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Heat stress affects milk yield, milk quality, and gene expression profiles in mammary cells of Girolando cows

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ABSTRACT

Heat stress during lactation affects the physiological responses, hormonal release, health, and productivity of dairy cows. However, the adverse effects of heat stress on milk synthesis, immune response, and cellular apoptosis in mammary cells remains unknown in *Bos indicus* cows. This study aimed to understand the relationship between milk yield, milk quality, and the expression of genes related to milk synthesis, cell apoptosis, and immune response in mammary cells of Girolando cows. A total of 24 Girolando cows (3/4 Holstein and 1/4 Gir) were subjected to control (CT; with a temperature-humidity index ranging from 60 to 74, n = 12) or heat stress treatments (HS; with a temperature-humidity index ranging from 60 to 85, n = 12), from 111 to 120 d of lactation. Heat stress significantly increased the expression of heat shock proteins (*HSPD1* and *HSPD90AA1*), insulin receptors (*INSR*), and prolactin receptor (*PRLRsf*) genes, and decreased the expression of glucocorticoid receptor (*NR3C1*) gene in mammary cells of the HS cows when compared with the CT cows. The HS cows exhibited significantly higher vaginal temperatures and cortisol release compared with the CT cows. Moreover, the HS cows had significantly lower DMI and milk yield than CT cows. Although, HS cows showed higher percentage of lymphocytes in milk when compared with that from CT cows. We found no effect of heat stress on other leukocyte counts, somatic cell counts, bacterial counts in milk, or milk composition. Finally, this study demonstrated that Girolando cows are susceptible to heat stress, which decreases milk yield and affects the expression of genes linked to milk synthesis in the mammary cells.

Key words: Girolando, heat stress, milk yield, mammary cells, gene expression

INTRODUCTION

The effects of climate change due to global warming, which pose a serious threat to sustainable development, human food security, and animal welfare, have been extensively discussed. Crossbreeding between Holstein and Gir cattle has been used as an effective strategy to enhance milk yield in pasture systems under tropical conditions (Franzoni et al., 2018; Henry et al., 2018; Sejian et al., 2018). The adaptability of Zebu cattle to high temperatures and humidity primarily stems from their lower basal metabolic rate and improved heat dissipation efficiency attained through increased peripheral vasodilation, sweating rate, and respiratory frequency (Aleena et al., 2016; Taye et al., 2017; de Vasconcelos et al., 2020). Presently, 80% of the Brazilian dairy herd comprises Holstein and Gir crosses, known as the Girolando breed, a composite breed product of selective crossbreeding (Otto et al., 2020; Silva et al., 2022). However, even the typically thermotolerant Zebu cattle can trigger behavioral and physiological changes brought on by heat stress (Nardone et al., 2010; dos Santos et al., 2021).

In general, dairy cows' resilience under heat stress is inversely related to metabolic heat production, rectal temperatures, respiratory frequency, DMI, and milk yield (Aleena et al., 2016; Sejian et al., 2018; Campos et al., 2022). Furthermore, a previous study showed a negative correlation between milk production and thermotolerance in Gir cows in subtropical conditions (Santana Jr. et al., 2015). This inverse relationship between milk yield and heat stress responses indicates that strategies aimed at increasing milk yield could undermine the thermoregulatory capacity of Zebu cows. The negative impact of heat stress on physiological characteristics, feed behavior, milk production, and health has been studied among Holstein cows (Collier et al., 2017; Corazzin et al., 2021; Kappes et al., 2022). Several studies have shown that heat stress less-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

ens both DMI and rumination rate, indirectly leading to reduced milk yield (Collier et al., 2017; Corazzin et al., 2021; Meneses et al., 2021). Other studies have proposed that heat stress alters key hormonal releases needed for milk production (Baumgard and Rhoads Jr., 2013; Ponchon et al., 2017; Hooper et al., 2020), and affects the gene expression of cortisol (*GR*), prolactin (*PRLR*), insulin-like growth factor 1 (*IGF1R)*, and insulin (*INSR*) receptors in the mammary tissue, suggesting its direct influence on mammary cells (Hooper et al., 2021; Ouellet et al., 2021). In particular, the negative effect of heat stress on milk yield has been related to a temperature-humidity index (**THI**) above 72. Notably, a 2.1% reduction in milk yield has been estimated for every 1-point increase in THI (Pramod, 2021). Considering that the THI frequently surpasses 72 during many months of the year in tropical and subtropical regions and the fact that milk yield in Girolando cows has significantly improved over the past 20 years (Silva et al., 2022), these cows may encounter increased challenges in maintaining homeothermy due to the consistent rise in their milk yield (Zimbelman et al., 2009; Cardoso et al., 2015; Santana Jr. et al., 2015).

