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Residual Effects of Heat Stress on Lactating Holstein Cows During Post-Heat Recovery

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Abstract

Heat stress is frequently associated with reduced dry matter intake (DMI), milk yield, and physiological alterations in Holstein dairy cows. The aim was to evaluate how prior exposure to heat stress affects feed intake, milk production, energy balance, blood metabolites, and infrared thermography, with special emphasis on the cows' ability to recover and re-establish normal physiological and productive responses. Thirty multiparous lactating Holstein cows (days in milk = 87 ± 19.6 days) and previous milk yield (30.8 ± 3.95 kg/d) were monitored over 21 days, divided into three 7-day phases: thermoneutral (TN), cows kept at a constant temperature–humidity index (THI) of 69; heat stress (HS), cows at a THI of 82 for 9 h and 69 for 15 h, and recovery (REC), cows at a constant THI of 69. Milk yield decreased by 16.1% during HS and remained 17.3% lower during REC compared with TN. Dry matter intake was reduced by 5.8 and 4.2 kg/d for HS and REC compared to TN cows. Plasma insulin was higher in REC compared to HS. Under the conditions of the present study, heat stress was associated with impaired productive performance, altered metabolism, and changes in the physiological status of mid-lactation Holstein dairy cows, with some effects persisting after the cows returned to thermoneutral conditions.

Keywords: energy balance; insulin sensitivity; milk yield; residual effects

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1. Introduction

Holstein dairy cows constantly experience some degree of heat stress, particularly in tropical regions. Heat stress occurs when environmental conditions impose a thermal load that exceeds the animal's capacity to dissipate heat, resulting in an increase in body temperature and the activation of physiological mechanisms aimed at maintaining core body temperature within the normal homeostatic range [1]. Dairy cows under heat stress (temperature–humidity index (THI) > 72; [2]) decrease dry matter intake (DMI) by 0.49 kg/d for every one-unit increase in THI (mean ± SD = 71.8 ± 10.4; [3]), show reduced milk yield [4,5] and exhibit increased respiration rate [6], all of which can severely compromise farm profitability [7,8].

The effects of heat stress include alterations in metabolism [9], shifts in nutrient partitioning [7], and impaired immune function in dairy cows [10,11]. In addition to the

global challenges of climate change and the increase in the frequency and severity of heat waves, there are still few studies evaluating the impacts of post-heat stress.

Additionally, to our knowledge, these few studies [12,13] demonstrate that heat stress exerts residual effects during the post-stress period in mid-lactation, although the precise temporal and physiological mechanisms remain insufficiently characterized in mid-lactation Holstein dairy cows. An early study has shown that dairy cows in a recovering phase for 7 days after heat stress exhibit higher blood glucose concentrations than cows maintained under heat stress. However, this metabolic improvement is not necessarily accompanied by changes in milk yield or milk composition [13]. In addition, specifically, how energy partitioning, metabolic adjustments, and DMI fluctuate during the immediate days following heat stress require further investigation.

We hypothesized that heat stress induces persistent physiological and metabolic alterations that impair the recovery of productive performance after return to thermoneutral conditions. Therefore, this study aimed to evaluate the carryover effects of heat stress on feed intake, milk yield, energy balance, blood metabolites, and infrared thermography, with a specific emphasis on the cows' capacity to recover and re-establish normal physiological and productive responses once restored to thermal comfort.

2. Material and Methods

2.1. Cows Feeding Management and Facilities

This study was conducted at the Multi-user Laboratory of Bioefficiency and Livestock Sustainability (https://www.embrapa.br/laboratorio-multiusuario-de-bioeficiencia-e-sustentabilidade-da-pecuaria, accessed on 6 April 2026), belonging to Embrapa Dairy Cattle, located in Coronel Pacheco city, Minas Gerais, Brazil. All procedures involving animals were approved by the Animal Care and Use Committee of Embrapa Dairy Cattle (CEUA protocol n° 4661190421).

Thirty lactating multiparous Holstein dairy cows were blocked by days in milk (DIM; mean ± SD = 87 ± 19.6 days) and previous milk yield (30.8 ± 3.95 kg/d), and parity (2.9 ± 1.12). Before the experiment began, all cows were adapted to the free-stall facilities and diet for 14 days under thermoneutral conditions. Due to the limited capacity of the climatic chamber (which accommodated up to six cows simultaneously), the cows were randomly allocated into five sequential groups (block) between June and September 2021. To minimize potential confounding factors, the order of exposure to heat stress was randomized across these groups, and cow nested within blocks was included as a random effect in the statistical models.

The study followed a longitudinal repeated-measures design using the same experimental units (cows) across three sequential periods. A total of 30 lactating cows were monitored over 21 days, divided into three distinct 7-day phases: thermoneutral (TN), kept at a constant THI of 69 (22 °C; 64% relative humidity (RH)); heat stress (HS), exposed to a THI of 82 for 9 h/d (30 °C; 77% RH), and recovery (REC) maintained at a constant THI of 69 (22 °C; 64% RH) (Figure 1). Environmental temperature and RH were recorded at 0600, 1000, 1400, and 1800 h to coincide with the physiological measurements. These values were used to calculate the THI according to the equation proposed by Dikmen and Hansen [1]:

$$THI = (1.8 \times T + 32) - ((0.55 - 0.0055 \times RH) \times (1.8 \times T - 26))$$

where T is the environmental temperature (°C) and RH is the relative humidity (%).

During the pre-heat-stress period, the cows were housed in a free-stall barn with rubber mats covered with wood shavings as bedding. The barn was provided with electronic automated systems (automatic feeders, water troughs, and a weighing platform; Ponta, Betim, MG, Brazil) and a cooling system consisting of fans and sprinklers over the feed bunk with an

automatic trigger to provide a uniform THI of 69. Conversely, to ensure that all cows were uniformly exposed to heat stress, the cows were moved to a climatic chamber (Consciente Ltd., SCAL, Belo Horizonte, MG, Brazil) and were maintained in the climatic chamber in the recovery period (THI = 69) for 7 days. Within the climatic chamber, cows were housed in individual pens (1.78 × 1.14 m) featuring WingFlex rubber mats (Kraiburg TPE GmbH & Co., Waldkraiburg, Germany) on the floor and wood shavings as bedding. Thermometers (FEPRO-MUT600S, Exbom, São Paulo, Brazil) and thermo-hygrometers (AK28 new, AKSO, Rio Grande do Sul, RS, Brazil) were positioned at the cows' withers height to record maximum and minimum temperatures and RH during the 7-day evaluation periods.

