

In utero hyperthermia impacts the histological and transcriptomic signature of the mammary fat pad of newborn heifers

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Abstract

Development of the mammary gland begins *in utero* and requires coordinated interactions between the epithelial rich parenchyma and the surrounding mammary fat pad (mFP). In this study we investigated how maternal heat stress (HS) during gestation impacts the development of the offspring mFP.

Mammary fat pad tissue was collected from Holstein heifers at birth that were gestated under either HS or active cooling conditions for the last 54 days of gestation in a subtropical summer. Heifers exposed to *in utero* HS exhibited a higher number of small adipocytes and fewer large adipocytes. Additionally, there were 122 differentially expressed genes between *in utero* HS and *in utero* cooled heifers. Genes and pathways impacted by *in utero* HS were related to lipogenesis, intracellular signaling, extracellular matrix remodeling, insulin signaling, and cytoskeleton remodeling. Nuclear receptors, transcription factors, and growth factors were predicted to be inactivated due to *in utero* HS. These histological and transcriptomic results indicate a coordinated suppression of cell differentiation, lipid metabolism, hormone/nutrient sensing, and stress adaptation. These early-life disruptions of adipose development may ultimately compromise mammary gland development and future lactation performance.

INTRODUCTION

Across a heifer's lifetime, she undergoes multiple stages of mammary gland development in preparation for successful lactation. The foundation for future development begins *in utero* through coordinated interactions between the developing mammary parenchyma (**mPAR**, which includes epithelial ducts, lobular structures, and associated intralobular stroma) and the surrounding tissue known as the mammary fat pad (**mFP**) [1, 2]. At birth, the mammary gland consists of a small mPAR with a rudimentary epithelial ductal tree whereas mFP tissue constitutes the majority of the gland's volume [3]. Across the preweaning period, the mPAR expands rapidly into the mFP, growing 25-fold in size from birth until ~ 60 d of age while the mFP doubles in size during the same time [4]. During the subsequent peripubertal period, both tissue types continue to grow, whereby the mPAR undergoes ductal elongation and alveolar expansion into the mFP tissue in response to hormonal, metabolic, and developmental cues [5]. Because each developmental stage builds upon prior mammary growth and development, perturbations during this period can have negative effects on growth trajectory and lactation potential, emphasizing the importance of understanding mammary development early in life.

The mFP is not merely a structural matrix but a metabolically active and hormonally responsive depot that supports epithelial morphogenesis. The ability of different subtypes of mammary mesenchymal tissue to drive divergent effects on mammary epithelium has been established in rodent models [6]. Additionally, transplanted mammary epithelium can form ductal growths in cleared mFP and other adipose depots, but not other locations, such as subcutaneous sites or the peritoneal cavity, providing further evidence of the necessity of adipose tissue for early mammary development [7]. The amount of mFP tissue is also thought to determine the extent of mPAR growth [7]. Additionally, mammary epithelial growth and proliferation can be impacted by the stiffness of its supporting extracellular matrix [8]. Matrix metalloproteinases are found in bovine tissues and have been suggested to degrade extracellular matrix

components to enable ductal branching [9, 10]. In addition to providing a structural scaffold for future parenchymal development, the mFP also serves as a metabolic reserve and a signaling hub [1]. Rodent models suggest that epithelial cells can directly metabolize fatty acids derived from the mFP [11]. The mFP can also respond to hormonal cues such as growth hormone, estrogen, epidermal growth factor, and progesterone [1]. In response, the mFP can produce IGF-I, adipokines, and other signaling molecules to drive epithelial growth [12]. Given the dynamically plastic nature of the mFP and its critical role in supporting mammary gland morphogenesis, early-life alterations in fat pad composition or adipocyte function may have lasting effects on mammary gland development and function later in life.

The intrauterine environment plays a critical role in programming tissue development, including the mammary gland. Environmental stressors such as maternal heat stress during fetal development can induce long-term alterations in metabolic, endocrine, and developmental pathways, with consequences for postnatal mammary gland function [13]. First-lactation heifers exposed to *in utero* heat stress produce less milk than their counterparts gestated by cows with access to heat abatement to maintain thermoneutrality [14, 15]. The negative effects of *in utero* heat stress on mPAR structure and function are long lasting and evident at birth, weaning, puberty, and first lactation [3, 16, 17]. Effects on mPAR include reduced epithelial structure development, reduced proliferation, reduced mammary signaling, increased connective tissue deposition, and altered signaling pathways [3, 13, 16, 17]. Additionally, *in utero* heat stress reduces the weight of the mFP at birth [3]. However, despite its importance for mammary gland development, the specific effects of *in utero* heat stress on mFP adipocyte development and its consequences for gene expression remain largely unexplored.