Studies have suggested that heat stress during the summer increases SCC and bacterial count in milk, as well as increasing clinical mastitis incidence in dairy cows (Zeinhom et al., 2016; Nasr and El-Tarabany, 2017). Thus, metabolic and respiratory diseases increased in summer compared with winter (Thompson and Dahl, 2012; Dahl and McFadden, 2022), suggesting that heat stress reduces immune system efficiency (Park et al., 2021). Holstein cows, known for their susceptibility to higher air temperatures and humidity, showed these immunological variations (Berian et al., 2019; Otto et al., 2020). Indeed, an in vitro study at 41°C demonstrated that this temperature suppressed the phagocytic capacity of neutrophils and boosted monocyte apoptosis in bovine polymorphonuclear cells (Catozzi et al., 2020). However, few studies have addressed heat stress impact on SCC, milk synthesis, or immunological responses in Girolando cows. In this context, this study addresses the hypothesis that heat stress could impair the milk synthesis in the mammary gland, increase SCC in milk, and decrease the milk yield of Girolando cows. Consequently, this study aims to investigated the effect of heat stress on milk yield, milk quality, hormone release, and gene expression related to milk synthesis, apoptosis, and immune response in Girolando cows.

MATERIALS AND METHODS

The experiment was done at the Embrapa Dairy Cattle Multi-User Laboratory for Livestock Bioefficiency and Sustainability (Coronel Pacheco, Brazil). In this region, the climate is humid subtropical, with an average annual air temperature of 20°C and relative humidity of 76%. The rainy season extends from October to March, with annual rainfall between 1,500 and 2,000 mm.

The Animal Ethics Committee of Embrapa Dairy Cattle, in compliance with Brazilian federal law, approved all experimental procedures and activities (protocol code 5557190520).

Animals, Diets, and Milking Routine

The experimental procedures involved 24 Girolando cows (3/4 Holstein and 1/4 Gir) that were randomly assigned in a block design to ensure homogeneous distribution within the thermoneutral control (**CT**) and heat stress (**HS**) treatments. The HS cows weighed 543 ± 74 kg, were 116 ± 33 d into lactation, and had a daily milk yield of 19.2 ± 4.8 kg. The CT cows weighed 563 ± 55 kg, were 110 ± 32 d into lactation, and had a daily milk yield of 19.7 ± 4.5 kg.

All cows were fed the same TMR twice daily at 0900 and 1600 h. This diet consisted of 85% corn silage and 15% concentrate, which included 42% soybean meal, 52.5% corn bran, 4% mineral blend, and 1.5% urea. The formulation of the diet was based on the NRC requirements (NRC, 2001), taking into account the cow's weight, BCS, lactation phase, and milk yield. The total diet provided was calculated to leave 10% refusals. The DMI (kg/d) was monitored by an individual feed intake system (Chizzotti, et al., 2015). The ear tag of the cows was recorded at each visit to automatic electronic feeders (frequency, initial and final times on feeder), and individual feed intake was recorded by the difference between feeding weight at the start and end of each 24-h period.

Cows were milked daily at 0600 and 1600 h. The parlor was equipped with fans, which were activated during warm days. The milking machine maintained a vacuum level of 44 kPa and a pulse rate of 60 cycles/min. The same milker followed a specific routine each time. Before milking, the cows' teats were pre-dipped and dried. Teat cups were attached at time 0 and detached automatically when milking was finished. After milking, the cows' teats were post-dipped, the individual milk yield was recorded, and the cows left the milking parlor.

Experimental Organization

The current study, drawing on data from the Brazilian National Meteorology Institute (**INMET**; Brasilia, DF, Brazil), was conducted during the cool season in August to September. The experiment was carried out in a

freestall barn with a cooling system (equipped with fans and misters, using 1 min of sprinkling with large water droplets and 4 min of blowing). This system allows active cooling of the cows and was considered the control treatment (CT treatment). Additionally, a climatic chamber contiguous to the freestall barn was used to induce heat stress in other cows (HS treatment).

In the current study, we subjected 12 Girolando cows to a heat stress condition in a climate-controlled room (HS treatment, n = 12), and another 12 cows experienced a cooling system in a freestall barn (CT treatment, n = 12). The study spanned 12 consecutive days, comprising 3 adaptation days and 9 d of CT and HS treatment imposition, coinciding with d 111 to 120 of lactation. Throughout the experiment, the CT cows remained in the freestall barn, whereas the HS cows underwent a heat stress challenge in the climate-controlled room from 0700 to 1500 h. Outside of these hours, the HS and CT groups were both housed in the freestall barn and milked together at 0600 and 1600 h.

Climate Data

Air temperature and humidity were recorded in both the climatic room (HS environment) and the freestall barn (CT environment) using digital thermometers and hygrometers (Unity Instruments, São Paulo, Brazil). The climatic room was regulated to maintain an air temperature of 35°C and a relative air humidity of 60%. In contrast, the freestall barn exhibited an air temperature range of 15.4 to 34.4°C and a relative humidity variance between 23% and 92%. The cooling system, including fans and misters in the freestall barn, was programmed to activate immediately once the air temperature reached 25°C. According to the INMET, in the last decade, the average maximum temperature during August and September was 25°C in the region of the study (THI from 67 to 72 at 1500 h). This is considered a cool temperature for this region, and this threshold was chosen to activate the cooling system.

