Effect of heat stress during the early and late dry period on mammary gland development of Holstein dairy cattle

Thiago F. Fabris,¹ Jimena Laporta,¹ Amy L. Skibiell,¹ Bethany Dado-Senn,¹ Stephanie E. Wohlgemuth,¹ and Geoffrey E. Dahl^{1*}

Department of Animal Sciences, University of Florida, Gainesville 32611

ABSTRACT

Dry period heat stress impairs subsequent milk yield. Our objective was to evaluate the effect of heat stress or cooling during the early and late dry period on mammary gland gene expression and microstructure. Cows were dried off ~45 d before expected parturition and randomly assigned to 1 of 2 treatments: heat stress (HT, $n = 39$) or cooling (CL, $n = 39$) during the first 21 d of the dry period. On d 22, cows were switched or remained on HT and CL and this yielded 4 treatments: heat stress during the entire dry period (HTHT, $n = 18$); cooling during the entire dry period (CLCL, $n = 20$); HT for the first 21 d dry, then CL until calving (HTCL, $n = 21$); or CL for the first 21 d dry, then HT until calving (CLHT, $n = 19$). Data were analyzed in 2 periods: first 21 d dry (early dry period) and from 22 d until calving (late dry period) and analyzed using PROC MIXED or GLM in SAS (SAS Institute Inc., Cary, NC). Mammary biopsies (5–8 cows/treatment) were collected at –3, 3, 7, 14, and 25 d relative to dry-off to evaluate mammary gland gene expression and histology [i.e., cellular apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling) and proliferation (Ki67)]. Mammary alveoli number and connective tissue were visualized by hematoxylin and eosin and Mason's trichrome staining, respectively. During the early dry period, CL upregulated expression of *CASP3*, *IGF1R*, *HSP90*, *HSF1*, *BECN1*, *ATG3*, *ATG5*, and *PRLR-LF* relative to HT. However, in the late dry period, CLHT treatment upregulated expression of *CASP3*, *CASP8*, *HSP70*, *HSP90*, *PRLR-LF*, *STAT5*, *CSN2*, and *ATG3* relative to CLCL. During the early dry period, cows exposed to HT had reduced mammary and stroma cell apoptosis and proliferation relative to CL. In addition to these findings, cows exposed to HT had lower connective tissue 3 d after dry-off relative to CL. However,

in the late dry period, HTHT cows had higher connective tissue relative to CLCL. Also, in the early dry period, cows exposed to HT had greater alveoli number relative to CL, and HT decreased expression of genes related to autophagy and apoptosis in the early dry period, consistent with a delay in involution with HT. Thus, cows exposed to HT have extended involution with delayed apoptosis and autophagy signaling. Also, HT compromises mammary gland cell proliferation and leads to higher connective tissue later in the dry period. These results provide evidence that heat stress impairs overall mammary gland turnover during the dry period, which then affects secretory activity and productivity in the next lactation.

Key words: apoptosis, proliferation, gene expression

INTRODUCTION

Since 1980, the global temperature has increased ~0.2°C per decade with no indication of a plateau or downward trend (Hansen et al., 2006). The direct negative effects of global warming on livestock include enormous economic loss with lower efficiency and impaired animal welfare. One of the most well-recognized negative effects of heat stress in lactating dairy cows is a decrease in milk yield (Fuquay, 1981; West, 2003). Also, Ferreira et al. (2016) estimated that if cows do not receive heat stress abatement during the dry period, the economic losses to the US dairy industry could be over \$800 million dollars per year. In contrast to lactating cows, nonlactating dry cows generate less metabolic heat (West, 2003) and have a higher upper critical temperature (Hahn, 1999). However, environmental heat stress during the entire dry period negatively influences lactational performance in the subsequent lactation (Tao et al., 2012; Fabris et al., 2017). Also, our recent study demonstrated that cows exposed to heat stress during early, late, or entire dry period have reduced milk yield in the next lactation relative to cows cooled during the entire dry period (Fabris et al., 2019). Yet the cellular and molecular mechanisms that drive the reduced mammary function are unknown.

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*Corresponding author: gdahl@ufl.edu

The dry period can be characterized by 2 distinct periods, the early phase of involution (i.e., the first ~3 wk) and the late phase associated with cell proliferation (i.e., the last ~3 wk; Hurley, 1989; Tao et al., 2011; Wohlgemuth et al., 2016). During involution, autophagy and apoptosis predominate and drive cell loss (Wilde et al., 1997; Sorensen et al., 2006). Apoptosis and autophagy are cellular mechanisms responsible for the programmed destruction and bulk recycling of cells, respectively. Apoptosis and autophagy are important mechanisms for mammary epithelial cell (MEC) renewal and cell proliferation throughout the dry period (Zarzyńska et al., 2007; Sobolewska et al., 2009). It has been speculated that heat stress may negatively affect mammary gland remodeling and thus affect milk yield in the subsequent lactation (Tao et al., 2011). Apoptotic activity is high during the first 3 d of the dry period (Annen et al., 2008; Piantoni et al., 2010; Dado-Senn et al., 2018) and decreases as parturition approaches (Sorensen et al., 2006). Relative to cows under heat stress, autophagy increased during involution when dry cows were exposed to cooling systems (soakers, fans, and shade; Wohlgemuth et al., 2016), and that may set the stage for mammary cell proliferation after active involution ends (Tao et al., 2011; Wohlgemuth et al., 2016).