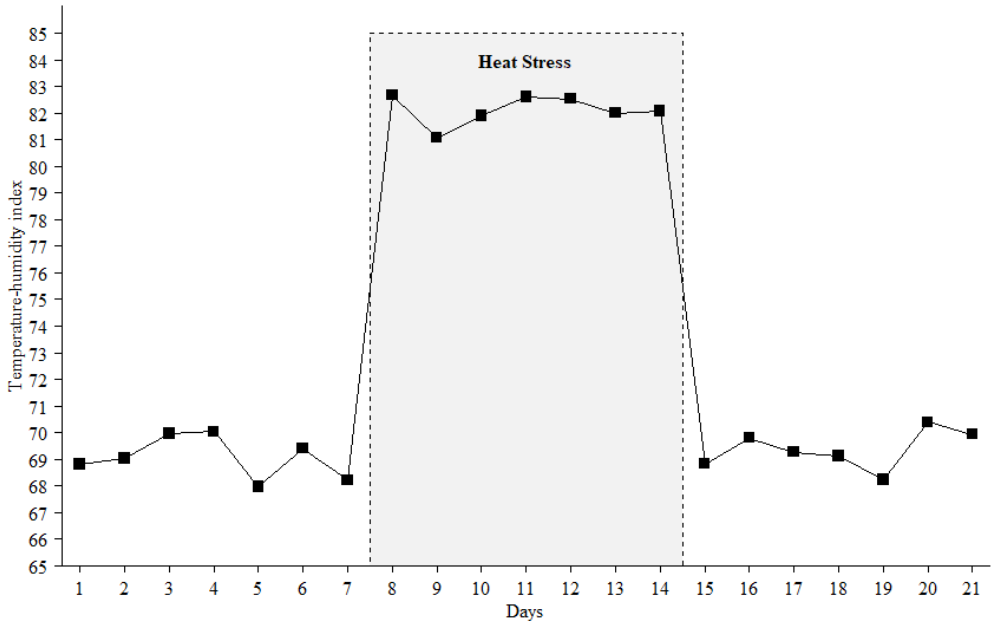


Figure 1. Temperature–humidity index (THI) of experimental periods. The grey box is the heat-stress period.

2.2. Diet and Chemical Analysis

The cows were fed a standardized total mixed ration (TMR; Table 1) twice daily at 0800 and 1600 h.

The diet was formulated according to the recommendations of NASEM [14] and 10% refusal was allowed. To avoid confounding effects, the same TMR was provided throughout all experimental periods. Samples of TMR ingredients, feed offered, and refusals were collected daily, dried in a forced-air oven at 55 °C for 72 h, ground through a 1 mm screen sieve (Wiley mill; A. H. Thomas Scientific, Swedesboro, NJ, USA), and subsequently pooled into weekly composites. Samples were analyzed for dry matter (DM), organic matter (OM), total nitrogen, and ether extract (EE) according to methods 930.15, 942.05, 984.13, and 920.39, respectively [15]. Crude protein (CP) was estimated by multiplying the total nitrogen content by a conversion factor of 6.25. Concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined sequentially using an Ankom 220 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). For the NDF analysis, a heat-stable α-amylase was used without the addition of sodium sulphide. The non-fibrous carbohydrate (NFC; g/kg DM) content was calculated by difference as follows:

$$NFC = 100 - [(CP + NDF + EE + ash) - NDF\ insoluble\ protein]$$

Table 1. Ingredient proportions and chemical composition of experimental diets.

Item, g/kg DM	Experimental Diet
Maize silage	524.6
Ground corn grain	179.8
Soybean meal	153.9
Bermuda grass	40.3
Whole cottonseed	70.1
Urea	3.66
Mineral mix	27.4
DM basis; ±SD	
DM, %	46.9 ± 2.86
CP, %	14.1 ± 1.08
NDF, %	29.9 ± 1.71
ADF, %	16.4 ± 1.14
Lignin, %	1.80 ± 0.30
Ash, %	5.98 ± 0.64
EE, %	3.51 ± 0.36
NFC, %	46.5 ± 2.53
NE_L Mcal/kg of DM	1.65 ± 0.055

Mineral mix: blend mineral containing (DM basis): 199 g of calcium; 30 g of phosphorus; 23 g of sulfur; 65 g of magnesium; 50 g of potassium; 24 g of sodium; 37.5 g of bentonite; 21 mg of cobalt; 626 mg of cooper; 25 mg of chrome; 26.2 g of iodine; 1830 mg of manganese; 25 mg of selenium; 3600 mg of zinc; 100 mg of biotin; 255,000 UI of vitamin A; 190,000 UI of vitamin D3; 1500 mg of vitamin E; 969 mg of D-limonene and 1.5×10^{11} UFC of *Saccharomyces cerevisiae*. NE_L: Estimated using the equation proposed by the NASEM [14] based on the chemical analysis of dietary ingredients.

2.3. Feed Samples Collections and Calculations

Samples of experimental diets were collected daily throughout the study. Refusals from each cow were also collected and weighed daily to determine DMI. Subsequently, weekly composite samples of the offered diets and individual refusals were prepared proportional to the daily amounts collected. These composite samples were stored and later analyzed for nutrient composition. Energy balance (EB) was calculated based on DMI, where the energy concentrations of the diets and refusals were determined according to NASEM [14]. Energy requirement for maintenance was calculated according to NASEM [14] using the metabolic BW ($0.10 \times BW^{0.75}$). Energy secreted as milk was calculated according to fat, protein, and lactose yields [14] as follows: milk yield × [(0.0929 × fat %) + (0.0563 × protein %) + (0.0395 × lactose %)]. Finally, the daily EB was calculated as follows [14]:

$$EB = \text{energy intake} - \text{energy for maintenance} - \text{energy secreted as milk}.$$

2.4. Body Weight and Body Condition Score

Body weight (BW) and body condition score (BCS) were evaluated at the beginning and end of the 7-day period for each phase, in the afternoon without prior fasting, and cows had ad libitum access to feed and water throughout the experimental period. The BCS was assessed by the same trained observer using a 1-to-5 scale [16] with 0.25-unit increments, as described in the Elanco BCS chart [17]. The daily BW change (ΔBW, kg/d) was calculated as the difference between the final and initial BW of each phase, divided by the number of days.