Due to the negative effects of *in utero* heat stress on mammary gland growth and lactation potential, as well as the importance of the mFP to mPAR development, we hypothesized that *in utero* heat stress disrupted normal mFP adipocyte development by altering genes regulating tissue growth and metabolism, adipogenesis and lipogenesis, and parenchymal-adipose interactions. The objective of this study was to characterize the microstructure and transcriptomic signature of mFP adipocytes by assessing adipocyte number and size distribution, and gene expression profiles in neonatal heifers exposed to prenatal hyperthermia or not. This approach aimed to identify genes and biological pathways dysregulated by prenatal hyperthermia, providing a foundation for understanding how environmental stressors may program early mammary gland development and influence future lactation performance.

MATERIALS AND METHODS

Animals and treatments

All procedures were approved by the University of Florida Institutional Animal Care and Use Committee (protocol #201910599). All experiments were performed in accordance with relevant guidelines and regulations. This study is reported in accordance with the ARRIVE guidelines. Treatment details and cow management protocols are extensively described by [18].

Briefly, multiparous Holstein cows from a single commercial farm (Alliance Dairies, Trenton, FL, USA) were dried off at 54 ± 5 d before expected calving and housed in a free-stall barn during a subtropical summer. The average temperature humidity index throughout the study was 79, with hourly average temperature humidity index ranging from 75–83 throughout the study which indicates environmental heat stress in the absence of active cooling [19]. Under these conditions, cows were considered heat-stressed (HS) with respiration rates of 79 breaths/min and skin temperatures of 36°C from dry-off to calving. In contrast, cows housed during the same time under similar conditions receiving active heat abatement (cooling, CL) through fans and water soakers, had respiration rates within thermoneutral physiological ranges (54 breaths/min and skin temperatures to 37.8°C). Female heifers born to these cows were classified as either *in utero* heat-stressed (**iuHS**; $n = 8$) or *in utero* cooled (**iuCL**; $n = 8$) during late gestation.

Fat pad dissection and tissue collection

Heifers were euthanized and mammary gland tissue collected at birth (4 ± 2 h after calving), before colostrum feeding. A detailed description of euthanasia procedures and tissue collections can be found in [3]. Briefly, heifers were euthanized via captive bolt and exsanguination. The udder was removed within 10 min after exsanguination and bisected into the right and left portions along the median suspensory ligament. The right rear quarter was further dissected to collect mPAR and mFP for histological analysis. Tissue samples were placed in 4% paraformaldehyde at 4°C overnight, rinsed in increasing concentrations of ethanol (25%, 50%, 70%, and 100%), and transferred to 70% ethanol and stored at 4°C until embedded in paraffin and sectioned at $5\text{ }\mu\text{m}$ onto poly-L-lysine coated slides. The mPAR macro and microstructure results have been reported previously by our group [3]. Two pieces ($0.5 \times 0.5 \times 0.5\text{ cm}$) of mFP from the remaining quarters were cut from a region in close proximity to the mPAR, rinsed in sterile PBS, and stored in RNAlater (ThermoFisher, Invitrogen; #AM7020) overnight at 4°C and at -80°C until RNA isolation.

Histological evaluation of the fat pad

The mFP slides were stained with Masson's trichrome by the Molecular Pathology Core Laboratory at the University of Florida according to the manufacturer's protocol at standard times for animal tissue with histology grade xylene and graded ethanol series (Masson Trichrome Kit, Epredia; #87019). The stained slides were used for morphometric analyses that included quantification of adipocyte number and size (area) distribution. Tissue section scans were captured at 20x using an Olympus SLIDEVIEW VS200 Research Slide Scanner at the Laboratory for Toxicologic and Environmental Pathology at Michigan State University (East Lansing, MI). Using the OlyVIA viewer software (v. 14), images of scanned slides were exported to the Gnu Image Manipulation Program for further processing. Backgrounds were removed and images were sliced into 16 sub-images of equal size using horizontal and vertical guides. Two-dimensional adipocyte size analysis was performed on digitized 20x images of tissue sections using a standardized imaging and analysis pipeline applied uniformly across all samples. Quantification was

performed across the available tissue area within each section, rather than selecting individual fields of view, to minimize bias related to section size or tissue heterogeneity. Adipocyte morphometric analysis was completed using the Adiposoft jar for Fiji's ImageJ (v.2.0.0). As reported in [20], adipocyte counts were separated by area into predefined bins ranging from 200 μm^2 to 8000 μm^2 in increments of 200 μm^2 (i.e. 200–400, 401–600, up to 7801–8000 μm^2).