To characterize the climatic conditions in both HS and CT environments, the THI was calculated using the NRC formula (NRC, 1971): $THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$, where T represents air temperature in degrees Celsius and RH is the relative humidity percentage. During experimental trials (from 0700 to 1500 h), the THI was 84 ± 1 in the climatic room and 71 ± 4 in the cooled freestall barn. According to previous studies on Zebu crosses (Berian et al., 2019), the THI values obtained in HS and CT conditions were categorized as thermal comfort (THI <72), moderate stress (THI between 72 and 78) and severe stress (THI between 79 and 91), respectively. The 24-h THI profile in the CT and HS environments is presented in Figure 1.

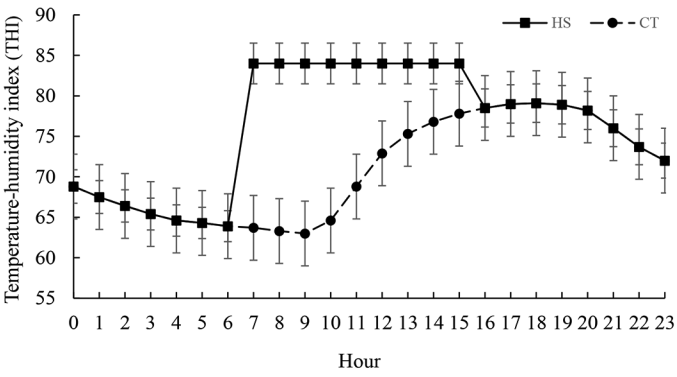

Figure 1. Temperature-humidity index (THI; ± SEM) on heat stress (HS) and control (CT) environments throughout 24 h. The THI was calculated as proposed by NRC (1971). The THI corresponds to the mean measured during the experimental period (111–120 d of lactation).

Respiratory Rate, Vaginal Temperature, and Behavior Measurements

We measured the respiratory rate (**RR**) and vaginal temperature (**VagT**) of Girolando cows daily as physiological markers of treatment effects during the experiment. We recorded RR twice daily at 0900 and 1400 h by tallying flank movements for 1 min. Using an intravaginal device and data loggers (iButton, Whitewater, WI), we recorded each cow's VagT every hour throughout the experiment. The daily DMI (kg/d) of Girolando cows was monitored throughout the entire experimental period.

A trained observer assessed the cows' temperament in the milking parlor during the morning milkings on d 2, 4, 6, and 8 of the experiment. The behaviors noted included the number of steps taken, kicks, and instances of urination, defecation, and rumination, based on prior studies with Girolando cows (Marçal-Pedroza et al., 2020; Marçal-Pedroza et al., 2021). Records were kept of steps and kicks performed by the hind legs during 2 phases: udder preparation (beginning with the milker's first contact with the cow's teats) and the attachment of the milk unit (from the initial to the final teat cup attachment). Behaviors such as urination, defecation, and rumination were tracked from the start of udder preparation up to the removal of the milk unit. These actions were marked as "yes" if they occurred at least once or "no" if they were not observed during milking.

Blood Sampling and Hormonal Analysis

Physiological measurements were taken at 0900 h, followed by collection of blood samples from the coccygeal vein on d 0, 1, 3, 5, and 7 of the treatments. These samples were placed in heparinized tubes and then centrifuged at 1,500 × *g* for 15 min at 4°C. The plasma was analyzed for cortisol (**CORT**), thyroxine (**T4**), triiodothyronine (**T3**),

IGF-1, and insulin (**INS**) using enzyme immunoassay kits (Monobind, Lake Forest, CA). The intra- and inter-assay CV for were less than 3.6% and 8.0%, respectively, for all hormones analyzed.

Milk Yield, Milk Sampling, and Analysis

Milk yields from morning and afternoon milking sessions were recorded individually throughout lactation. Milk samples were collected from each cow during the morning milking on d 0, 1, 3, 5, and 7 of the experimental treatment (from 111 to 120 d of lactation). Milk samples from each cow were aseptically collected from the 4 teats into sterile tubes for bacteriological analysis. After milking each cow, 50 mL of milk was aseptically collected from the milk meter into sterile vials for components, SCC, and differential cell count (**DCC**) analysis.

The milk composition (fat, protein, lactose, total solids, casein, and urea percentage) and SCC were analyzed using Somacount equipment (Bentley, Chaska, MN) in accordance with the guidelines of the International Dairy Federation (IDF, 1995, 1996). Milk composition was expressed as mass/mass (%), and SCC results were recorded as the number of somatic cells $\times 10^3$ mL^{-1} of milk. For DCC count analysis, milk samples were smeared on slides (Paape et al., 1965). Precisely, 2 smears were made with 0.01 mL of the sample, air-dried, fixed with Carnoy fixative, and stained with pyronin y-methyl green. Each smear was then examined under bright-field microscopy at 100× magnification. Milk leukocytes were categorized into neutrophils, monocytes, lymphocytes, basophils, eosinophils, and unidentified cells. These unidentified cells were considered epithelial cells, as established by previous research (Boutinaud and Jammes, 2002). For each DCC smear, 200 cells were counted, and the total and individual numbers of each cell type were recorded. Subsequently, the percentage for each cell type was calculated.