2.5. Milk Yield and Composition

The cows were milked twice daily at 0700 and 1500 h, and the milk yield was automatically recorded using the Delpro Manager System software (Delpro, DeLaval, Tumba, Sweden), which was equipped with MM23 electronic milk meters, MPC 580/680 control

units, and automatic take-offs modified for milk sampling. During the heat-stress challenge, cows were temporarily transferred from the climatic chamber to an external parlor for milking and promptly returned to their respective indoor pens immediately thereafter.

Individual milk samples were collected from two sequential milkings (morning and afternoon) on days 6 and 7 of each phase. The samples were stored at 4 °C with a preservative (2-bromo-2-nitro-1,3-propanediol; bronopol) and analyzed within 24 h for fat, protein, lactose, milk urea nitrogen (MUN) and somatic cell count (SCC) using a Bentley Fourier-Transform Infrared Spectrometer System (Bentley FTS, Bentley Instruments, Chaska, MN, USA). To satisfy the assumption of normality, the SCC data were log10-transformed prior to analysis.

Furthermore, milk yield and composition data were used to calculate the fat-corrected milk (FCM), energy-corrected milk (ECM), and net energy (NE) as follows: FCM = 0.4324 × milk yield + (16.218 × fat yield); ECM = [(0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield)]; and NE = (0.0929 × milk fat) + (0.0563 × milk protein) + (0.0395 × milk lactose) [14].

2.6. Vaginal Temperature, Respiration Rate and Infrared Thermography

Vaginal temperature (VT) was continuously monitored throughout the study using automated data loggers (model DS1921H2kb; Thermodata Corporation, Whitewater, WI, USA) inserted into the vagina and attached to a blank intravaginal CIDR device. The VT data were exported to an Excel spreadsheet (Microsoft office Professional Plus 2016) using Thermodata Viewer software (v.3.2.0; Thermodata Corporation, Whitewater, WI, USA). The VT recordings were retrieved every 2 h daily over the 7-day period for each phase. These values were then averaged across the 7 days of each evaluation period to perform the statistical comparisons.

Respiration rate (RR; breaths/min) was measured by visual observation of thoracoabdominal movements for 15 s, recorded by the same trained observer at 0600, 1000, 1400 and 1800 h. The 15 s counts were subsequently multiplied by four to extrapolate the values to a per-minute basis. Additionally, infrared thermography images of several body regions (eye, snout, left scapula, flank, rump, and vulva surface) were captured using a thermal imaging camera (FLIR T420; FLIR Systems Inc., Wilsonville, OR, USA).

The images were captured at 0600, 1000, 1400 and 1800 h. The camera was positioned perpendicular to the animals at a standardized distance of approximately 1.5 m. To obtain specific thermal images, the camera was oriented toward the ocular area and snout (frontal view), the left scapula, the left flank (paralumbar fossa), the rump, and the vulva surface. Settings were calibrated using a reflected temperature of 20 °C and an emissivity value of 0.98, as recommended for biological tissues [18,19]. All thermal images were subsequently processed using FLIR Tools software (v.5.6; FLIR Systems Inc., Wilsonville, OR, USA).

2.7. Blood Sample Collection and Processing

Blood samples were collected 3 h after the morning feeding at day 7 of each phase via puncture of the coccygeal blood vessels. Samples were drawn into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) with no anticoagulant for serum separation, or containing K_3-EDTA, sodium heparin, or lithium heparin for plasma separation. Following collection, tubes were centrifuged at 2500× *g* for 15 min (LS-3 Plus, CELM, São Paulo, SP, Brazil) for subsequent analyses of glucose, insulin, non-esterified fatty acids (NEFAs), and β-hydroxybutyrate (BHBA). Aliquots of 2.5 mL were then stored at −20 °C until further analysis.

Plasma glucose concentrations were determined via enzymatic oxidation using commercial glucose oxidase kits (Glucose PAP Liquiform, Ref.: 84, Labtest, Lagoa Santa, MG, Brazil) and a microplate spectrophotometer managed by Gen5 interphase software (Eon

Biotek, Winooski, VT, USA). Non-esterified fatty acids and BHBA were analyzed using commercial colorimetric enzymatic assays (Randox Laboratories Ltd., Kearneysville, WV, USA). Insulin concentrations were quantified using a bovine-specific ELISA kit (Bovine Insulin ELISA, Mercodia, Winston Salem, NC, USA).

Additionally, whole blood samples from the tubes containing lithium heparin were used without prior centrifugation for immediate determination of albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), total Ca (tCa), total P (tP), sodium (Na), potassium (K), creatinine (CREA), total protein (TP), and globulin (GLOB). These parameters were quantified using a Vetscan VS2 automated biochemical analyzer (Zoetis, Parsippany, NJ, USA).

2.8. Statistical Procedure

Prior to analysis, the assumptions of normality of residuals (Shapiro Wilk; $p \leq 0.05$) and homogeneity of variance (Likelihood Ratio Test; $p \leq 0.05$) were verified for all dependent responses following model fitting. Variables that violated these assumptions were transformed using the Box–Cox procedure [20] with PROC TRANSREG of SAS (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA). Data were subsequently analyzed using the MIXED procedure of the SAS 9.4 (SAS Institute Inc., Cary, NC, USA; 2013) with repeated measures over time (days or hours). The AR(1) and UN covariance structure was selected based on the smallest corrected Akaike's information criterion (AIC).

The experimental design accounted for the dependency of observations within each animal across three consecutive 7-day phases. The statistical model included the fixed effects of treatment (TN, HS, and REC), day (1 to 7), and their interaction. The effect of cow nested within blocks was included as a random effect to account for individual variation and the inherent correlation of repeated measurements within the same subject. Heterogeneous residual variances across treatments were modeled using the GROUP option in PROC MIXED. The Kenward–Roger approximation was applied to calculate the denominator degrees of freedom for the fixed effects. Least square means (LSM) were evaluated through the F and Tukey tests for LSM multiple comparisons. Statistical significance was declared at $p \leq 0.05$.