Therefore, cooling pregnant dairy cows might lead to optimal mammary gland involution and consequently improve cell proliferation when active involution ends, which may explain the greater milk yield of cooled cows relative to heat-stressed cows (Tao et al., 2011; Fabris et al., 2019). However, there is a gap in our knowledge about the underlying biological mechanisms of heat stress that drive negative outcomes on mammary gland development, and the temporal relationship of those events relative to parturition. Thus, in the current study we investigated cellular events induced by environmental heat stress that occur in the mammary gland during the early and the late dry period. Our overall hypothesis is that heat stress negatively affects mammary involution in the early dry period and diminishes subsequent proliferation of mammary tissue during the late dry period.

MATERIALS AND METHODS

Treatments, Experimental Design, and Animals

The experiment was conducted over one summer season (2016) at the University of Florida Dairy Unit (Hague, FL). Data presented in this manuscript are additional information to the production-related data from the same study, which was published by Fabris et al. (2019). Cows were dried off ~45 d before expected

parturition and randomly assigned to 1 of 2 treatments: heat stress (HT, $n = 39$) or cooling (CL, $n = 39$) during first 21 d of the dry period. On d 22, cows were switched or remained in a HT or CL pen and yielded 4 treatments: heat stress during the entire dry period (HTHT, $n = 18$); cooling during the entire dry period (CLCL, $n = 20$); HT for the first 21 d dry, then CL until calving (HTCL, $n = 21$); or CL for the first 21 d dry, then HT until calving (CLHT, $n = 19$). Data were analyzed in 2 periods: first 21 d dry (early dry period; CL vs. HT) and from 22 d until calving (late dry period; HTHT, CLCL, HTCL, and CLHT treatment) using PROC MIXED or GLM in SAS (version 9.4, SAS Institute Inc., Cary, NC).

Cows were housed in a sand-bedded freestall barn during the dry period and lactation and housing facilities were as described by Fabris et al. (2019). Briefly, temperature-humidity index was recorded, and it was higher than 72 during the whole period. All cows were fed a common TMR during the entire dry period and daily DMI of individual cows was measured from dry-off to calving. Rectal temperature (RT) and respiration rate (RR) were measured and reported as follows: (RT: CL, 38.8; HT, $39.1 \pm 0.04^\circ\text{C}$, early dry period) and (RR: CL, 52.9; HT, 70.5 ± 1.9 breaths/min, early dry period). In the late dry period, RT and RR were as follows: (RT: CLCL, 38.7; HTHT, 39.1; CLHT, 39.1; HTCL, $38.9 \pm 0.05^\circ\text{C}$; RR: CL, 47; HT, 64; CLHT, 66; HTCL, 53 ± 2.1 breaths/min, late dry period). In addition to these effects, gestation length was evaluated and reported as follows: (CLCL, 277.4; HTHT, 275.1; CLHT, 272.9; HTCL, 274.0 ± 1.18 d). After parturition cows were kept under the same environment and provided with shade, fans, and soakers, and milk yield was recorded up to 30 wk into lactation and was reported as follows: CLCL, 40.2; HTHT, 36.3; CLHT, 36.1; HTCL, 36.3 ± 1.36 kg/d.

Mammary Gland Biopsy and Histology

Mammary gland biopsies were collected at -3 (before treatments were applied), 3, 7, 14 (early dry period), and 25 d (late dry period) relative to dry-off to evaluate mammary gland gene expression and microstructure from a subset of cows (early dry period; HT, $n = 13$, CL, $n = 13$; and late dry period; HTHT, $n = 5$, CLCL, $n = 7$, HTCL, $n = 8$, CLHT, $n = 6$). Mammary gland tissue collection was performed as described by Tao et al. (2011). Briefly, mammary gland quarters were checked to confirm they were healthy and without indication of mastitis. The right rear quarter of the cow was biopsied (~500-mg sample) 3 d before dry-off (d -3), and on d 7 and 25 relative to dry-off. The left rear quarter was biopsied on d 3 and 14 rela-