Bacteriological analyses were conducted following the guidelines of the National Mastitis Council (NMC, 1987) with modifications proposed by Brito and Brito (1999) to identify the primary mastitis-causing agents: *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*, and coagulase-negative *Staphylococcus* species.

Gene Expression

Milk samples collected on d 7 of the experiment were used for gene expression analysis. Milk cell separation was conducted through centrifugation at 1,500 × *g* for 15 min at 4°C, with subsequent washings using a sterile PBS solution. Afterward, the mammary epithelial cells were separated using an immunomagnetic method (Herve et al., 2017) and then stored at −80°C. Using the PureLink RNA Mini Kit (Invitrogen, Toronto, ON, Canada), the RNA from these samples was extracted and purified. The quality of each RNA sample was assessed by the optical density at the 260 and 280 nm absorbance waves, with an average absorption ratio of ~2. The RNA concentrations were measured using Qubit Fluorometric Quantification (Thermo Fisher, Waltham, MA). To eliminate potential genomic DNA contamination, the total RNA samples were treated with RNase-Free DNase (Promega, Madison, WI). Finally, the RNA was converted into cDNA by using the GoScript Reverse Transcriptase kit (Promega).

The gene amplification study for the target genes of interest was carried out using real-time quantitative PCR and the SybRGreen system (Life Technologies, Carlsbad, CA). Each gene was individually amplified in separate, duplicate reactions. The 20-µL reaction solution contained 1 µL of cDNA sample (mean concentration: 10 ng/µL), 10 µL of SYBR Green PCR Master Mix, 0.8 µL of the primer pairs (final concentration: 0.4 µ*M*) and 8.2 µL of ultrapure water. All these reactions were processed in a StepOnePlus thermocycler (Life Technologies). The thermocycling protocol comprised an incubation stage at 95°C for 10 min, followed by 40 cycles of alternation between 95°C for 15 s and 60°C for 1 min, ending with a dissociation (melt) curve. Real-time PCR efficiency was validated for all primer pairs, along with specific product verification through melt curve analysis and 1.5% agarose gel electrophoresis.

Three housekeeping genes (*GAPDH*, *ACTB*, *UBC*) were evaluated, with *UBC* and *GAPDH* chosen based on their efficiency and stable expression across treatments. Supplemental Table S1 (see Notes) provides a detailed description of the target primers. Expression levels of target genes were determined through the $2^{-\Delta\Delta CT}$ method by comparing them with reference genes and control cycle thresholds at each specific point (Schmittgen and Livak, 2008).

Statistical Methods

The Statistical Package for the Social Sciences (version 10; SPSS, 1998) was used to conduct statistical analyses. The data normality distribution was evaluated with the Kolmogorov–Smirnov or Shapiro–Wilk test, depending on the number of observations. For the analysis, were used an ANOVA within a mixed linear model, taking into account the treatment, day, hour, and their interactions as fixed effects, with the animal as a random effect. Gene expression data were compiled using ANOVA with a generalized linear model, considering treatment as a fixed effect and the animal as a random effect. The Fisher's test was used to compare parametric data and the Mann-Whitney test to compare nonparametric data. Data

Table 1. Vaginal temperature (VagT), respiratory rate (RR), cortisol (CORT), thyroxine (T4), triiodothyronine (T3), insulin-like growth factor 1 (IGF-1), and insulin (INS) concentration (mean ± SEM, n = 12) of the Girolando cows subjected to heat stress and control treatments

	Heat stress	Control		*P*-value[1]			
Item	Mean (n = 12)	Mean (n = 12)	SEM	T1	D	T × D	T × H
VagT[2] (°C)	39.4[a]	38.8[b]	0.03	0.02	0.84	0.77	0.01
RR[2] (breaths/min)	48	49	1.46	0.63	0.72	0.90	0.87
CORT[3] (ng/mL)	18.65	15.46	1.83	0.57	0.18	0.05	
T4[3] (ng/mL)	4.67	4.50	0.19	0.90	0.01	0.22	
T3[3] (ng/mL)	1.44	1.41	0.04	0.70	0.01	0.18	
IGF-1[3] (pg/mL)	24.16	23.09	0.80	0.79	0.03	0.20	
INS[3] (ng/mL)	0.36	0.35	0.02	0.89	0.82	0.94	

[a,b]Means within a row with different letters differ ($P \leq 0.05$).

[1]T = treatment effect, D = day effect, T × D: treatment × day interaction, T × H: treatment × hour interaction.

[2]Data correspond to the mean of the experimental period (111–120 d of lactation).

[3]Data correspond to the mean of 0, 1, 3, 5, and 7 d from the experimental period.

were represented as the mean ± SEM and considered a result statistically significant with a significance level set at $P \leq 0.05$.

The bacteriological data and frequency of urination, defecation, and rumination behaviors were expressed as the percentage occurrence within the HS and CT treatments. These data were analyzed these data using the frequency procedure (FREQ) and the chi-squared test, with a significance level set at $P \leq 0.05$; afterward, the odds ratios (**OR**) were analyzed. These variables were represented as percentages.