3. Results

3.1. Dry Matter Intake and Energy Balance

A treatment × day interaction was detected for DMI ($p < 0.01$), driven by the DMI of REC cows returning to baseline levels only by day 5 post-heat stress and was lower than that of TN cows at day 7 (Figure 2A). In contrast, caloric intake began to recover by the fourth day of HS exposure ($p < 0.01$; Figure 2B), although energy secreted as milk did not recover during the evaluated period (Figure 2C). Additionally, EB remained negative throughout the HS phase ($p < 0.01$; Figure 2D; Table 2).

Table 2. Dry matter intake, caloric intake, energy secreted as milk, and energy balance of Holstein cows exposed to thermoneutral, heat-stress, and recovery conditions.

	Treatments				*p*-Value		
Response	**TN**	**HS**	**REC**	**SEM**	**Treat**	**Day**	**T × D**
DMI (kg/d)	23.8 ^{a}	18.0 ^{c}	19.6 ^{b}	0.78	<0.01	0.16	<0.01
Caloric intake (Mcal/d)	37.4 ^{a}	29.1 ^{c}	31.8 ^{b}	0.77	<0.01	0.25	<0.01
Energy secreted as milk (Mcal/d)	20.4 ^{a}	16.6 ^{b}	16.1 ^{b}	0.58	<0.01	<0.01	<0.01
EB (Mcal/d)	4.10 ^{a}	−0.190 ^{b}	3.27 ^{a}	0.71	<0.01	0.53	<0.01

DMI = dry matter intake; EB = energy balance; TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean; *p*-value: Treat = fixed treatment effect; Day = fixed effect of day; T × D = treatment × day interaction. ^{a,b,c} Means with different letters differ.

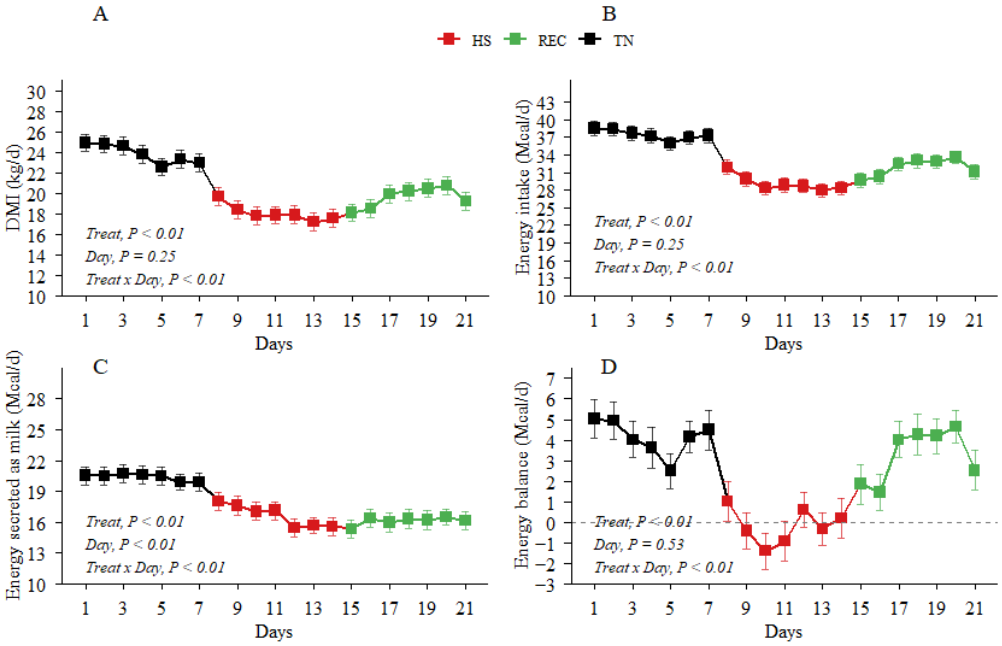


Figure 2. DMI (**A**), caloric intake (**B**), energy secreted as milk (**C**), and energy balance (**D**) of Holstein cows exposed to thermoneutral (TN), heat-stress (HS), and recovery (REC) conditions. Error bars denote SEM. The dashed line indicates zero reference for negative values.

3.2. Body Weight, Body Condition Score, and Body Weight Change

Body weight tended to be lower in HS and REC periods (p = 0.07), whereas BCS remained unaffected by treatments (p = 0.13). Although cows lost weight during both TN and HS, REC cows gained BW. Specifically, weight loss was greater in HS cows compared with TN cows (TN = −0.433 vs. HS = −1.53 vs. REC = 1.40 kg/d; p < 0.01) (Table 3).

Table 3. Body weight, body condition score, and BW change of Holstein cows in thermoneutral, heat-stress, and recovery conditions.

	Treatments				
Response	**TN**	**HS**	**REC**	**SEM**	***p*-Value**
BW (kg)	651.9	629.3	624.1	11.64	0.07
BCS (1–5 scale)	2.85	2.69	2.77	0.058	0.13
BW change (kg/d)	−0.433 ^{b}	−1.53 ^{c}	1.40 ^{a}	0.233	<0.01

BW = body weight; BCS = body condition score; TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean. ^{a,b,c} Means with different letters differ.

3.3. Milk Yield and Milk Composition

An interaction between treatment and day was observed for milk yield, FCM, and ECM (p < 0.01). Specifically, milk yield was significantly reduced from the third day of heat-stress exposure onward and began to recover by the second day of the recovery phase, although did not recover to baseline level before the heat-stress period (p < 0.01) (Table 4 and Figure 3A–C).

Table 4. Milk yield and composition of Holstein cows exposed to thermoneutral, heat-stress, and recovery conditions.