tive to dry-off. The biopsy procedure was based on the method reported by Farr et al. (1996). For each biopsied quarter, 2 consecutive biopsies were separated by at least 10 d, which minimizes the effects of biopsy-related tissue damage on the cellular events in mammary tissue. Indeed, within 3 d after biopsy, the intramammary blood accumulated during the biopsy clears from the quarter and the mammary function recovers to the pre-biopsy level (Farr et al., 1996; Dover et al., 2008). After collection, mammary tissue was washed in sterile saline, trimmed of visible fat, and sectioned and stored for immunohistochemistry and gene expression analysis (Skibieli et al., 2018). Briefly, for staining and immunohistochemistry, one portion of mammary gland tissue was placed in cassettes and fixed overnight at 4°C in 4% paraformaldehyde, and then submitted to a series of ethanol rinses (25%, 50%, 70%, and 100%) and transferred to 70% ethanol and kept at 4°C until all samples were collected and paraffin embedded following the procedures of the University of Florida Molecular Pathology Core. Tissues were then sectioned at 5 µm onto slides coated with poly-L-lysine and stained with hematoxylin and eosin, or Masson's trichrome to visualize alveolar morphology and connective tissue, respectively. Immunohistochemistry was used to detect apoptotic (terminal deoxynucleotidyl transferase dUTP nick end labeling; **TUNEL**) and proliferating (Ki67) cells as described by Dado-Senn et al. (2019). Briefly, the Ki67 assay was performed using the UltraVision One Detection System: HRP Polymer/DAB Plus Chromogen kit (catalog number TL-015-HDJ, Thermo Fisher Scientific, Waltham, MA) with primary antibody (Monoclonal Mouse Anti-Human Ki67 Antigen Clin MIB-1, catalog number M724029-2, Dako Agilent, Glostrup, Denmark). In addition, positive and negative controls were established using calf jejunum (high proliferation rates) and bovine mammary tissue at 25 d dry without primary antibody incubation, respectively. Briefly, the TUNEL assay was performed using ApopTag Plus Peroxidase in situ Apoptosis Kit (catalog number S7101, Millipore, Billerica, MA) according to the manufacturer's instructions. Mammary alveoli number and luminal area were quantified from hematoxylin and eosin-stained slides. Connective tissue area was measured in Masson's trichrome-stained slides. Image analysis was performed using Image J software (Abramoff et al., 2004). Quantification of histological sections were performed as described by Skibieli et al. (2018) and Dado-Senn et al. (2019). Briefly, histological sections from Ki67 and TUNEL were used to capture 3 photomicrographs using the 40× objective with EVOS XL Core imaging system (Advanced Microscopy Group, Bothell, WA). In each section, every cell was counted and defined as positive (stained brown) and negative

(stained blue) and at least 2,000 cells were counted per section, and 2 main cell types were considered: MEC or stromal cells.

For gene expression analysis, samples were stored in RNAlater Stabilization Solution (catalog number AM7021, Invitrogen, Carlsbad, CA) for 24 h at 4°C and then kept at −80°C until RNA extraction. The RNA was extracted from 50 to 70 mg of mammary gland tissue using RNeasy Plus Universal Mini Kit (catalog number 74134, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantity and purity of each RNA sample were measured using a NanoDrop1000 Spectrophotometer (Wilmington, DE) and samples were evaluated by optical density measurement (A_{260}/A_{280} ratio), where 260/280 absorption rate was between 1.98 and 2.2. For cDNA synthesis, 1 µL of RNA was used for cDNA synthesis using reverse transcription kit, iScript Bio-Rad (catalog number 1708841, Bio-Rad, Hercules, CA). Then, SsoAdvanced Universal SYBER Green Supermix (catalog number 1725272, Bio-Rad) quantitative real-time PCR was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The following 3-step PCR program was used: denaturation for 3 min at 95°C, 49 cycles of amplification (each consisting of 10 s at 95°C, the primer-specific annealing temperature and extension at 60°C for 30 s, and quantification of fluorescence), and finally a melting curve program (65–95°C). To evaluate the stability of reference genes, 3 different genes (*RSP9*, *GAPDH*, and *K8*) were analyzed as housekeeping candidates, tested with NormFinder software (<https://moma.dk/normfinder-software>) and the best combination of 2 genes were identified as housekeeping genes (*RSP9* and *GAPDH*). Data (cycle threshold, *Ct*) were normalized to the geometric mean of 2 reference genes (*RSP9* and *GAPDH*) to obtain ΔCt values (target gene *Ct* – reference gene mean *Ct* = ΔCt) and ΔCt values were analyzed statistically. The $\Delta\Delta Ct$ values were used for visual representation (Livak and Schmittgen, 2001). Autophagy-related genes, *ATG3* and *ATG5*, and beclin-1 (*BECN1*); apoptosis-related genes, caspase 3 and 8 (*CASP3*, *CASP8*; respectively), heat shock proteins (*HSP70* and *HSP90*), and heat shock factor 1 (*HSF1*); insulin growth factor 1 receptor (*IGF1R*); and prolactin signaling-related genes, prolactin receptor long form (*PRLR-L*), *STAT5a*, and *CSN2* were analyzed by quantitative real-time PCR (Table 1).