RESULTS

Heat Stress Increases Vaginal Temperature and Cortisol Concentration Without Changing the Respiratory Rate

Figure 2 shows the 24-h evolution of the VagT in Girolando cows over 9 d of the experimental period. During challenging conditions from 0700 to 1500 h, the VagT in HS cows registered notably higher than in CT cows (39.4°C ± 0.02 compared with 38.8°C ± 0.04 respectively). However, we found no significant difference in the RR between HS and CT cows (48 ± 2.0 compared with 49 ± 1.0 breaths/min) throughout the study (Table 1).

A significant interaction was observed between the treatment and the day of CORT release ($P = 0.05$). The HS cows presented higher CORT concentrations on d 5 and 7 than CT cows (Figure 3). Nevertheless, HS did not significantly affect the concentrations of T4, T3, IGF-1, and INS throughout the experimental period (Table 1).

Heat Stress Decreased Milk Yield Without Changing Milk Composition, Milk Cells, and Bacteriological Analysis in Milk

During the experimental period, the milk yield of HS cows was notably lower than that of CT cows (17.6 ± 0.20 vs. 18.9 ± 0.25 kg/d). However, the treatment had no significant impact on milk composition and SCC between

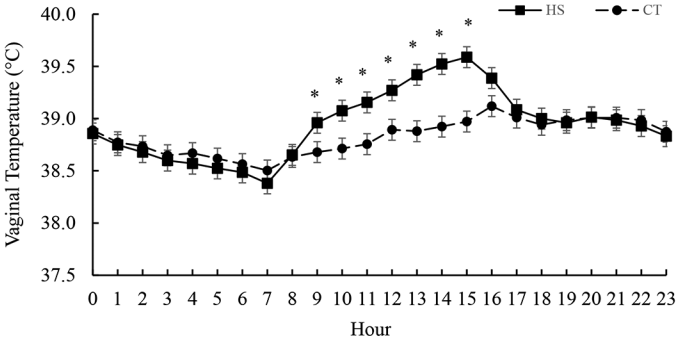

Figure 2. Vaginal temperature (VagT: mean ± SEM, n = 12) of Girolando cows subjected to heat stress (HS) and control (CT) treatments throughout 24 h. Data concerning VagT correspond to the mean measured during the experimental period (111–120 d of lactation). Means with * differ (treatment × hour interaction, $P \leq 0.05$).

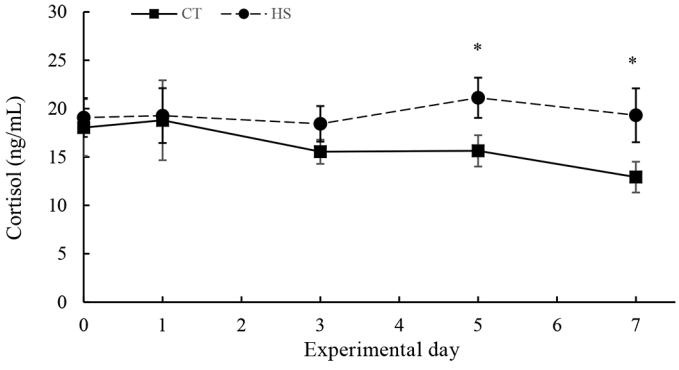

Figure 3. Cortisol (CORT) release by Girolando cows subjected to heat stress (HS) and control (CT) treatments (mean ± SEM, n = 12). Data concerning CORT correspond to the mean measured on d 0, 1, 3, 5, and 7 from the experimental period (111–120 d of lactation). Means with * differ (treatment × day interaction, $P \leq 0.05$).

Table 2. Different cell types measured in the milk of the Girolando cows subjected to heat stress and control treatments

Cell type[1]	Heat stress Mean (n = 12)	Control Mean (n = 12)	SEM	*P*-value
Neutrophils (%)	68	69	2.5	0.87
Lymphocytes (%)	6[a]	3[b]	0.6	0.05
Monocytes (%)	1	1	0.3	0.34
Basophils[2] (%)	—	—	—	—
Eosinophils[2] (%)	—	—	—	—
Epithelial (%)	25	27	2.0	0.45

[a,b]Means within a row with different letters differ ($P \leq 0.05$).

[1]Data correspond to the mean on 0, 1, 3, 5, and 7 d from the experimental period (111–120 d of lactation).

[2]Insufficient data for statistical analysis.

HS and CT cows (96 ± 8.9 vs. 114 ± 10.0 × 10^3 cells/mL; details in Supplemental Table S2, see Notes).

The HS cows exhibited a higher percentage of lymphocytes in milk compared with CT cows (6% vs. 3%, Table 2). However, the treatment did not affect the percentage of various other types of cells in the milk, including neutrophils, monocytes, basophils, eosinophils, and epithelial cells (Table 2). Finally, heat stress did not affect the bacterial count in milk (Supplemental Table S3, see Notes).