	Treatments						
	TN	**HS**	**REC**	**SEM**	***p*-Value**		
Response					**Treat**	**Day**	**T × D**
Milk yield (kg/d)	28.4 ^{a}	23.8 ^{b}	23.5 ^{b}	1.25	<0.01	<0.01	<0.01
3.5%FCM (kg/d)	29.7 ^{a}	24.9 ^{b}	23.9 ^{b}	1.34	<0.01	<0.01	<0.01
ECM (kg/d)	29.2 ^{a}	24.5 ^{b}	23.8 ^{b}	1.26	<0.01	<0.01	<0.01
Milk composition							
Milk fat (%)	3.80	3.78	3.61	0.087	0.22	-	-
Milk protein (%)	3.06	3.08	3.18	0.046	0.07	-	-
Milk lactose (%)	4.55 ^{a}	4.47 ^{ab}	4.39 ^{b}	0.030	<0.01	-	-
Milk fat (kg/d)	1.08 ^{a}	0.90 ^{b}	0.82 ^{b}	0.053	<0.01	-	-
Milk protein (kg/d)	0.86 ^{a}	0.73 ^{b}	0.73 ^{b}	0.027	<0.01	-	-
Milk lactose (kg/d)	1.29 ^{a}	1.07 ^{b}	1.00 ^{b}	0.047	<0.01	-	-
MUN (mg/dL)	13.8 ^{c}	19.0 ^{a}	16.6 ^{b}	0.91	<0.01	-	-
SCC	1.95	2.06	2.14	0.153	0.48	-	-
ECM/DMI	1.25 ^{b}	1.35 ^{a}	1.22 ^{b}	0.047	<0.01	0.46	0.34

FCM = fat-corrected milk; ECM = energy-corrected milk; MUN = milk urea nitrogen; SCC = somatic cell count; TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean; *p*-value: Treat = fixed treatment effect; Day = fixed effect of day; T × D = treatment × day interaction. ^{a,b,c} Means with different letters differ.

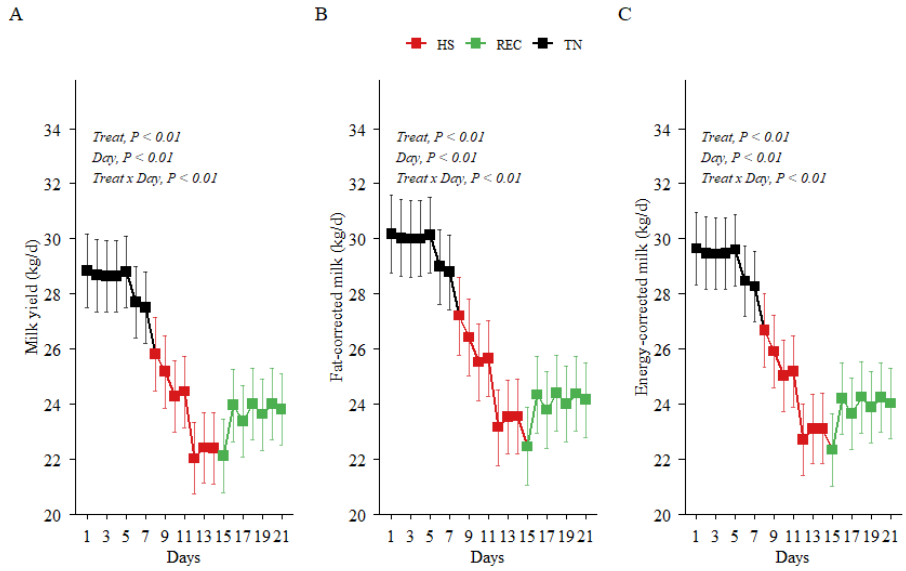


Figure 3. Milk yield (**A**), 3.5%FCM (**B**), and ECM (**C**) of Holstein dairy exposed to thermoneutral (TN), heat-stress (HS), and recovery (REC) conditions. Error bars denote SEM.

Heat stress did not influence milk fat ($p = 0.22$), SCC ($p = 0.48$) or milk protein content ($p > 0.05$) compared to TN and HS cows. Conversely, milk lactose in REC cows decreased by 3.51% compared to TN cows ($p < 0.01$). Additionally, HS cows showed a 37.6 and 14.4% increase in MUN compared to TN and REC cows, respectively ($p < 0.01$). Finally, feed efficiency was greater for HS cows compared to the other treatments ($p < 0.01$) (Table 4).

3.4. Vaginal Temperature and Respiration Rate

Heat-stressed cows exhibited elevated VT for an additional 6 h compared with TN and REC cows ($p < 0.01$; Figure 4A; Table 5). Peak VT occurred at 1400 h, with HS cows reaching the highest value (TN = 39.4 vs. HS = 40.5 vs. REC = 39.5 °C). Additionally, cows exposed

to HS displayed greater RR at 1000 h (+20.4 breaths/min) and 1400 h (+30 breaths/min) compared to TN and REC cows ($p < 0.01$; Table 5; Figure 4B).

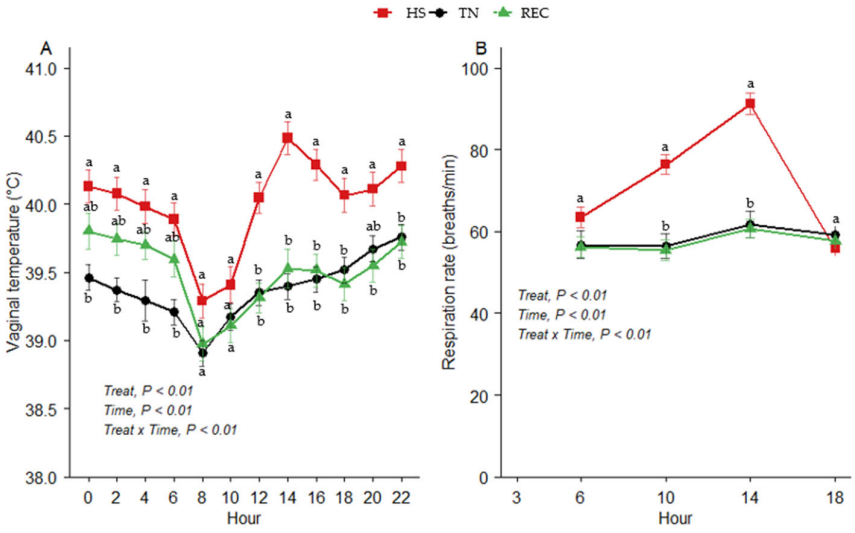


Figure 4. Vaginal temperature (**A**) and respiration rate (**B**) of Holstein cows in thermoneutral (TN), heat-stress (HS), and recovery (REC) conditions. Error bars denote SEM. [a,b] Means with different letters within each hour differ.

Table 5. Vaginal temperature and respiration rate of Holstein cows in thermoneutral, heat-stress, and recovery conditions.

