (Magnum 52," GEA; height: 3 m, diameter: 1.3 m). The 3 yards (one for each treatment) were adjacent to the milking parlor (1.6 ha, 100 m^2/cow) and exclusive for the cows of the correspondent treatment. Artificial shade was provided with black plastic cloth with 80% UV block.

The shade structure had an east–west orientation, a height of 3.5 m in the southern extreme, and a 15% declination toward the north. The shade area available per cow was 4.5 m^2. Animals were milked at 0500 and 1500 h. All cows received the same partial mixed ration (**PMR**). Diet was formulated to meet the nutrient demand proposed by the NASEM for a milk yield of 22 kg/d (NASEM, 2001). In the milking parlor, cows received a commercial concentrate (Prolesa) at a rate of 3.6 kg of DM/cow per day. After morning milking, animals were returned to their respective yards and received a PMR. After p.m. milking (~1700 h), cows returned to their respective confinement yards. From 2000 h to the next morning milking, all cows grazed the same alfalfa-based pasture (daily grazing strips). Daily grazing area was calculated to achieve an intake of 8 kg of DM of pasture per cow.

Temperature–humidity index was calculated as described by Armstrong (1994):

$$\text{THI} = (1.8 \times \text{T} + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times \text{T} - 26)],$$

where T is air temperature (°C) and RH is relative humidity (%). Data were obtained from the automatic meteorological station (temperature and humidity sensor: HMP45C model, Campbell Scientific Inc.) located 1 km away from the experimental area. Dry matter and chemical composition of concentrate, pasture, and PMR are shown in Table 1.

Data Collection and Laboratory Analyses

DMI. Individual DMI was measured in 10 cows per treatment throughout 3 consecutive days (starting on d 32 of the experimental period). To measure PMR, cows were housed in individual pens (60 m^2/cow) and intake was estimated by difference between offered and refused DM. Concentrate was offered at individual feeders in the milking parlor, and intake was estimated by difference between offered and refused DM. For pasture intake measurements, cows were placed in individual grazing plots (1 for each day of measurement). The area per cow was determined to provide an allowance of 10 kg of DM of pasture per cow per day. Intake was calculated by difference between offered and refused biomass according to Haydock and Shaw (1975). Ten replicates per individual plot were registered to determine pre- and postgrazing biomass.

All feeds were sampled every week. Samples were dried at 65°C in a forced-air oven for 48 h and ground to pass through a 1-mm screen for NDF (Van Soest et al., 1991), ADF, ash, and CP determination (AOAC, 1980). Acid detergent lignin content of PMR samples was also determined (Van Soest, 1963).

Milk Yield and Composition. Individual milk production was recorded daily (#7161-9005-062; Metatron P21, GEA). Milk samples were collected from each milking 3 times each week (Monday, Wednesday, and Friday) and composited by cow for analysis of components. Milk fat, protein, lactose, and urea were determined by Fourier transform infrared spectroscopy (Combi-Foss FT+, Foss Electric).

BW and BCS. All animals were weighed (#ID3000, Tru-Test; 500-g accuracy) every 2 wk at 0700 h without fasting. Body condition score (1-to-5-point scale; Edmonson et al., 1989) was monitored every 2 wk by the same operator during the experimental period.

Cow Activity. Cow activity was monitored by 4 trained observers during four 24-h periods on d 15, 21, 41, and 56. However, data from d 15 and 42 were excluded from the statistical analysis due to a marked decrease in THI caused by rain. The average THI of d 21 was 76.2 and of d 56 was 71.6 (both days included in the analysis). All animals were observed every 15 min. Their activities were classified as follows: staying under the shade (only in SH and SHplus treatments), eating PMR, grazing, lying, ruminating, or drinking water. Grazing activity was defined as the cow properly grazing and standing or walking with her head below back level, not necessarily foraging. It was assumed that between observations cows continued the same activity. Cow activity was not registered when cows were in

Table 1. Chemical composition of pasture, concentrate, and partial mixed rations (PMR) offered to late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

Item, % of DM ± SD	Concentrate	Pasture	PMR[1]
DM, % ± SD	89.6 ± 0.3	24.6 ± 0.5	35.7 ± 0.8
OM	93.5 ± 0.1	89.2 ± 1.0	82.0 ± 1.1
NDF	39.3 ± 2.6	42.7 ± 4.8	47.6 ± 0.9
ADF	21.1 ± 1.9	27.0 ± 2.6	33.4 ± 2.5
ADL	—	6.8 ± 1.1	11.5 ± 1.3
CP	21.2 ± 1.2	21.5 ± 2.7	11.0 ± 0.6

[1]The PMR composition was as follows: 56% of DM of oat silage, 11% of DM of pasture haylage, and 33% of DM of ground corn grain.

the holding pen of the milking parlor, during milking, and while they were walking from pasture or paddocks.

Physiological Variables. Rectal temperature and respiratory rate were determined twice each week at 0700 and 1300 h (after morning milking and before afternoon milking). The RT was recorded using a digital thermometer (MC–245, Omron Healthcare Inc.; 0.1°C accuracy). The RR was determined by visual observation of the right flank of the cow for 1 min and expressed as breaths per minute.