Prolactin Analysis

Blood samples were collected 1 d before switching treatments (approximately 20 d dry) and 1 d before the biopsy performed on d 25 (3 d after cows were switched; n = 8 cows/treatment). In the late dry

period, contrast analysis was performed among treatments (HTHT, CLCL, HTCL, CLHT). Also, prolactin concentrations were pooled to compare the exposure to heat stress (HTHT + CLHT) versus cooling (CLCL + HTCL) during the late dry period. Blood samples were collected from coccygeal vessels into EDTA tubes (BD Vacutainer spray-coated K2EDTA Tubes, 10 mL, Becton Dickinson, Franklin Lakes, NJ). Samples were then placed on ice and centrifuged at $2,619 \times g$ at 4°C for 20 min within 1 h after collection and plasma samples were then stored at -20°C. Plasma samples were used for prolactin analysis. Prolactin was measured by using Enzyme-Linked Immunosorbent assay kit (catalog number SEA846Bo, Cloud-Clone Corp., Katy, TX) according to the manufacturer's protocol. The intra- and interassay coefficients of variation were <5% and 7.8%, respectively.

Statistical Analysis

Data were analyzed as a completely randomized design using PROC MIXED (early dry period) or GLM (late dry period) procedures of SAS, and least squares

means $\pm$ standard error of the mean are reported. Gene expression data (delta cycle threshold) was analyzed and presented as $\Delta\Delta Ct \pm SEM$. For histological and gene expression analysis during the early dry period, the statistical model included treatment (HT and CL), time, and treatment by time (3, 7, and 14 d relative to dry-off) interaction with cow (treatment) as random effect. In the late dry period, contrast analysis (HTHT, CLCL, HTCL, CLHT) was performed. Also, for mammary cell proliferation and apoptosis proportion, the value at 3 d before dry-off was used as baseline and included in the model as covariate if significant. Differences between $0.05 \leq P \leq 0.10$ were considered as a tendency and differences with $P \leq 0.05$ were considered significant.

RESULTS

Mammary Gland Gene Expression

Early Dry Period (Before Switch). There was no effect of time ($P \geq 0.11$) or treatment by time interaction ($P \geq 0.14$) during the early dry period. However,

Table 1. Primers used in quantitative real-time PCR assay of gene expression in cows exposed to heat stress or cooling in the dry period

Gene	Primer	Sequence (5'-3')	Accession no.
<i>GAPDH</i>	Forward	TGACCCCTTCATTGACCTTC	NM_001034034.2
	Reverse	TACTCAGCACCAGCATCACC	
<i>RSP9</i>	Forward	GGAGACCCTTCGAGAAGTCC	NW_021639847.1
	Reverse	CTTTCTCATCCAGCGTCAGC	
<i>CASP3</i>	Forward	TGAAATACGAAGTCAGGATTAAA	NM_001077840.1
	Reverse	GTCCGTTGGTTCCAAAAATG	
<i>CASP8</i>	Forward	TTAGCATAGCACGGAAGCA	NM_001045970.2
	Reverse	TATCCAAAGCGTCTGCATCA	
<i>HSF1</i>	Forward	CAGCTGATGAAGGGGAAGCA	NM_001076809.1
	Reverse	TGGATGAGCTTGTGACGACT	
<i>HSP70</i>	Forward	GGGGAGGACTTCGACAACAGG	NM_203322.3
	Reverse	CGGAACAGGTCGGAGCACAGC	
<i>HSP90</i>	Forward	AGGCAGAGGCTGACAAGAATGACA	NM_001079637.1
	Reverse	AGCCAGAAGACAGGAGAGCTGTTT	
<i>PRLR-SF</i>	Forward	AGGTGACACTATAGAATAAGCAAC	NM_174155.3
	Reverse	GTACGACTCACTATAGGGAAGGC	
<i>PRLR-LF</i>	Forward	AAGGCCATGTGGAAGATTG	NM_174155.3
	Reverse	GATGACTGTGAGGACCAGCA	
<i>STAT5a</i>	Forward	GAAACATCACAAGCCCCATT	NM_001012673.1
	Reverse	TGAAGCGCAACAAGAAGGTA	
<i>CSN2</i>	Forward	GCTATGGCTCCTAAGCACAAAGA	KC993858.1
	Reverse	GGAAACATGACAGTTGGAGGAAG	
<i>BECN1</i>	Forward	GATGGAATAGGAACCACCAC	NM_001033627.2
	Reverse	AGTTGAGAAAGGCGAGACAC	
<i>ATG3</i>	Forward	GGTTGTTTCGGCTATGATGAG	NM_001075364.1
	Reverse	GGGAGATGAGGGTGATTTTC	
<i>ATG5</i>	Forward	TTTGAATATGAAGGCACACC	NM_001034579.2
	Reverse	TGTAAACCCATCCAGAGTTG	
<i>IGFR1</i>	Forward	GGGCTGAGTTGGTGGATG	NM_001244612.1
	Reverse	CTCCAGCCTCCTCAGATCAC	

¹*RSP9* = ribosomal protein S9; *CASP3* = caspase 3; *CASP8* = caspase 8; *HSF1* = heat shock factor 1; *HSP70* = heat shock protein 70; *HSP90* = heat shock protein 90; *PRLR-SF* = prolactin receptor short form; *PRLR-LF* = prolactin receptor long form; *STAT5a* = signal transducer and activator of transcription 5A; *BECN1* = beclin-1; *ATG3* = autophagy related 3; *ATG5* = autophagy related 5; *IGFR1* = insulin-like growth factor 1 receptor.