	Treatments				*p*-Value		
Response	**TN**	**HS**	**REC**	**SEM**	**Treat**	**Hour**	**Treat × Hour**
VT (°C)	39.4 [b]	40.0 [a]	39.5 [b]	0.09	<0.01	<0.01	<0.01
RR (breaths/min)	58.5 [b]	71.7 [a]	57.5 [b]	2.02	<0.01	<0.01	<0.01

VT = vaginal temperature; RR = respiration rate; TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean; *p*-value: Treat = fixed treatment effect; Hour = fixed effect of hour; Treat × Hour = treatment × hour interaction. [a,b] Means with different letters differ.

3.5. Thermography Analysis

A treatment × hour interaction was observed for all infrared thermography responses ($p < 0.01$; Table 6).

Table 6. Infrared thermography of Holstein cows exposed to thermoneutral, heat-stress, and recovery conditions.

	Treatments				*p*-Value		
Response (°C)	**TN**	**HS**	**REC**	**SEM**	**Treat**	**Hour**	**T × H**
Eye	36.9 [b]	37.9 [a]	37.3 [b]	0.12	<0.01	<0.01	<0.01
Snout	35.2 [b]	36.1 [a]	35.1 [b]	0.16	<0.01	<0.01	<0.01
Scapula	35.3 [b]	36.7 [a]	35.4 [b]	0.10	<0.01	<0.01	<0.01
Flank	35.4 [b]	36.6 [a]	35.3 [b]	0.14	<0.01	<0.01	<0.01
Rump	35.4 [b]	36.6 [a]	35.4 [b]	0.14	<0.01	<0.01	<0.01
Vulva	36.9 [b]	38.0 [a]	37.1 [b]	0.14	<0.01	<0.01	<0.01

TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean; *p*-value: Treat = fixed treatment effect; Hour = fixed effect of hour; Treat × Hour = treatment × hour interaction. [a,b] Means with different letters differ.

Specifically, HS cows had greater surface temperatures at 1000 and 1400 h compared to TN and REC cows. However, temperatures were similar among all treatments at 0600 and 1800 h for most body regions (Figure 5A–E), with the exception of the vulva; vulva surface temperature in HS cows remained higher than in TN cows at 1800 h ($p < 0.01$; Figure 5F).

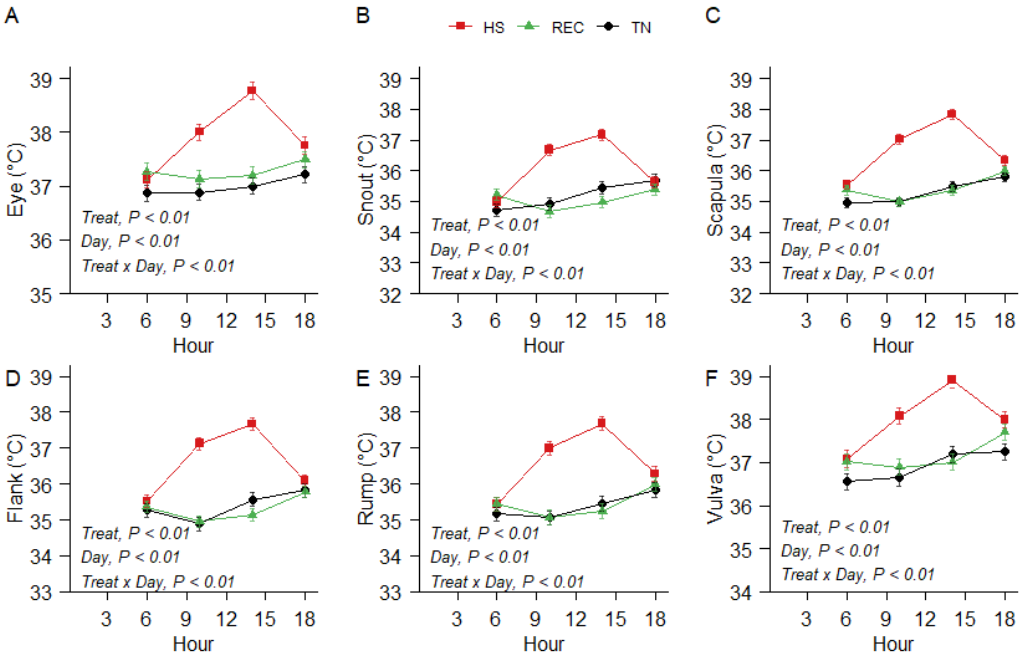


Figure 5. Eye (**A**), snout (**B**), scapula (**C**), flank (**D**), rump (**E**), and vulva (**F**) of Holstein cows exposed to thermoneutral (TN), heat-stress (HS), and recovery (REC) conditions. Error bars denote SEM.

3.6. Blood Metabolites and Mineral Concentration

Recovery (REC) cows had a greater insulin concentration compared to TN cows, but concentrations were like those of HS cows ($p = 0.02$). In contrast, glucose, NEFA, and BHBA were not influenced by treatments ($p > 0.05$). Plasma albumin (ALB) concentrations tended to be greater during the TN phase ($p = 0.06$), whereas HS cows tended to exhibit higher creatinine concentrations ($p = 0.08$). Total protein (TP), GLOB, and K were unaffected by treatments ($p > 0.10$). Blood ALP, P, and Na concentrations decreased in both HS and REC cows compared to TN cows ($p < 0.01$). Interestingly, REC cows had lower BUN compared to both TN and HS cows ($p = 0.01$), while Ca concentrations were not influenced by treatments ($p > 0.05$, Table 7).

Table 7. Blood metabolites and blood mineral concentration of Holstein cows in thermoneutral, heat-stress, and recovery conditions.