Metabolites and Hormones. Two blood samples were collected every 7 d throughout the trial from the jugular vein into vacuum tubes starting at 0700 h. The first sample was collected using a glass tube for serum separation (BD Vacutainer, Becton Dickinson) to determine concentrations of IGF1, insulin, BHB, nonesterified fatty acids (**NEFA**), cholesterol, albumin, total proteins, urea, aspartate transaminase enzyme (**AST**), and haptoglobin. The second sample was collected using a glass tube with sodium fluoride and potassium oxalate for glucose determination (BD Vacutainer). Samples were immediately placed on ice and then centrifugated at 1,500 × *g* for 15 min at 18 to 25°C. After centrifugation, serum was harvested, and samples were stored at −20°C until analysis. Concentrations of serum glucose, BHB, NEFA, cholesterol, albumin, total proteins, urea, and AST were determined by spectrophotometry (Vitalab Selectra II autoanalyzer; Vital Scientific) using enzymatic commercial kits. Kits for glucose (GOD-POD method; #1400071), cholesterol (CHE-CHOD method; #1221221), albumin (BCG method; #1009300), total proteins (Biuret method; #1009327), urea (GLDH method; #1810054), and AST (GOT-MDH method; #1009811) were from Wiener Laboratory. The BHB determination kit (D-3-Hydroxybutyrate method; #RB1007) was from Randox Laboratories. The nonesterified fatty acids determination kit (ACS-ACOD method; #99934691) was from Fujifilm Wako Pure Chemical Corporation. The intra-assay CV were for glucose 0.5%, cholesterol 1.5%, NEFA 2.2%, BHB 2.4%, urea 2.6%, albumin 2.3%, total proteins 1.4%, and AST 4.7%. The inter-assay CV were for glucose 0.9%, cholesterol 2.1%, NEFA 3.0%, BHB 2.6%, urea 3.1%, albumin 3.0%, total proteins 1.5% and AST 6.8%. Serum insulin concentration was determined by radioimmunoassay with a commercial kit (#KIP1251; DIAsource Immuno Assays S.A.). The minimal detectable concentration of insulin was 0.5 IU/mL. The intra-assay CV was 7.2%, and the inter-assay CV was 7.4%. Serum free IGF1 concentration was determined by radioimmunoassay with a commercial kit (#72-IGF1-RIACT; Cis Bio International). The minimal detectable concentration of IGF1 was 0.3 ng/mL, the intra-assay CV 7.9%, and the inter-assay CV 14.9%. Haptoglobin concentrations were determined by a commercial ELISA kit (#TP801; Tridelta Development Ltd.). The sensitivity of the method according to the manufacturer was 0.005 mg/mL. The intra-assay CV was 13% and the inter-assay 16%.

Hepatic Gene Expression. To assess hepatic expression of 7 target genes associated with carbohydrate metabolism, heat-shock response, chronic stress, and inflammatory processes (Table 2), liver biopsies were collected from a subset of cows (n = 7 per treatment). Biopsies were performed on d 40 of the experimental period. A 14-gauge biopsy trocar (16 cm long with a 20-mm notch; True Core Angiotech) was inserted between the 11th and 12th ribs to take a liver sample as described by Hoff et al. (2012). Local anesthesia was applied (5 injections of 2.5 mL of 2% lidocaine). Tissue samples were first frozen in liquid nitrogen and then stored at −80°C until quantitative real-time PCR analysis. The tissue was homogenized by hand with an Eppendorf Autoclavable Safe-Lock Micropestle in tubes that were kept on ice or liquid nitrogen. The RNA extraction was performed following the TRIzol method according to manufacturer's instructions (Invitrogen, #15596-026). Liver sample weight was 107.6 ± 22.7 mg. One milliliter of TRIzol was used for each extraction. The mean Abs at 260/280 was 2 ± 0.058. The RNA concentration was determined using a Nano Drop ND–1000 spectrophotometer (NanoDrop Technologies). Genes tested in the current study are listed in Table 2. Primers were previously used (Graugnard et al., 2013; Khan et al., 2014). The *GAPDH* and *RPS9* were selected as internal control genes (housekeeping genes). The geometric mean of these genes was used to normalize gene expression data. Both *GAPDH* and *RSP9* were previously used in several experiments (Hosseini et al., 2015). Both genes are known to be more stable expressed across different stages of lactation in liver tissue (Janovick-Guretzky et al., 2007). Transcript abundance for each gene was determined by relative quantitative real-time PCR. Reactions were performed in duplicate using 2 μL of cDNA, 0.4 μmol of each primer, and 12.5 μL of Kapa sybr Fast One-Step qRT-PCR Master Mix kit (#KK4651; KAPABIOSYSTEMS) in a 25-μL reaction volume. Reaction cycling conditions were 95°C for 3 min followed by 45 cycles at 94°C for 15 s, annealing temperature for 30 s, and 72°C for 30 s with fluorescence measurement during the extension step. Results were analyzed using the REST software (relative expression software tool), which allows the comparison of 2 groups with up to 4 reference genes. For more details, please see Pfaffl et al. (2002). Briefly, the following equation was used to estimate the relative expression of the gene of interest.

$$\text{Ratio} = (\text{Etarget})\ \Delta\text{CTtarget (control – sample)}/$$
$$(\text{Eref})\ \Delta\text{CTref (control – sample)}$$

The relative expression ratio of a target gene is computed based on its real-time PCR efficiencies (E) and the cycle threshold (CT) difference (Δ) of an unknown sample versus a control (ΔCTcontrol – sample), and mean comparison was performed (Proc GLM, SAS, version 9.2; SAS Institute).