Adipocyte areas were compared between treatment groups using a chi-square test to evaluate differences in the proportion of adipocytes across bins (distribution-level comparison). Data were analyzed in SAS (v. 9.4 SAS Institute, Cary, NC) using PROC FREQ. For further analysis a *t* test was used to evaluate differences between treatments at the bin level. The percentage of adipocytes which fell into each bin was calculated by taking the number of adipocytes within the bin range divided by the total number of adipocytes of that animal. For visualization, data are presented as least square means (LSM) $\pm$ standard error (SEM), and significance was declared at $P \leq 0.05$ and tendency at $0.10 \geq P > 0.05$. Data was visualized using GraphPad Prism (v.9.0, Dotmatics, Boston, MA; <https://www.graphpad.com>).

RNA-sequencing and bioinformatics analysis

Fat pad samples (80–100 mg) were homogenized (Omni Tissue Master 125) using 1 mL Qiazol, and RNA was extracted using Qiagen RNeasy Plus Universal Mini Kit (Qiagen; #73404) following the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and a final amount of 1 μg was utilized for analysis. Samples with 28S/18S > 1 and an RNA integrity number ≥ 7.5 were used for library construction. Libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (#E7775; New England BioLabs) following the manufacturer's protocol. The libraries were sequenced using the Illumina NovaSeq 6000 platform (Illumina Inc.), generating 150 base-pair paired-end reads. All 16 samples were processed using the same library preparation workflow and sequenced within a single run, with randomization during library pooling and lane assignment to minimize potential batch and lane effects. Sequencing depth was targeted at approximately 10 Gb per sample (9.01 ± 0.77 ; average $\pm$ SD). The resulting RNA-sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE316291.

RNA-seq data were analyzed using a standardized bioinformatics pipeline on the High-Performance Computing Cluster (HPCC) at Michigan State University, utilizing modules and SLURM-based job scheduling for scalable processing. Raw pair-end FASTQ files were first assessed for base quality, duplication levels, and adapter contamination using FastQC v0.11.9 (Andrews, 2010). Summary reports were generated using MultiQC v1.14 [21]. Reads were trimmed using Trimmomatic v0.40, [22] with parameters set to remove low-quality bases (Phred score < 20) and adapter sequences. Only reads ≥ 20 bp in length were retained. Transcript quantification was performed with Salmon (v1.10.2) using the decoy-aware selective-alignment workflow: a "gentrome" index was built by concatenating the species transcriptome with the reference genome and supplying a decoy list (k-mer 31); quantification ran with --

validateMappings, sequence-specific and GC-bias correction (`--seqBias --gcBias`), automatic library-type detection, and 30 bootstrap replicates to capture inferential uncertainty. When needed, transcript FASTA was generated from the genome and GTF using gffread prior to indexing. Transcript-level estimates (TPM and counts) were imported into R with tximport and summarized to genes using effective-length-aware aggregation (`countsFromAbundance = "lengthScaledTPM"`). Software versions, command lines, Salmon logs, and QC reports were archived for reproducibility [23, 24].

Statistical power for differential expression analysis was estimated using the RNASeqPower R package, which models RNA-seq count data with a negative binomial framework and incorporates mean sequencing depth, gene-level biological variability (median CV across expressed genes), minimum detectable effect size, and an FDR of 0.05. Power was calculated for the original design ($n = 8$ animals per group). Power curves were also generated for sample sizes ranging from 3 to 20 per group to evaluate power for detecting genes with fold changes of 1.5–2.0.