	Treatments				
Response	**TN**	**HS**	**REC**	**SEM**	***p*-Value**
Glucose (mmol/L)	3.52	3.46	3.38	0.084	0.65
Insulin (µg/L)	0.159 ^{b}	0.195 ^{ab}	0.223 ^{a}	0.0171	0.02
NEFA (mmol/L)	0.076	0.071	0.077	0.0055	0.56
BHBA (mmol/L)	0.752	0.776	0.715	0.0594	0.49
ALB (g/dL)	2.69	2.61	2.57	0.056	0.06
ALP (U/L)	51.4 ^{a}	38.4 ^{b}	42.5 ^{b}	2.74	<0.01
ALT (U/L)	22.7 ^{a}	20.2 ^{b}	20.5 ^{ab}	0.75	0.05
BUN (mg/dL)	17.1 ^{a}	17.7 ^{a}	15.4 ^{b}	0.84	0.01
Ca (mg/dL)	9.42	9.55	9.64	0.08	0.07
P (mg/dL)	7.10 ^{a}	6.32 ^{b}	6.60 ^{b}	0.167	<0.01

Table 7. *Cont.*

	Treatments				
Response	**TN**	**HS**	**REC**	**SEM**	***p*-Value**
Na (mg/dL)	144.7 [a]	142.2 [b]	142.4 [b]	1.53	<0.01
K (mg/dL)	4.46	4.38	4.35	0.072	0.41
CREA (mg/dL)	0.793	0.905	0.792	0.073	0.08
TP (mg/dL)	8.39	8.48	8.31	0.171	0.34
GLOB (g/dL)	5.70	5.87	5.66	0.147	0.30

NEFA = non-esterified fatty acids; BHBA = β-hydroxybutyrate; ALB = albumin; ALP = alkaline phosphatase; ALT = aminotransferase; BUN = blood urea nitrogen; Ca = calcium; P = phosphorus; Na = sodium; K = potassium; CREA = creatinine; TP = total protein; GLOB = globulin; TN = cows in thermoneutral conditions seven days before heat stress; HS = cows exposed to heat stress for seven days; REC = recovering cows seven days after heat stress; SEM = standard error of the mean. [a,b] Means with different letters differ.

4. Discussion

Interestingly, REC cows maintained a similar VT to that of HS cows from 0 h to 1000 h. This indicates that, despite returning to thermoneutral conditions, heat dissipation mechanisms, such as increased respiration rate, were insufficient to reduce core body temperature. Conversely, Cowley et al. [12] observed lower rectal temperatures in Holstein cows following a 7-day exposure to heat stress. On the other hand, it may reflect a lag in circadian rhythm resetting that potentially impairs the return to normal core body temperature after the heat-stress challenge, as observed by [21].

The physiological VT typically ranges from 37.0 to 39.0 °C in Holstein dairy cows [22]. In the present study, HS cows remained above this threshold for a longer duration and exhibited a greater magnitude of elevation compared to TN and REC cows. This reinforces the finding that heat dissipation pathways were ineffective and that this deficit persisted even after heat-stress exposure, likely due to an impaired thermoregulatory capacity [23]. In contrast, Garner et al. [24] reported that the VT of dairy cows exposed to heat stress did not differ from those under thermoneutral conditions 14 days after a 4-day heat challenge.

Dry matter intake declined by 24.4% (5.8 kg/d) under HS and remained 17.6% (4.2 kg/d) lower during the REC phase compared with pre-heat-stressed cows. In comparison, a recent meta-analysis reported an average DMI reduction of 19.3% for cows under heat stress relative to those in thermal comfort [25]. The more pronounced intake depression observed in our study is likely attributed to the lower THI threshold (i.e., 76.6 ± 2.92 for 9 ± 4.4 h) utilized by Chen et al. [25] to define heat-stress conditions (studies in climate chambers only).

Alongside acute physiological alterations, DMI is one of the primary parameters of the decline when dairy cows are exposed to heat stress. This reduction is driven by the over-activation of the hypothalamic–pituitary–adrenal (HPA) axis [26], which suppresses the appetite center in the hypothalamus [27] to minimize metabolic heat production from the heat increment of feeding. Furthermore, rumen motility is reduced by approximately 20% in heat-stressed cows [27], further contributing to the depression in feed intake.

More importantly, DMI did not fully return to pre-heat-stress baseline values, even after cows returned to thermoneutral conditions. This demonstrates a distinct residual effect of thermal stress on underlying physiological mechanisms. Although DMI recovery progressively across days, it remained incomplete. In contrast to our findings, Cowley et al. [12] reported no differences in DMI among treatments during a 7-day post-heat-stress (~78 THI) in multiparous Holstein-Friesian cows.

The observed declines in milk yield of 4.6 and 4.9 kg/d for HS and REC cows, respectively–were primarily driven by the drastic reduction in DMI. This reduction was

less pronounced than that reported by Onan-Martinez et al. [28], who observed an 8 kg/d decrease in milk yield in high-producing cows subjected to heat stress (73.5 THI). Beyond the direct effects of depressed nutrient intake, these effects of heat stress can possibly be explained by compromised mammary tissue integrity, causing cellular damage, decreasing mammary epithelial cell proliferation, and downregulating the expression of genes associated with milk protein synthesis, as demonstrated by Sammad et al. [29].

Furthermore, FCM and ECM were reduced in the HS treatment and did not fully recover during the REC period. This response was primarily associated with the lower milk protein and lactose concentrations, whereas milk fat concentration was not affected by the treatments. As discussed previously, heat stress impairs mammary synthesis of milk components through metabolic and physiological alterations, including reduced amino acid availability associated with oxidative stress and changes in mammary nutrient partitioning. Although milk fat concentration remained unchanged in the present study, the reduction in milk yield contributed to the lower energy-corrected and fat-corrected milk production [30].

Although a trend toward higher milk protein content was observed in the recovery period, this outcome does not seem to be a direct physiological consequence of the thermal challenge itself, as no significant differences in protein concentrations were detected between the thermoneutral and heat-stress phases. These findings contrast with recent literature reporting decreased milk protein concentrations in cows exposed to heat stress [25,28], whereas another study found no such reduction [30].

Although cows exposed to HS exhibited greater feed efficiency than those in the TN and REC treatments, this finding should not be interpreted as a true improvement in productive efficiency. The apparent increase in efficiency primarily resulted from the marked reduction in dry matter intake relative to the decrease in ECM during heat stress, rather than from enhanced nutrient utilization. In this context, when heat stress induces only modest changes in milk composition, efficiency indices based on milk yield or even ECM may overestimate productive efficiency because they do not fully account for the metabolic constraints imposed by thermal stress. This interpretation is consistent with the meta-analysis of Chen et al. [25], which demonstrated that feed efficiency estimates based on milk yield should be interpreted with caution under heat-stress conditions.