Table 2. Name and primer sequences of each target gene analyzed by quantitative real-time PCR in bovine liver of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

Gene	Name	Sequence		Accession number	Reference
PCK1	Phosphoenolpyruvate carboxykinase 1	Forward	AAGATTGGCATCGAGCTGACA	NM_174737	Khan et al. (2014)
		Reverse	GTGGAGGCACTTGACGAACTC		
PDK4	Pyruvate dehydrogenase kinase 4	Forward	CAGGTGGACAGGGCAGTCTAG	NM_001101883	Khan et al. (2014)
		Reverse	TCCTCCTTTTCCATCTTTCTTCTTT		
HP	Haptoglobin	Forward	GGTTCGGAAAACCATCGCTA	NM_001040470	Khan et al. (2015)
		Reverse	CACTCGTGTCCCCTCCACTC		
HSPA1B	Heat shock protein family A (Hsp70) member 1A	Forward	CCCTGGATTGCTCATGTTTGT	NM_203322	Khan et al. (2015)
		Reverse	TCAACATCTCAAACAGCTTGCA		
NFKB1	Nuclear factor kappa B subunit 1	Forward	TTCAACCGGAGATGCCACTAC	NM_001076409	Khan et al. (2015)
		Reverse	ACACACGTAACGGAAACGAAATC		
XBP1	X-box binding protein 1	Forward	GAGAGCGAAGCCAATGTGGTA	NM_001034727	Khan et al. (2015)
		Reverse	ACTGTGAATTCAGGGTGATCTTTCT		
SOCS2	Suppressor of cytokine signaling 2	Forward	GGGATGCTTCCCTTCCTAAG	NM_177523	do Amaral et al. (2009)
		Reverse	GTGCTGGGACCTTTCACCTA		

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS with repeated measures (version 9.2; SAS Institute). The model for all variables (except for DMI and cow activity) was as follows:

$$Y_{ijk} = \mu + \text{Cov} + B(A)_i + \text{Treat}_j + \text{Week}_k + (\text{Treat} \times \text{Week})_{jk} + E_{ijk},$$

where Y_{ijk} is the response, μ is the mean, Cov is the covariate effect (milk yield or composition of the previous month), B_i is the block effect (1 to 16), Treat_j is the treatment effect (CTL; SH; SHplus), Week_k is the effect of the week of sampling, when necessary (1 to 10), $(\text{Treat} \times \text{Week})_{jk}$ is the interaction effect, and E_{ijk} is the error term. Animal (A) nested into block was used as a random effect.

The same model was used for cow activity, but the week effect was replaced with the day of visual observation effect.

The model for DMI was as follows:

$$Y_{ijk} = \mu + \text{Cov} + B(A)_i + \text{Treat}_j + E_{ijk},$$

where Y_{ijk} is the response, μ is the mean, Cov is the covariate effect (milk yield or composition of the previous month), B_i is the block effect (1 to 16), Treat_j is the treatment effect (CTL; SH; SHplus), and E_{ijk} is the error term. Animal (A) nested into block was used as a random effect.

Normality of the residuals was tested for all variables following Shapiro-Wilk test. Covariance structures were examined and selected according to the Bayesian information criterion statistic. The structures that fitted well were ARH (heterogeneous autoregressive) for milk yield and composition data; CS (compound symmetry) for RR, RT, blood metabolites, and hormones data; UN (unstructured) for DMI data; and AR (autoregressive) for BW, BCS, its variation, and cow activity data. Data were reported as least squares means. Differences were considered significant when $P \leq 0.05$ and trends when $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

Our hypothesis that cows with access to shade and shade combined with EC would produce more milk and have less body condition losses, which would be reflected in better physiological parameters, compared with control cows was partially accepted. We were unable to increase milk yield. However, SHplus cows had lower BW losses than SH and control cows.