Figure 1. Mammary gland gene expression during the early dry period of Holstein dairy cows. Multiparous cows were exposed to either heat stress or cooling (HT vs. CL, $n = 13/\text{treatment}$) during the early dry period (3, 7, and 14 d relative to dry-off). Quantitative real-time PCR was performed to assess the expression of *CASP3*, *CASP8*, *HSF1*, *HSP70*, *HSP90*, *IGF1R*, *PRLR-SF*, *PRLR-LF*, *STAT5a*, *BECN1*, *CSN2*, *ATG3*, and *ATG5* genes. Data are presented as fold change (HT relative to CL). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, † $P = 0.10-0.05$.

the analysis of mRNA extracted from mammary gland tissue showed effects of treatment on genes related to apoptosis and cell proliferation. Cows exposed to HT during early dry period showed downregulation of autophagy-related genes, such as *ATG3*, *ATG5*, and *BECN1* relative to CL cows ($P \leq 0.05$, respectively; Figure 1). Heat-stressed cows tended to have down-

regulation of *CASP3* ($P \leq 0.10$; Figure 1). Also, cows exposed to HT tended to have downregulation of *IGF1* ($P \leq 0.10$; Figure 1) and downregulated expression of *PRLR-LF*, *HSF1*, and *HSP90* ($P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.05$, respectively; Figure 1) relative to CL. During early dry period, no differences were observed in expression of *CASP8*, *STAT5a*, *CSN2*, and *PRLR-SF* ($P \geq 0.16$).

Late Dry Period (After Switch). Analysis of mRNA extracted from mammary gland tissue showed that cows exposed to heat stress only during the proliferative phase late in the dry period (CLHT) had higher expression of genes related to apoptosis, autophagy, and proliferation relative to CLCL and HTCL. Relative to CLCL, contrast analysis showed that CLHT-treated cows tended to upregulate expression of genes related to autophagy (*ATG3*, $P \leq 0.10$). Genes associated with apoptosis (*CASP3* and *CASP8*; $P \leq 0.01$) and *PRLR-LF*, *STAT5a*, and *CSN2* expression were significantly upregulated in CLHT relative to CLCL ($P \leq 0.05$; Table 2). Relative to CLCL and HTCL, CLHT cows upregulated expression of *HSP70* and *HSP90* ($P \leq 0.01$). In addition, CLHT cows had upregulation of *PRLR-LF*, *STAT5a* ($P \leq 0.10$), *ATG5*, *BECN1*, *IGF1R* (all $P \leq 0.05$), *ATG3*, *CASP3*, *CASP8*, and *CSN2* expression relative to HTCL (all $P \leq 0.01$; Table 2).

Mammary Gland Histology

TUNEL. We did not observe a time effect ($P \geq 0.43$) or treatment by time interaction in the response ($P \geq 0.18$) in the early dry period. However, epithelial,

Table 2. Summary of $\Delta\Delta\text{Ct} \pm \text{SEM}$ of genes related to prolactin signaling, development, apoptosis, and heat shock in the mammary gland of cows exposed to heat stress (HT) or cooling (CL) in the dry period¹

Gene ²	HTHT	CLHT	HTCL
<i>ATG3</i>	-0.68 ± 1.21	$-2.10 \pm 1.15^\dagger$	1.08 ± 1.08
<i>ATG5</i>	-0.16 ± 1.51	-1.43 ± 1.44	1.38 ± 1.35
<i>BECN1</i>	0.12 ± 1.13	-1.70 ± 1.08	0.92 ± 1.01
<i>CASP3</i>	-0.90 ± 1.27	$-3.78 \pm 1.21^{**}$	0.31 ± 1.14
<i>CASP8</i>	-1.37 ± 0.61	$-2.53 \pm 0.67^{**}$	-0.08 ± 0.67
<i>HSF1</i>	0.66 ± 0.42	0.28 ± 0.42	0.34 ± 0.38
<i>HSP70</i>	0.47 ± 0.57	$-1.63 \pm 0.54^{**}$	0.12 ± 0.52
<i>HSP90</i>	-0.76 ± 0.78	$-2.08 \pm 0.74^{**}$	0.31 ± 0.70
<i>IGF1R</i>	0.67 ± 0.87	-0.85 ± 0.87	1.01 ± 0.78
<i>PRLR-LF</i>	0.63 ± 0.91	$-1.99 \pm 0.91^*$	0.29 ± 0.83
<i>PRLR-SF</i>	-0.19 ± 2.88	-1.94 ± 2.48	1.75 ± 2.28
<i>STAT5a</i>	-0.09 ± 0.46	$-1.19 \pm 0.42^*$	-0.27 ± 0.45
<i>CSN2</i>	-1.56 ± 1.19	$-2.53 \pm 1.14^*$	-0.63 ± 1.12

¹Multiparous cows were exposed to 1 of 4 treatments: heat stress (HTHT, $n = 5$) or cooling (CLCL, $n = 7$) during the entire dry period; HT first 21 d dry, then CL until calving (HTCL, $n = 8$); or CL first 21 d dry, then HT until calving (CLHT, $n = 6$) during the late dry period (25 d relative to dry-off).