Heat Stress Decreased Feed Intake and Rumination Frequency

Heat stress significantly affected DMI (kg/d; $P = 0.05$), with HS cows exhibiting a decrease in intake compared with the CT cows (Table 3). Additionally, a significant difference ($P = 0.01$) was recorded in rumination frequency between HS and CT cows (Table 3). The likelihood of HS cows ruminating in the milking parlor was 23% lower than that of CT cows, reflected in an OR of 1.7 (OR = 2.76). However, we observed no differences between the 2 groups in terms of other behaviors measured during milking (Table 3).

Table 3. Total DMI and steps, kicks, urination, defecation, and rumination frequency measured during milking (mean ± SEM, n = 12) of the Girolando cows subjected to heat stress and control treatments

Item	Heat stress Mean (n = 12)	Control Mean (n = 12)	SEM	*P*-value
DMI[1] (kg/d)	17.2[a]	18.1[b]	0.62	0.05
Steps[2] (number)	2.5	2.0	0.86	0.55
Kicks[2,3] (number)	—	—	—	—
Urination[2] (%)	17	10		0.37
Defecation[2] (%)	21	17		0.60
Rumination[2] (%)	25[a]	48[b]		0.01

[a,b]Means within a row with different letters differ ($P \leq 0.05$).

[1]Data correspond to the mean of the experimental period (111–120 d of lactation).

[2]Data correspond to the mean of 2, 4, 6, and 8 d from the experimental period.

[3]Insufficient data for statistical analysis.

Heat Stress Affected the Expression of Genes Related to Heat Shock Proteins and Milk Synthesis Without Changing Cell Apoptosis and Immune Response Genes

Heat stress significantly increased the gene expression of *HSPD1* ($P = 0.01$), *HSPD90AA1* ($P = 0.02$), insulin receptors (*INSR*; $P = 0.01$), and prolactin receptors (*PRLRsf*; $P = 0.01$) in HS cows compared with CT cows, as shown in Table 4. Simultaneously, the expression of the glucocorticoid receptor (*NR3C1*) significantly decreased ($P = 0.001$) in HS cows relative to CT cows (Table 4).

DISCUSSION

The heat challenge in this study significantly affected VagT, cortisol release, DMI, rumination frequency, gene expression, and milk yield in HS cows. Similar physiological responses and a negative impact on milk yield

Table 4. Gene expression (mRNA, $2^{-\Delta\Delta CT}$ method) of target genes in mammary cells of Girolando cows subjected to heat stress or control treatments

Gene (mRNA)[1]	Heat stress (Mean, n = 12)	Control Mean, n = 12)	SEM	*P*-value
HSP40	1.02	1.00	0.22	0.96
HSPD1	1.56[a]	1.00[b]	0.13	0.01
HSPA1A	1.47	1.00	0.22	0.34
HSPD90AA1	2.03[a]	0.99[b]	0.20	0.02
BAX	1.19	0.99	0.12	0.49
BCL2	0.88	1.00	0.07	0.45
CASP3	0.99	1.00	0.03	0.92
CASP8	1.03	1.00	0.32	0.97
NR3C1	0.47[b]	1.00[a]	0.08	0.001
NRC3C2[2]	—	—	—	—
11BHSD2	0.83	1.00	0.20	0.88
CREB1	0.86	1.00	0.15	0.74
GHR	1.63	1.00	0.57	0.69
IGF1R	0.83	1.00	0.15	0.76
INSR	2.17[a]	1.00[b]	0.25	0.01
PR	0.92	1.00	0.25	0.93
ERα	4.06	1.00	1.06	0.21
Erβ[2]	—	—	—	—
PRLRlf	1.03	1.00	0.20	0.95
PRLRsf	2.37[a]	1.00[b]	0.45	0.01
TGFβ1	0.55	1.00	0.25	0.48
PTGFR	0.88	1.00	0.26	0.44
NFKB1	1.29	1.01	0.22	0.53
NFKBIA	0.85	1.00	0.17	0.68
PTGES	0.59	1.00	0.17	0.35
APAF1	1.04	1.00	0.18	0.91
PTEN	1.68	1.01	0.18	0.41
FAS	0.67	1.00	0.20	0.49

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

[1]Data correspond to gene expression on d 7 from the experimental period (111–120 d of lactation).

[2]Insufficient data for statistical analysis.

in Zebu cows under heat stress have been reported in previous studies (da Costa et al., 2015; Santana et al., 2015; Carvalheira et al., 2021). However, the heat stress imposed in the present study did not affect the respiratory frequency (49 ± 1 movements/min), indicating that Girolando cows did not increase their RR to handle severe heat stress. On the other hand, Holstein cows typically raise their respiratory rates under heat stress to re-establish homeothermy (Rhoads et al., 2009; Collier et al., 2017). Some researchers argued that the adaptation of Zebu cattle to tropical conditions can be attributed to a more effective body heat dissipation, efficient and gradual respiratory responses, increased peripheral vasodilation, and higher sweating rate (Alfonzo et al., 2016; Ribeiro et al., 2020; dos Santos et al., 2021). Most heat stress studies focus on body temperature as it is often deemed the most reliable heat stress marker in dairy cows (Tao et al., 2018). In the present study, the VagT of the HS cows was 39.5 ± 0.1°C in the climate chamber, which is higher than that of CT cows and the physiological standard established for Holstein cows (Berman et al., 1985; West, 2003; Smith et al., 2013). These findings collectively indicate that Girolando cows, often thought to be thermotolerant, are susceptible to severe heat stress.