Environmental conditions during the study are illustrated in Figure 1. The average, minimum, and maximum THI from the experimental period were 69.8, 53.1, and 82.6, respectively. On average, during 14 h/d (0800 to 2200 h), the THI remained above 68 (Figure 2), and for 9 h, the THI remained above 72 (1000 to 1900 h). These data dem-

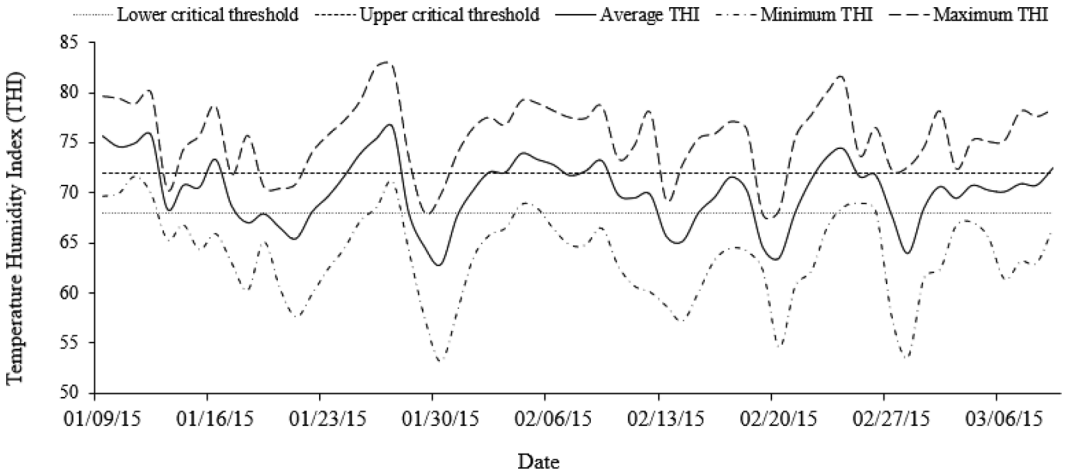

Figure 1. Environmental conditions [average, maximum, and medium temperature–humidity indexes (THI)] of the experimental period and THI lower and upper critical thresholds (68 and 72, respectively). Date = mo/d/yr.

onstrate that environmental conditions across the study predisposed cows to suffer HS.

In the present experiment there was no effect of shade on daily milk yield. In addition, we did not observe any effect of shade or shade combined with EC on fat yield and MUN (Table 3). Daily ECM yield tended to be greater in CTL and SHplus compared with SH ($P = 0.07$). Our results differ from those of Kendall et al. (2006), who reported improved milk yield in late-lactation cows even they had lower THI values (THI around 63).

Milk fat concentration tended to be greater ($P = 0.09$) in SH compared with SHplus and similar with CTL. Milk protein percentage was not different among treatments ($P = 0.72$), but milk protein yield was greater ($P = 0.03$) for SHplus and CTL compared with SH. Treatments affected lactose content: SHplus had greater lactose content ($P < 0.01$). Interactions of treatment by week of sampling were observed ($P < 0.05$) for yield of ECM and milk and concentrations of fat, lactose, and MUN. In an effort to explain those interactions, we observed the behavior of the variables and THI (Supplemental Figures S1–S5; https://doi.org/10.15232/aas.2020-02109) and could not identify any consistent pattern between them. The THI had high variation within and between week of sampling; the response of each HS mitigation strategy is difficult to explain and might be related to the adaptation mechanisms of the cows to the weather conditions previous to or during the experimental period.

Results related to DMI are shown in Table 4. Total DMI (kg/d), DMI as a proportion of BW, and feed conversion efficiency were similar among treatments ($P = 0.22$, $P = 0.18$, and $P = 0.19$, respectively). Estimated pasture intake (kg of DM/d) tended to be greater in SH compared with CTL ($P = 0.09$) but was similar between SH and SHplus. We found no differences in DMI, which would partially explain the absence of effects on milk yield. Another explanation for the absence of response in milk yields when cows were provided with HS-abatement strategies could be the productive level of cows, which is a consequence of the nutritional management applied. In our experiment, cows

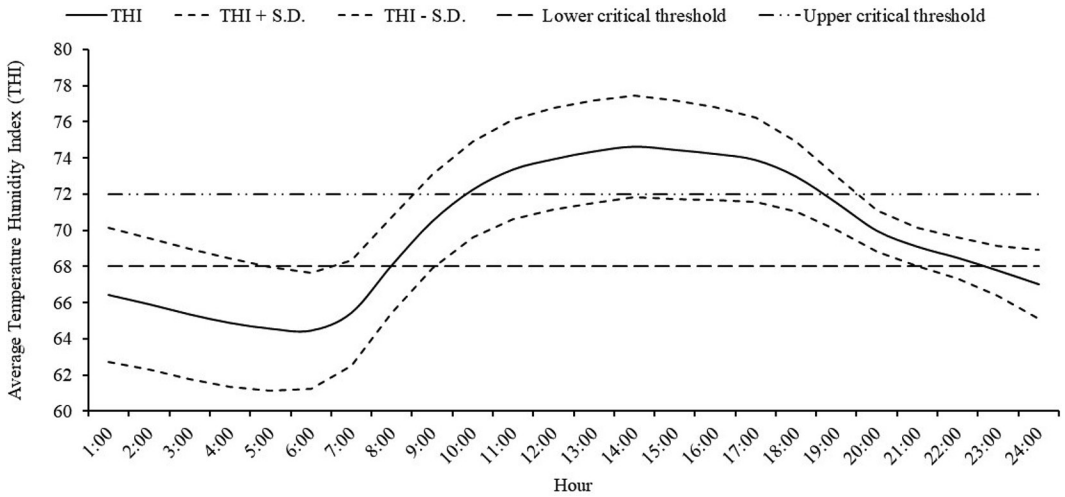

Figure 2. Average daily temperature–humidity index (THI) ± SD and lower and upper critical thresholds (68 and 72, respectively).