Milk lactose concentration was lower in REC cows than in TN cows. This depression is likely related to heat stress compromising mammary epithelial cell integrity, as previously discussed. Mechanistically, lactose acts as the primary osmotic regulator of milk volume, and the lower DMI observed in REC cows may have restricted glucose availability to the mammary gland for lactose synthesis. Furthermore, these findings suggest that the cows were unable to fully restore mammary epithelial cell proliferation post-heat stress. In contrast, milk lactose content was unaffected during thermal stress or throughout the 7-day post-challenge period in Australian Holstein dairy cows [12].

Consistent with the potential damage to mammary epithelial cells, MUN concentrations were markedly increased in HS cows and remained elevated throughout the 7-day post-heat-stress period. This response suggests a dysregulation of protein metabolism, possibly driven by enhanced mobilization and utilization of endogenous amino acids [31]. Similarly, Ellett et al. [30] reported increased MUN in heat-stressed Holstein cows; however, this elevation was restricted to days 3 and 4 post-heat exposure under a cyclical THI regimen (THI = 74 from 1800 to 0600 h, and 80 from 0600 to 1800 h).

In the present study, this mechanism is supported by the more pronounced negative BW change observed in HS cows. Although absolute BW and BCS did not differ among treatments, and BUN concentrations were similar between TN and HS cows, the rate of BW loss was altered. The negative changes in body weight observed in the TN and HS were likely attributable to the weighing procedure rather than the experimental treatments, given

that the animals had unrestricted access to feed and water throughout the experimental period and were weighed without prior fasting, which possibly influenced gastrointestinal tract fill.

In comparison, Guo et al. [32] reported 26.4% greater plasma amino acid concentrations in heat-stressed dairy cows exposed to a THI of 78.1 during summer. In our study, REC cows exhibited a decrease in BUN concentrations, indicating that the partial recovery of DMI was sufficient to attenuate endogenous protein mobilization, a shift further supported by their transition to a positive energy balance.

Blood glucose concentrations were unaffected by heat stress in the current study, contrasting with previous findings [30,32] that reported lower circulating glucose in heat-stressed cows. On the other hand, ALP decreased in both HS and REC cows, suggesting potential liver impairment in response to thermal stress and a lingering residual effect on this enzyme's kinetics. In contrast to those results, Blond et al. [33] observed no alterations in ALP in heat-stressed cows. Furthermore, the lower serum ALT activity observed in the HS group suggests heat-stress-induced alterations in hepatic metabolism. Similarly, Vasconcelos et al. [34] reported lower serum ALT activity in Sindi dairy cows during the summer than during the winter.

Blood phosphorus decreased in both HS and REC cows, potentially driven by the concurrent increase in insulin concentration observed in the current study, which is known to promote an intracellular shift of phosphorus [35]. The elevated circulating insulin in REC cows may be linked to the energy deficit experienced during heat-stress exposure and subsequently caused alterations in insulin signaling, which directly modulate the mobilization of tissue reserves [36].

Furthermore, the higher insulin in REC cows possibly reflects enhanced peripheral tissue sensitivity, which may have favored insulin-dependent glucose uptake by these tissues, thereby reducing glucose availability to the mammary gland, although this hypothesis can only be confirmed in futures studies taking dynamic endocrine adaptations into consideration.

This shift in nutrient partitioning likely accounts for lower milk lactose and reduced milk yield observed in HS and REC cows. Supporting this hypothesis, Zeng et al. [37] reported that high-producing dairy cows (41.4 ± 0.47 kg/d) under severe heat stress ($79 < \text{THI} \leq 88$) had lower mammary glucose supply, blood flow, and glucose uptake compared to cows under thermoneutral or moderate heat-stress conditions ($68 \leq \text{THI} \leq 79$). Concurrently, the hyperinsulinemia observed in the current study suggests a suppression of the adipose tissue mobilization, partially explaining why heat stress failed to alter plasma NEFA and BHBA concentrations.

Infrared thermography successfully detected heat load in HS cows, confirming its efficacy as a valuable, non-invasive tool for identifying heat stress in Holstein dairy cows. This aligns with the findings of Blond et al. [33], who reported that infrared thermography effectively monitors thermal status regardless of the region evaluated in Holstein multiparous cows under a THI > 80. Conversely, no residual effect on body surface temperature was observed post-exposure, indicating that surface temperatures promptly returned to baseline once the heat-stress episode ceased in the current study.

The results of the present study partially confirm our hypothesis that heat stress exerts a residual effect on DMI, milk yield, FCM, ECM, energy secreted as milk, vaginal temperature, insulin, ALP, and BUN that persists for at least seven days post-exposure.

Notably, this is the first study to directly evaluate the legacy effects of heat stress in mid-lactation Holstein dairy cows. Although our study makes a valuable contribution to the topic, the study design without a time-matched control treatment under thermoneutral comfort represents a limitation when interpreting variables with slower dynamic responses

as a potential temporal drift, such as the natural progression of the lactation curve, which cannot be completely ruled out for DMI and milk yield. Therefore, the residual effects of heat stress found here should be interpreted with caution.

5. Conclusions

In conclusion, under the conditions of the present study, heat stress impaired productive performance, altered metabolism, and changes in the physiological status of mid-lactation Holstein dairy cows, with some effects persisting after the cows returned to thermoneutral conditions. Infrared thermography successfully identified the acute thermal load but showed no residual effect. Future studies should focus efforts on evaluating which insulin signaling pathways are impaired, and how alterations in insulin sensitivity occur in cows experiencing the residual effects of heat stress.

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Abbreviations

The following abbreviations are used in this manuscript:

ADF	Acid detergent fiber
AIC	Akaike's information criterion
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
BCS	Body condition score
BHBA	β-hydroxybutyrate
BUN	Blood urea nitrogen
BW	Body weight
CP	Crude protein
CREA	Creatinine
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ECM	Energy-corrected milk
EE	Ether extract
FCM	Fat corrected milk
GLOB	Globulin
HPA	Hypothalamic–pituitary–adrenal
HS	Heat stress
K	Potassium
LSM	Least square means
MUN	Milk urea nitrogen
Na	Sodium
NDF	Neutral detergent fiber
NE	Net energy
NEFA	Non-esterified fatty acids
NFC	Non-fibrous carbohydrates
OM	Organic matter
RH	relative humidity
RR	Respiration rate
SCC	somatic cell count
tCa	Total Ca
THI	Temperature–humidity index
tP	Total P
TP	Total protein
TMR	Total mixed ration
TN	Total nitrogen
VT	Vaginal temperature

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