Conversely, heat stress did not affect the release of T4, T3, IGF-1, and INS in Girolando cows. Various studies have accredited the superior adaptation of the Zebu breed to tropical climates to their diminished metabolic rate and heat production (da Costa et al., 2015; dos Santos et al., 2021; Carvalheira et al., 2021). Hence, it could be argued that the maintenance of these hormone levels assists in controlling heat production by Girolando cows under heat stress (Itoh et al., 1998; Nardone et al., 2010; Aleena et al., 2016). However, in our study, the higher cortisol concentrations were recorded on the fifth and seventh days in a climate-controlled chamber. This observation implies that HS cows attempt to regain their thermal equilibrium, suggesting robust resilience in Girolando cows against enforced heat stress. Cortisol release has been linked to metabolic adjustments needed to generate accessible energy and restore balance (Itoh et al., 1998; Mormède et al., 2007; Bomfim et al., 2022). This correlation could partially clarify the enhanced heat production and VagT in Girolando cows. Thus, the reduced milk yield indicates that Girolando cows, and other dairy breeds, divert nutrients and energy from milk production to endorse adaptive mechanisms to regain their heat balance (Nardone et al., 2010; Collier et al., 2017). Consequently, the heat tolerance of Girolando cows should be factored into their selection process for tropical environments.

Indeed, heat stress notably reduced the milk yield of HS cows, making it 7% less than that of the CT cows. This finding aligns with our initial hypothesis, proposing that heat stress reduces the milk yield of Girolando cows despite them being known for their adaptation to tropical climates. Although limited studies have compared Girolando cows under HS against those under thermal control (Ribeiro et al., 2020), several studies have reported negative heat stress effects on the milk yield of Girolando cows in summer compared with winter (da Costa et al., 2015; de Vasconcelos et al., 2020; dos Santos et al., 2021). Studies involving high milk-producing Holstein cows subjected to heat stress highlighted significant milk losses between 15% and 30% (Wheelock et al., 2010; Das et al., 2016; Polsky and von Keyserlingk, 2017). In line with the reduced milk yield, HS cows also experienced a significant reduction in their DMI and rumination frequency compared with CT cows. These lower rates potentially affect the nutrient supply reaching the mammary gland for milk production, negatively affecting milk yield during HS. Studies have previously linked changes in ingestive behavior to lower heat production (Bouraoui et al., 2002; Rhoads et al., 2009; Moretti et al., 2017). However, in the current study, HS cows remained stressed and exhibited higher vaginal temperatures when compared with CT cows. Although some research reveals that heat stress can either positively or negatively affect the milk composition of Holstein cows (Bernabucci et al., 2010; Carabaño et al., 2016; M'Hamdi et al., 2021), our study found no heat stress effect on the milk composition of Girolando cows. These shifting results highlight the need for further research to better understand heat stress effect on dairy cow milk composition.

The current study found that heat stress significantly elevated the expression of *HSPD1* and *HSPD90AA1* genes in the mammary cells of HS cows compared with CT cows. These previous studies reported a similar increase in the expression of heat shock proteins (**HSP**) in cattle subjected to heat stress and linked this heightened expression to cell survival (Collier and Benesch, 2020; Fu et al., 2021; Masroor et al., 2022). Typically, increased expression of *HSPD1* and *HSPD90AA1* genes is viewed as an immediate cellular response to stress (Masroor et al., 2022) and could be tied to the animals' adaptability to environmental challenges (Aleena et al., 2018; Sejian et al., 2019; Garner et al., 2020). Indeed, HSP enhance the cell's capacity to resist stress, repair damaged proteins, preserve cellular functional integrity, and hinder the apoptotic process (Hu et al., 2015; Collier and Benesch., 2020). Our findings of heightened *HSPD1* and *HSPD90AA1* expression in mammary cells suggest these cells initiate local responses to manage imposed heat stress, indicative of an adaptive response in mammary cells from Girolando cows.

In the same way, none of the apoptosis-related genes (*BAX*, *BCL2*, *CASP3*, *CASP8*, *APAF1*, and *FAS*) were affected by heat stress in the HS cows. Our study also

showed a lower expression of the *NR3C1* gene, known as the primary cortisol receptor, in mammary cells (Bomfim et al., 2018, 2022). This suggests that mammary cells alter their responsiveness to locally counter the effects of increased cortisol release triggered by heat stress in Girolando cows. Although further research is necessary, our findings suggest that the expression of *HSPD1*, *HSPD90AA1*, and *NR3C1* genes in mammary cells can be useful markers of the Girolando resilience to heat stress and ongoing climate challenges brought on by global warming.