Table 3. Milk yield and composition of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				*P*-value[1]		
Variable	CTL	SH	SHplus	SEM	Treatment	Week	Treatment × week
Milk yield, kg/d	19.0	18.9	19.6	0.27	0.12	<0.01	0.07
ECM, kg/d	19.7^{A}	19.1^{B}	19.5^{A}	0.19	0.07	<0.01	0.01
Fat, %	4.14^{AB}	4.16^{A}	3.96^{B}	0.038	0.09	<0.01	0.01
Fat, kg/d	0.76	0.77	0.75	0.012	0.67	<0.01	0.01
Protein, %	3.34	3.31	3.35	0.042	0.72	<0.01	0.55
Protein, kg/d	0.64^{a}	0.61^{b}	0.67^{a}	0.081	0.03	<0.01	0.17
Lactose, %	4.67^{b}	4.67^{b}	4.92^{a}	0.041	<0.01	<0.01	<0.01
Lactose, kg/d	0.88	0.87	0.96	0.227	0.12	<0.01	0.06
MUN, mg/dL	19.87	19.23	19.29	0.49	0.35	<0.01	0.02

^{a,b}Means within a row with different superscripts significantly differ ($P < 0.05$).

^{A,B}Means within a row with different superscripts show a trend ($0.05 \leq P \leq 0.10$).

[1]Significance of treatment, week, and the interaction of treatment × week.

were fed to produce about 22 kg/d (in concordance with their stage of lactation), whereas in the study conducted by Román et al. (2017), who reported differences in milk yield, cows were fed to produce about 50% more. Kadzere et al. (2002) stated that, due to the combination of genetic merit and nutrition, high-producing dairy cows are more sensitive to thermal stress because their body heat production is increased (West, 2003). Cows with access to shade and shade combined with EC tended to consume more pasture than CTL cows (Table 4). This could be partially explained by the longer grazing time observed by the animals in those treatments. There were no effects of the use of shade and shade combined with EC on the time dedicated to other activities (Table 5). Control cows spent less time grazing than SH and SHplus cows ($P < 0.01$). Previous results from Tapki and Şahin (2006) established that heat-stressed cows in confined systems spent less time eating. Breinholt et al. (1981) found that cows exposed to stressful thermal conditions, even when provided with shade in the grazing areas, doubled pasture intake during the cooler hours (1800 to 0600 h) compared with daytime. Fisher et al. (2002) reported that cows with access to shade spent more time grazing at night to compensate their reduced feed intake during the hottest part of the day (when they preferred to stay under the shade). In our experiment, the grazing session was allocated at night (with lower THI values). We expected a compensatory effect in CTL cows in grazing time and pasture intake; how-

Table 4. Dry matter intake and conversion efficiency of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				
Variable	CTL	SH	SHplus	SEM	*P*-value[1]
DMI, kg/d	15.7	16.8	16.1	0.43	0.22
DMI, % BW	2.9	3.1	3.0	0.09	0.18
Pasture intake, kg of DM/d	5.9^{B}	7.4^{A}	6.7^{AB}	0.43	0.09
PMR[2] intake, kg of DM/d	6.1	5.9	5.8	0.04	0.10
Concentrate intake, kg of DM/d	3.6	3.6	3.6	0.01	0.10
Conversion efficiency[3]	1.09	1.35	1.29	0.082	0.19

^{A,B}Means within a row with different superscripts show a trend: $0.05 \leq P \leq 0.10$.

[1]Treatment significance.

[2]PMR = partial mixed ration.

[3]Conversion efficiency = ECM (kg/d)/DMI (kg/d).

Table 5. Cow activity (min/d) of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				*P*-value[1]		
Variable	CTL	SH	SHplus	SEM	Treatment	Day	Treatment × day
Eating PMR[2]	90	77	89	4.9	0.10	<0.01	0.01
Grazing	198[b]	250[a]	240[a]	6.8	<0.01	<0.01	0.01
Lying	381	375	360	11.7	0.44	<0.01	0.32
Ruminating	477	480	479	10.0	0.97	0.27	0.08
Drinking	11	16	16	2.6	0.39	0.01	0.05

[a,b]Means within a row with different superscripts differ ($P < 0.01$).

[1]Significance of treatment, day, and the interaction of treatment × day.