²*CASP3* = caspase 3; *CASP8* = caspase 8; *HSF1* = heat shock factor 1; *HSP70* = heat shock protein 70; *HSP90* = heat shock protein 90; *PRLR-SF* = prolactin receptor short form; *PRLR-LF* = prolactin receptor long form; *STAT5a* = signal transducer and activator of transcription 5A; *BECN1* = beclin-1; *ATG3* = autophagy related 3; *ATG5* = autophagy related 5; *IGF1R* = insulin-like growth factor 1 receptor.

Fold changes are shown CLCL relative to HTHT, CLHT, and HTCL. † $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$.

Figure 2. Mammary immunohistochemistry of apoptotic and proliferating cells during the early dry period of Holstein dairy cows. Multiparous cows were exposed to either heat stress or cooling (HT vs. CL, $n = 13/\text{treatment}$) during the early dry period. Mammary biopsies were collected at d 3, 7, and 14 d relative to dry-off. For apoptosis analysis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. For cell proliferation analysis, Ki67 assay was performed. Mammary gland tissue was visualized at 40 $\times$ for apoptosis and proliferation proportion. Proportion of cells undergoing apoptosis (panels A, C, and E) and proliferation (panels B, D, and F) in mammary epithelial cells (MEC), stromal cells, and total mammary cells (including MEC and stromal cells) are presented. During the late dry period, no differences were found among treatments. Data are presented as LSM $\pm$ SEM. $\dagger P \leq 0.10$; $*P \leq 0.05$.

stromal, and the total proportion of cells undergoing apoptosis were decreased by HT in the early dry period ($P \leq 0.05$, Figure 2). In the late dry period, no differences were observed among treatments for epithelial, stromal, or total proportion of cells undergoing apoptosis ($P \geq 0.44$, Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17911>).

Ki67 Antigen Localization. Similar to apoptosis, we did not observe an effect of time ($P \geq 0.35$) or treatment by time interaction ($P \geq 0.34$) in the early dry period. However, epithelial, stromal, and total pro-

portion of cells undergoing proliferation were decreased with HT in the early dry period (Figure 2). In the late dry period, we did not observe differences among the treatments for epithelial, stromal, and total proportion of cells undergoing proliferation ($P \geq 0.56$; Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17911>).

Trichrome Staining. Analysis of connective tissue showed a treatment by time interaction in the early dry period ($P \leq 0.05$). Cows exposed to HT treatment had less connective tissue in their mammary glands 3 d after dry-off ($P \leq 0.05$, Figure 3). However, in the late dry

Figure 3. Mammary connective tissue area in dry Holstein dairy cows. Multiparous cows were exposed to either heat stress or cooling (HT vs. CL, $n = 13/\text{treatment, trt}$) during the early dry period (panel A). In the late dry period (panel B), multiparous cows were exposed to 1 of 4 treatments: heat (HTHT, $n = 5$) or cooling (CLCL, $n = 7$) during the entire dry period; HT first 21 d dry, then CL until calving (HTCL, $n = 8$); or CL first 21 d dry, then HT until calving (CLHT, $n = 6$). For trichrome analysis, Masson's trichrome stained mammary gland tissue was visualized at $20\times$ and the proportions of connective tissue in the mammary gland are represented as $\text{LSM} \pm \text{SEM}$. Cows exposed to HT had a lower proportion of connective tissue 3 d after dry-off relative to CL cows ($P \leq 0.05$). In the late dry period, contrast analysis showed that HTHT and HTCL had a higher proportion of connective tissue relative to CLCL ($P \leq 0.05$). $*P \leq 0.05$.

period HTHT and HTCL cows had higher connective tissue compared with CLCL cows ($P \leq 0.05$; Figure 3). Also, CLHT cows tended to have lower connective tissue relative to HTHT and HTCL ($P \leq 0.10$).

Alveoli Number and Area. Alveoli number showed no treatment ($P = 0.25$) or time effect ($P = 0.82$), but we observed a treatment by time interaction ($P \leq 0.01$) in the early dry period. The HT cows had more mammary alveoli number 14 d after dry-off relative to CL cows ($P \leq 0.01$; Figure 4). However, in the late dry period no differences were observed among treatments ($P = 0.52$; Figure 4). We did not observe a treatment, time, or treatment by time interaction effect ($P \geq 0.43$) for alveoli area in the early dry period. In contrast, in the late dry period, the mammary glands of HTHT cows had larger alveoli compared with CLCL ($P \leq 0.05$; Figure 4).

Plasma Prolactin Concentrations

No difference was observed between treatments when prolactin concentration was measured 1 d before the switch (CL = 77.1 ± 10.4 ng/mL, HT = 61.4 ± 10.4 ng/mL; $P = 0.29$). However, in the late dry period, contrast analysis showed that CLHT cows had higher prolactin concentration relative to HTCL cows ($P \leq 0.05$, Figure 5), but no differences were detected for any other comparison ($P = 0.21$). Also, when prolactin (PRL) concentration values from HTHT and CLHT,

and CLCL and HTCL were combined to compare exposure to heat stress (HTHT + CLHT = 89.1 ± 12.0 ng/mL) or cooling (CLCL + HTCL = 59.9 ± 12.0 ng/mL) in the late dry period, heat stress was associated with elevated concentrations of PRL relative to cooling ($P \leq 0.10$).