The HS cows presented an increase in *INSR* and *PRLRsf* gene expression in mammary cells compared with CT cows. However, this rise in gene expression did not mitigate the adverse effects of heat stress on milk synthesis, resulting in lower milk production in HS cows than in CT cows. In this study, insulin concentrations remained unaffected by imposed heat stress, yet the heightened expression of the *INSR* gene in Saanen goat mammary tissue had previously been linked to increased milk yield (Bomfim et al., 2022). Our findings hint at the ability of heat stress to adjust insulin responsiveness in the mammary gland, possibly altering glucose metabolism (Wheelock et al., 2010; Ribeiro et al., 2020; Sammad et al., 2020). Likewise, HS cows exhibited elevated expression of *PRLRsf* in mammary cells compared with CT cows. However, pregnant Saanen goats and dry-off Holstein cows under heat stress showed a decreased expression of the *PRLRlf* gene in mammary tissue, a result that also correlated with lower milk production in subsequent lactation (Fabris et al., 2019; Hooper et al., 2020; Ouellet et al., 2021). Although previous studies have argued that PRL binds to PRLRlf to induce mammary tissue remodeling, milk protein synthesis, and the maintenance of lactation, PRL binding to PRLRsf apparently has an inhibitory action on these processes (Berlanga et al., 1997; Yang et al., 2021). Our results show that under heat stress, the PRL action in mammary tissue is also regulated by its interaction with *PRLRsf*. In fact, our study supports the concept that heat stress modifies the expression of *INSR* and *PRLRsf* in mammary cells, affirming the notion that it alters the mammary gland's responsiveness toward milk synthesis. Furthermore, the PRLR mutation has been related to thermotolerance in cattle (Sosa et al., 2022). For these reasons, more research is needed to fully understand how heat stress negatively affects INS and PRL responsiveness in mammary tissue and milk synthesis.

In this study, we found no effect of heat stress on the expression of genes associated with inflammatory-immune response (such as *TGFB1*, *PTGFR*, *NFKB1*, *NFKBIA*, *PTGES*, *APAF1*, *PTEN*, and *FAS*). Nevertheless, HS cows displayed a higher lymphocyte percentage in milk compared with CT cows. It has been suggested that stress-induced cortisol increases can potentially affect the balance between Th1 and Th2 cells (Webster et al., 2002; Lacetera et al., 2005; Bagath et al., 2019). This shift can partially explain the heightened lymphocyte presence in milk under heat stress. In the same way, several authors have linked the summer season to lower immune system efficacy, increased SCC, and higher mastitis rates in Holstein cows (Ferreira and De Vries, 2015; Becker et al., 2020; Li et al., 2021). Others authors propose that increased SCC during summer may result from greater exposure to environmental pathogens (Das et al., 2016; M'Hamdi et al., 2021). As reported by other authors (Tao et al., 2018; Lengi et al., 2022), in the present study, we found no heat stress effect on SCC, neutrophils, monocytes, basophils, eosinophils, and epithelial cell percentage in milk. Moreover, no heat stress effect was observed on bacterial count in milk, and the Girolando cows showed no subclinical or clinical symptoms of mastitis throughout the entire experiment. Taking into account these findings, it is not feasible to argue that heat stress modifies the immune response in the mammary gland of Girolando cows.

In this study, the expression of genes relative to cellular apoptosis (*BAX*, *BCL2*, *CASP3*, *CASP8*, *APAF1*, and *FAS*) was not affected by either HS or CT treatments. Some authors demonstrated that heat stress influences the balance between apoptosis and mammary cell proliferation, negatively changing the growth of the alveolar structure during the dry period (Fabris et al., 2019; Ouellet et al., 2021). A greater expression of the *PRLRsf* gene in HS cows can correlate with mammary cell survival during lactation. As prolactin reportedly promotes cell proliferation and safeguards mammary cells from apoptosis (Accorsi et al., 2002; Herve et al., 2016; Ouellet et al., 2021), it is possible to speculate that higher expression of *PRLRsf* in HS cows changed the exfoliation of mammary cells as a compensatory response of Girolando cows to tropical conditions. Additionally, higher SCC rates in a healthy animal's milk are modulated by natural exfoliation of mammary epithelial cells (Boutinaud and Jammes, 2002; Herve et al., 2016). Considering our findings revealed no heat stress effect on the apoptosis-related genes or SCC in milk, links cannot be drawn between heat stress and an uptick in mammary cell apoptosis in Girolando cows.

Finally, the heat stress applied in this study resulted in increased VagT and cortisol release, as well as decreased DMI, rumination frequency and milk yield in Girolando cows, demonstrating their susceptibility to global warming. Furthermore, heat stress upregulated the *HSPD1*, *HSPD90AA1*, *INSR*, and *PRLRsf* genes and downregulated the *NR3C1* gene in mammary cells, presenting a localized response to cope heat stress. These findings only partially confirmed our initial hypothesis, because there were no effects of heat stress on the expression of

genes associated with cell apoptosis or immune response, and SCC in milk.

NOTES

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Nonstandard abbreviations used: CORT = cortisol; CT = control treatment; DCC = differential cell count; HS = heat stress treatment; INMET = Brazilian National Meteorology Institute; INS = insulin; OR = odds ratio; RR = respiratory rate; T3 = triiodothyronine; T4 = thyroxine; THI = temperature-humidity index; VagT = vaginal temperature.

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