[2]PMR = partial mixed ration.

ever, we did not observe it. A possible explanation for this could be that those cows that suffered HS during the day could not recover by night and were unable to compensate pasture intake. The treatment-by-day interaction was statistically different for activities: eating PMR, grazing, ruminating, and drinking. On d 21, when THI was higher, SHplus consumed more PMR and ruminated more time than SH and CTL, although the overall treatment effect was not significant. Regarding eating PMR and ruminating, on the day with the higher THI (d 21), SHplus ate and ruminated more time than SH and CTL (in spite of the absence of effects of treatments). These results suggest that under higher THI conditions, we would expect more effects of the use of shade and EC.

Increased p.m. RR and RT in CTL are reported as the most evident and immediate signs of HS. Treatments SH and SHplus did not affect RT in the morning ($P = 0.11$; Table 6). However, SHplus cows had the lowest RT in the afternoon ($P < 0.01$). We found no effects of shade and shade combined with EC on a.m. RR ($P = 0.13$), but in the afternoon, CTL cows had the highest rate compared with SH and SHplus cows ($P < 0.01$). These results are consistent with those obtained by Brown-Brandl et al. (2003) and Román et al. (2017), indicating that the evaluated HS-mitigation strategies had beneficial effects on RT and RR in the moments of the day when THI values were higher. Several authors reported a reduction of 30 to 70% in RR with respect to control cows, suggesting that the combination of the 2 strategies could be even more effective in reducing RR than when used alone (Tarazón-Herrera et al., 1999; Fike et al., 2002; Valtorta and Gallardo, 2004; Kendall et al., 2007). Regarding p.m. RR, we obtained a better response in both shade treatments, although p.m. RT was lower only for SHplus, indicating a better response when shade was combined with sprinkling and air circulation.

Shade and shade combined with EC affected insulin concentration ($P = 0.01$; Table 7). Treatments SH and SHplus had lower insulin concentrations than CTL ($P = 0.01$). On the other hand, SHplus cows had the highest glucose concentration ($P = 0.02$) and the lowest BHB and urea concentrations ($P = 0.01$). We did not observe differ-

Table 6. Rectal temperature (°C) and respiration rate (breaths per minute) of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				*P*-value[1]		
Variable	CTL	SH	SHplus	SEM	Treatment	Week	Treatment × week
Rectal temperature a.m.	37.8	37.9	37.9	0.04	0.11	<0.01	0.03
Rectal temperature p.m.	38.9[a]	38.9[a]	38.5[b]	0.07	<0.01	<0.01	<0.01
Respiration rate a.m.	35.7	35.0	36.9	0.67	0.13	<0.01	<0.01
Respiration rate p.m.	67.8[a]	56.1[b]	59.4[b]	0.95	<0.01	<0.01	<0.01

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Significance of treatment, week, and the interaction of treatment × week.

ences in serum concentrations of IGF1, NEFA, cholesterol, albumin, total proteins, AST, and haptoglobin between treatments (Table 7). Lactose concentration was increased by SHplus (Table 3), in line with serum glucose, a precursor of milk lactose in the mammary gland, suggesting that SHplus cows could be more efficient using glucose for lactose synthesis (Rhoads et al., 2009). In fact, the CTL and SH cows had the lowest lactose concentrations in coincidence with the observed highest p.m. RT values, indicating that both treatments were affected by HS. At the same time, insulin, a key hormone in the regulation of carbohydrate and lipid metabolism, was increased in CTL ($P = 0.01$). It is known that under HS, glucose, which is normally used by the mammary gland, is redirected to the muscle to be used in place of fatty acids producing less metabolic heat (O'Brien et al., 2010; Baumgard and Rhoads, 2013).

The use of shade and shade combined with EC did not affect BW ($P = 0.49$), BCS ($P = 0.33$), or variation in BCS ($P = 0.11$; Table 8). Control and SH cows had greater BW losses across the experimental period, compared with SHplus cows ($P = 0.05$). Some authors established that HS cows are unable to oxidize as much NEFA as early-lactation or undernourished cows (Baumgard and Rhoads, 2013), and so differences in BW or BCS are not fully explained by fat losses. Shwartz et al. (2009) and Wheelock et al. (2010) suggested that BW losses in HS cows comes mainly from muscle breakdown provoking an increase in plasma urea nitrogen concentration. In our study, CTL and SH had greater serum urea levels than SHplus in coincidence with the greater BW losses observed in the former treatments. Elevated serum urea levels sometimes indicate muscle breakdown, so we can hypothesize that BW losses of CTL and SH cows came from muscle. Notwithstanding, BHB values were within physiological values; however, they followed the pattern of serum urea (smaller in SHplus, $P < 0.01$ and $P = 0.01$, respectively). These data suggest that even though they were provided with shade, SH cows were experiencing some degree of HS.