DISCUSSION

It is well known that heat stress during the dry period has a negative effect in the next lactation (Tao et al., 2012; Fabris et al., 2017). Recently, our group reported that cows exposed to heat stress at any time, either during the early or late dry period, have reduced milk yield in the next lactation when compared with cows that were exposed to cooling during the entire dry period (Fabris et al., 2019). This study was conducted to examine some of the mechanisms whereby heat stress during the early and late dry period compromises mammary gland cellular turnover and reduces milk yield in the subsequent lactation.

After dry-off, the mammary gland goes through a period of involution that is driven by autophagy and apoptosis (Wilde et al., 1997; Sorensen et al., 2006). Thus, our focus was to determine how heat stress affects involution by altering expression of genes related to autophagy and apoptosis during the early dry period. Our data demonstrate that HT downregulates expression of genes related to autophagy and apoptosis

compared with CL. These results are in accord with Wohlgemuth et al. (2016), where it was demonstrated that heat stress decreases expression of microtubule-associated protein light chain 3. Also, it is known that heat stress increases the expression of heat shock proteins that have an anti-apoptotic effect (Seigneuric et al., 2011). However, in the present study, cows exposed to CL upregulated expression of *HSF1* and *HSP90* during involution (early dry period), but this increase did not appear to have a negative effect on cell loss, perhaps because cooled cows also had upregulation of *CASP3*, an apoptotic regulator during involution. Thus, our results indicate that HT drives changes in gene expression consistent with a delayed process of involution and, perhaps, redevelopment.

Apoptosis and autophagy are cellular mechanisms responsible for programmed destruction and recycling of cells (Capuco et al., 1997; Tao et al., 2011). Apoptotic activity is higher immediately after dry-off (Annen et al., 2008) and declines as parturition approaches

(Sorensen et al., 2006). Caspase 3 is a key factor in the apoptotic response to breast cancer, where loss of expression or function can lead breast cancer cell to apoptotic resistance in response to certain apoptotic stimuli (Devarajan et al., 2002). Thus, the lower MEC, stromal, and total proportion of cells undergoing apoptosis in HT cows is consistent with downregulation of *CASP3* expression that we observed in our study. Also, in our study we found that HT downregulates expression of *ATG3* and *ATG5*. A recent study reported that *ATG5* modulates the immune system and there is cross talk with apoptosis (Xin et al., 2018). Further, *ATG5* is indispensable for autophagic vesicle formation, and if *ATG5* is reduced, there is downregulation or total inhibition of autophagy (Pierdominici et al., 2012). Therefore, apoptosis and autophagy are important for MEC renewal and cell proliferation during the late dry period (Zarzyńska et al., 2007; Sobolewska et al., 2009), and our data suggest that HT compromises the progression of both processes.

Figure 4. Multiparous cows were exposed to either heat stress or cooling (HT vs. CL, $n = 13/\text{group}$) during the early dry period. In the late dry period, multiparous cows were exposed to 1 of 4 treatments: heat (HTHT, $n = 5$) or cooling (CLCL, $n = 7$) during the entire dry period; HT first 21 d dry, then CL until calving (HTCL, $n = 8$); or CL first 21 d dry, then HT until calving (CLHT, $n = 6$). Alveoli number (panels A and C) showed no treatment ($P = 0.25$) or time effect ($P = 0.82$), but we observed a treatment (trt) by time interaction ($P \leq 0.01$) in the early dry period. The HT cows had more alveoli number 14 d after dry-off relative to CL ($P \leq 0.01$). We did not observe a treatment, time, or treatment by time interaction effect ($P \geq 0.43$) for alveoli area (panels B and D) in the early dry period. However, in the late dry period, HTHT cows tended to have larger alveoli relative to CLCL cows ($P \leq 0.10$). Data presented as LSM $\pm$ SEM. $\dagger P \leq 0.10$; $**P \leq 0.01$.

During the early dry period, our data indicate that HT downregulates expression of *IGF1R* and *PRLR-LF*. The IGF1 pathway plays a critical role in cell growth (ter Braak et al., 2017) and downregulation of *IGF1R* expression caused by HT might compromise cell growth. Whereas cell loss predominates as involution progresses, it is clear that there is also a component of proliferation during this early dry period and heat stress compromises its initiation. Perhaps the suppression of *IGF1R* with HT slows that proliferative process initiated early in the dry period in comparison with cows that are CL. In addition to its role in mammary development, prolactin signaling is important for lactogenesis (Freeman et al., 2000; Tucker, 2000). The observation that PRL tended to increase in the cows that were exposed to heat stress late in the dry period (CLHT) may be associated with an early initiation of lactogenesis, and thus reduced proliferation compared with cooled cows. The increase in PRL may also be associated with the earlier calving date of the cows exposed to heat stress in late gestation, as reported in Fabris et al. (2019). However, when days before calving was included in the model the significant difference remained, indicating that the difference in PRL was likely not due to the earlier calving date. In addition, the cellular effectiveness of increased PRL signaling was confirmed by upregulation of *CSN2* expression, which is the most pronounced PRL-induced gene in mammary tissue (Bole-Feysot et al., 1998). Also *STAT5a*, which is the major downstream mediator involved in PRL signaling, was upregulated in CLHT

cows relative to CLCL, although we recognize the limitation of *STAT5A* mRNA expression as an indicator of activity of the phosphorylated ligand.