Hepatic mRNA expression (Figure 3) of *PCK1* ($P = 0.01$), *PDK4* ($P = 0.01$), and *HP* ($P = 0.01$) genes was downregulated in SH and SHplus relative to CTL. Hepatic mRNA expression of *NFKB1* was downregulated in SHplus cows ($P = 0.05$) in comparison with CTL, and no differences were found between SH and CTL ($P = 0.26$). Hepatic mRNA expression of *SOCS2* was upregulated in SHplus compared with CTL ($P = 0.01$), but no differences were found in SH expression relative to CTL ($P = 0.86$). No differences were found in hepatic mRNA expression of *HSPA1B* ($P > 0.10$) and *XBP1* ($P > 0.10$) in SH and in SHplus relative to CTL. The SHplus and SH cows had a lower relative hepatic expression of *PCK1* and *PDK4* genes compared with CTL cows. In general, the expression of these genes increases the output of glucose from the liver by different mechanisms (Baumgard and Rhoads, 2013). The *PDK*s genes inhibit glycolysis, and their activity is mainly regulated at the transcriptional level by intracellular energy status (Harris et al., 2002). The *PCK* stimulates gluconeogenesis in liver and kidneys (Yang et al., 2009) through phosphoenolpyruvate carboxykinase

Table 7. Blood variables of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				*P*-value[2]		
Variable[1]	CTL	SH	SHplus	SEM	Treatment	Week	Treatment × week
IGF1, ng/mL	86.0	87.0	83.0	8.59	0.98	<0.01	0.93
Insulin, µIU/mL	8.8[a]	7.1[b]	6.8[b]	0.44	0.01	0.14	0.64
Ratio IGF1:insulin	10.4	12.9	13.1	1.21	0.14	0.90	0.90
Glucose, mmol/L	2.8[a]	2.8[a]	3.0[b]	0.05	0.02	0.001	0.50
BHB, mmol/L	0.6[a]	0.6[a]	0.4[b]	0.27	<0.01	<0.01	<0.01
NEFA, mmol/L	0.2	0.2	0.3	0.01	0.11	<0.01	0.31
Cholesterol, mmol/L	5.1	5.4	5.2	0.17	0.43	0.05	0.81
Albumin, g/L	33.5	33.5	33.9	0.59	0.79	0.34	0.50
Total proteins, g/L	85.0	90.1	88.5	2.19	0.23	0.03	0.49
Urea, mmol/L	6.5[a]	6.2[a]	5.6[b]	0.18	<0.01	<0.01	<0.01
AST, UI	76.2	79.1	79.5	3.53	0.77	0.04	0.42
Haptoglobin,[3] mg/mL	0.2	0.1	0.2	1.08	0.34	0.40	0.03

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]NEFA = nonesterified fatty acids; AST = aspartate transaminase enzyme.

[2]Significance of treatment, week, and the interaction of treatment × week.

[3]Because the variable does not have a normal distribution, the significance of the transformed variable is reported.

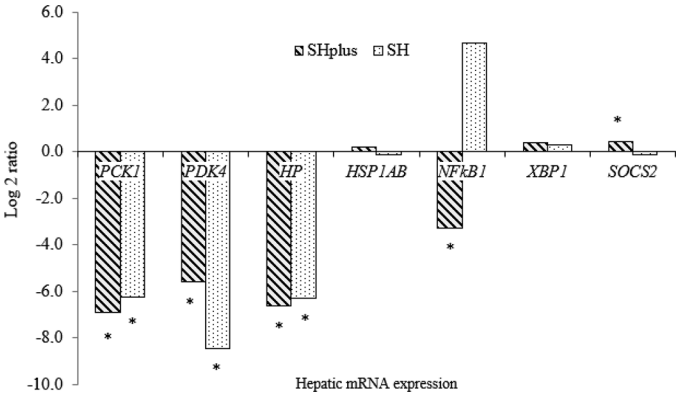

Figure 3. Hepatic mRNA expression of *PCK1*, *PDK4*, *HP*, *HSP1AB*, *NFKB1*, *XBP1*, and *SOCS2* genes in late-lactation Holstein dairy cows with access to shade (SH) and shade combined with evaporative cooling (SHplus) relative to control cows. Asterisks indicate significant differences ($P < 0.05$) between the treatment and control.

synthesis, a key enzyme in gluconeogenesis (Proft and Grzesitza, 1995). In addition, insulin upregulates *PCK1* expression (White, et al., 2012), which agrees with the increased insulin concentration in CTL cows.