Of interest to the present observations, it has previously been demonstrated that photoperiod manipulation has similar effects to the responses demonstrated in the current study. Similar to the heat stress effect, cows exposed to long day photoperiod have higher circulating PRL and lower mammary cell proliferation during the dry period relative to cows exposed to short day photoperiod (SDPP; Wall et al., 2005; Dahl et al., 2012). Cows exposed to SDPP produced approximately 3 kg/d more in the subsequent lactation relative to cows exposed to long day photoperiod during the dry period (Auchtung et al., 2005). The higher milk yield of cows exposed to SDPP during the dry period is associated with increased mammary gland development during the dry period (Wall et al., 2005). However, cows did not improve milk yield in the subsequent lactation if provided with SDPP only during late dry period (i.e., last 21 d; Reid et al., 2004). Similarly, milk yield was not improved in cows provided with cooling during the last 21 d of the dry period (Fabris et al., 2019).

Heat stress during involution reduced the proportion of MEC, stromal, and total cell proliferation, which agrees with previous data that showed that cows exposed to HT from the time of dry-off had lower total cell proliferation –20 d relative to calving (Tao et al., 2011). A switch from CL in the early dry period to HT after 21 d triggered upregulation of autophagy and apoptosis-related gene expression, consistent with a reduction in proliferative activity. This induction of autophagy and apoptosis during a period where cows are shifting to a more rapid proliferation rate might explain why these cows produced less milk in the next lactation as demonstrated previously (Fabris et al., 2019). Also, early activation of genes related to cell proliferation might be related to the increase in PRL discussed above. Further, the fact that cows exposed to HT at any time during the dry period calve approximately 5 d before cows exposed to CL treatment for the entire dry period (Fabris et al., 2019) is additional support for the idea that HT cows may initiate lactogenesis earlier than in CL cows. Thus, enhanced autophagy and apoptosis and a shorter duration of proliferation appear to combine to reduce mammary growth with HT late in the dry period.

The lack of differences in mammary gland microstructure after the switch from CL to HT as the proliferative phase accelerates are inconsistent with the observed effects on PRL and gene expression. However, it may be that the timing of mammary gland biopsy collection was early enough to observe differences in gene expression, yet too soon to observe changes at the histological

Figure 5. Prolactin concentration during the late dry period of Holstein dairy cows exposed to heat stress (HTHT and CLHT; $n = 8$ cows/treatment) or cooling (CLCL and HTCL; $n = 8$ cows/treatment) in the late dry period. Blood samples were collected from coccygeal vessels and plasma samples were used for prolactin concentration analysis. Samples were collected 1 d before the switch and 3 d after cows were switched. The sample collected before the switch was used as a baseline and included in the model as a covariate. Following the switch, CLHT had greater circulating prolactin relative to HTCL cows. Data presented as LSM $\pm$ SEM. * $P \leq 0.05$.

level. Further studies to resolve the temporal relationship of gene expression, hormonal shifts, and ultimate effects on mammary development are necessary to fully understand the later dry period effects of heat stress.

CONCLUSIONS

Cows exposed to HT during involution have down-regulation of *CASP3*, *ATG3*, *ATG5*, *IGF1R*, and *PRLR-LF* gene expression in the mammary gland, which leads to compromised mammary gland turnover as observed in lower proportion of MEC, stromal, and total cell apoptosis and development. Later in the dry period, in the proliferative phase, abrupt exposure to HT activated autophagic, apoptotic, and proliferative gene expression, and increased circulating prolactin concentrations relative to CL. Therefore, lower milk yield after dry period HT appears to be driven by slower mammary cell loss immediately after cessation of milk removal, continued enhancement of mammary cell loss later in the dry period, and an overall reduction in the duration of mammary growth with earlier calving when compared with cows that are cooled throughout the dry period.

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ORCIDS

- Thiago F. Fabris <https://orcid.org/0000-0001-8197-2159>
- Jimena Laporta <https://orcid.org/0000-0002-3186-5360>
- Amy L. Skibiel <https://orcid.org/0000-0002-2729-0402>
- Bethany Dado-Senn <https://orcid.org/0000-0002-6104-7334>
- Stephanie E. Wohlgemuth <https://orcid.org/0000-0002-2900-2777>
- Geoffrey E. Dahl <https://orcid.org/0000-0002-2182-6317>