The hepatic gene expression of *SOCS2* was greater in cows at SHplus compared with CTL but similar between SH and CTL. This gene encodes a protein that acts as an inhibitor of growth hormone (**GH**) signaling, among other functions (Masuzaki et al., 2016). On the other hand, Wolfenson and Roth (2018) reported that dairy cows under HS-abatement strategies had a greater concentration of estradiol-17β, and this hormone upregulates the expression of *SOCS2* (Winkelman et al., 2008). It is possible that cows under HS (CTL and SH) had lower concentration of estradiol-17β, and consequently, *SOCS2* gene expression was downregulated. It is known that *SOCS2* decreases the GH action by targeting the GH receptor for ubiquitination and degradation (Masuzaki et al., 2016). We hypothesize that when *SOCS2* expression was decreased in CTL and SH cows, the GH action could have been enhanced, potentially increasing gluconeogenesis. In fact, *PCK1* gene expression was greater in CTL cows. We did not find differences in the relative hepatic expression of *XBP1* and *HSPA1B* genes, which can be related to the degree of stress. Heat shock proteins (**HSP**) are a family of proteins produced by cells when exposed to stressful conditions. The *HSPA1B* encodes synthesis of HSP70, which has an important role preventing cell damage cause by inflammation, oxidative stress, and heat (Morimoto and Santoro, 1998; Roti Roti, 2008). Insulin also upregulates HSP synthesis (Lee et al., 2006). Shahzad et al. (2015) found greater expression of the *HSPA1B* gene in heat-stress conditions during peripartum, but there could be a confounding effect because gene expression may be caused by heat load or by other factors such as energy mobilization (Gessner et al., 2013) or by inflammatory and immunological conditions typical of these stages of lactation (Catalani et al., 2010). The *XBP1* gene is related to endoplasmic reticulum stress; however, there is not enough information about *XBP1* expression in dairy cows exposed to HS. Adachi et al. (2009) worked with cultivated cells and reported more expression of *XBP1* in heat-stressed cells. It is possible that under moderate HS conditions like those of this study, some physiological responses to stress are not activated as would probably would be under more severe conditions. We found differences in *NFKB1* expression, a classical inflammatory signaling gene (Khan et al., 2015). The SHplus had lower *NFKB1* hepatic expression relative to CTL, but hepatic expression of this gene in SH was similar to that in CTL. It appears that CTL and SH cows could had been having an inflammatory process with the consequent immune system activation, which might be explained by the altered gastrointestinal-tract permeability during HS (Baumgard et al., 2015). When cows are un-

Table 8. Body weight and BCS of late-lactation Holstein cows without heat-stress mitigation (CTL) or provided shade (SH) or shade combined with evaporative cooling (SHplus)

	Treatment				*P*-value[1]		
Variable	CTL	SH	SHplus	SEM	Treatment	Week	Treatment × week
BW, kg	551	546	553	8.1	0.49	0.03	0.97
BCS[2]	2.7	2.8	2.8	0.15	0.33	<0.01	0.12
BW change,[3] kg	−18.9[a]	−16.6[a]	−6.1[b]	3.83	0.05	—	—
BCS change[4]	0.07	0.23	0.14	0.051	0.11	—	—

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Significance of treatment, week, and the interaction of treatment × week.

[2]BCS on a 1-to-5-point scale.

[3]Body weight variation through the experimental period, initial versus final BW.

[4]BCS variation through the experimental period, initial versus final BCS.

der HS, gastrointestinal-tract blood flow is diverted from the respiratory and gastrointestinal-tract systems to the periphery of the body to dissipate heat through convection (Kadzere et al., 2002; Lambert et al., 2002). Therefore, gastrointestinal-tract cells might become hypoxic (Rollwagen et al., 2006), allowing the infiltration of lipopolysaccharides into systemic blood (Hall et al., 2001; Pearce et al., 2013), triggering a local inflammatory response (Mani et al., 2012). The immune system recognizes molecular patterns from pathogens or toxins through specific receptors and produces cytokines, which induce inflammation and generate an acute phase response (Daha, 2011; Ceciliani et al., 2012). When the immune system is activated, its cells become glucose-obligate utilizers (Kvidera et al., 2017). Although expression of genes involved in the inflammatory response were increased in CTL, we did not find any difference in serum haptoglobin concentration, a well-known proinflammatory indicator (Vailati-Riboni et al., 2016). However, the hepatic expression of the *HP* gene was lower in SHplus and SH relative to CTL, suggesting some degree of stress or inflammation due likely to thermal load.

APPLICATIONS

In summary, we provided evidence that grazing dairy cows during late lactation experienced HS even when average THI values were moderate (average THI <72). Providing shade alone or in combination with EC did not improve milk yield, and only the combination of both reduced BW losses. Hepatic expression of genes involved in glucose metabolism and haptoglobin synthesis, an immune system protein, was downregulated in SH and SHplus compared with CTL. The hepatic expression of the *NFKB1* gene (related to inflammatory processes) was downregulated in SHplus in comparison with CTL. The alterations in the hepatic expression of these genes seemed to be a strategic response to mitigate the thermal load in a coordinated manner. From our results, it seems that shade combined with EC is necessary to mitigate the negative effects of HS and to prevent cows from potential health problems in temperate regions.

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ORCIDS

Rocío Soledad Martínez https://orcid.org/0000-0001-5687-6042
Georgget Banchero https://orcid.org/0000-0002-1146-3612
Alejandro La Manna https://orcid.org/0000-0002-1223-5099

APPENDIX

Table A1. Animal characteristics of cows randomly assigned to the treatments at the beginning of the experimental period

	Treatment[1]				
Item	**CTL**	**SH**	**SHplus**	**SEM**	***P*-value[2]**
DIM	186	206	200	12.1	0.51
Lactation number	2.6	2.4	2.2	0.33	0.62
BCS	3.19	3.22	3.19	0.036	0.77
BW	531	532	541	12.0	0.80

[1]Least squares means of treatments: CTL = without heat-stress mitigation strategies; SH = access to shade; SHplus = access to shade combined with evaporative cooling.

[2]Treatment effect.