Differential expression, visualization, and pathway analysis

Differential gene analyses were performed in R v4.3.1 using DESeq2 v1.38.3 [25]. Count matrices were filtered to remove low-expression genes and normalized using variance-stabilizing transformation. Principal component analysis was conducted to assess sample clustering, and two samples from each treatment were flagged. The four samples exhibited markedly sparse expression profiles, with approximately 40–50% (iuCL group: CL3-40.1%, CL6-44.3%; iuHS: HS1-44.95%, HS6-40%) of genes showing zero counts after alignment and summarization. These samples were removed resulting in $n = 6$ for both iuHS and iuCL groups. A post hoc power analysis confirmed that statistical power remained > 0.8 following sample exclusion. Differential gene expression was assessed using DESeq2's Wald test, and we considered differentially expressed genes (DEG) as those which had a false discovery rate (FDR) ≤ 0.05 and $|\log_2 \text{fold-change}| \geq 1.5$. Using a more permissive significance cutoff, nominally significant genes (NSG) with an unadjusted P -value ≤ 0.05 and $|\log_2 \text{fold-change}| \geq 1.5$ were used for functional enrichment in Ingenuity Pathway Analysis (IPA; QIAGEN Inc.; <https://www.qiagenbioinformatics.com>) to explore biologically relevant pathways. Core analysis was performed using default parameters, with pathway significance evaluated using Fisher's exact test. IPA output was used to interpret regulatory networks, upstream regulators, and canonical pathways. Pathways and upstream regulators with a P -value ≤ 0.05 and $|z\text{-score}| \geq 2$ and at least four NSG were retained within our dataset.

RESULTS

Adipocyte histology

Histological analysis revealed a difference in frequency distributions between iuHS and iuCL heifers (X^2 , $P < 0.01$). Heifers from the iuHS group exhibited a greater percent of small adipocytes ($400\text{--}600 \mu\text{m}^2$, $P = 0.04$; $600\text{--}800 \mu\text{m}^2$, $P = 0.08$) and smaller percent of large adipocytes ($1600\text{--}1800 \mu\text{m}^2$, $P = 0.05$;

2000–2200 μm^2 , $P = 0.06$; 2400–2600 μm^2 , $P = 0.06$; 2600–2800 μm^2 , $P = 0.09$; and 5000–5200 μm^2 , $P = 0.06$) within the mFP compared with iuCL heifers (Fig. 1).

Differentially expressed genes

Transcriptomic analysis identified 122 DEG in the mFP of iuHS heifers ($\text{FDR} \leq 0.05$, $|\log_2 \text{fold-change}| \geq 1.5$), including 40 upregulated and 82 downregulated genes. The downregulated genes were primarily related to lipid metabolism, mitochondrial activity, and adipocyte differentiation (e.g., *ACACA*, *PPARGC1A*, *GPAM*), whereas the upregulated genes were associated with signaling and cytoskeletal regulation (e.g., *PRKACA*, *GNB1*, *PLD2*). The top 30 DEG ranked by fold change, including the gene name and mean expression are presented in Table 1. The full list of DEG is provided in **Supplementary Table S1**.

Table 1

Top differentially expressed genes in the mammary gland fat pad of *in utero* heat-stressed (iuHS) Holstein heifers at birth compared to *in utero* cooled counterparts (iuHS; n = 6/treatment). Genes are ranked by log₂ fold change (FC; |log₂FC| ≥ 1.5) and have a false discovery rate (FDR) < 0.05.

Symbol	Gene Name	Mean ¹	log ² FC	FDR
PDE2A	Phosphodiesterase 2A	93.7	-25.0	6.75E-13
GOLGB1	Golgin B1	82.8	-24.8	6.75E-13
ACACA	Acetyl-CoA Carboxylase Alpha	72.3	-24.6	7.61E-13
TTC7A	Tetratricopeptide Repeat Domain 7A	68.1	-24.5	7.92E-13
TNS3	Tensin 3	63.1	-24.4	8.95E-13
EYA3	EYA Transcriptional Coactivator and Phosphatase 3	61.8	-24.4	8.95E-13
ARHGAP17	Rho GTPase Activating Protein 17	83.0	-24.2	1.20E-12
UTP20	UTP20 Small Subunit Processome Component	80.4	-9.9	9.54E-05
ALMS1	Alstrom Syndrome 1	62.4	-9.5	0.0053
REN	Renin	360.6	-3.4	0.0025
TGM3	Transglutaminase 3	510.4	-2.9	0.0065
TFRC	Transferrin Receptor	403.0	-2.5	0.0004
AKAP12	A-Kinase Anchoring Protein 12	1047.2	-2.2	0.0086
CLINT1	Clathrin Interactor 1	50.3	-2.2	0.0068
ATP13A3	ATPase 13A3	94.9	-2.1	0.0023
TREM2	Triggering Receptor Expressed on Myeloid Cells 2	245.8	1.5	0.0005
RASGRP4	RAS Guanyl Releasing Protein 4	244.3	1.5	0.0004
C2	Complement Component 2	450.9	1.5	7.03E-05
SELENBP1	Selenium Binding Protein 1	572.1	1.5	0.0005
¹ Mean represents the average normalized expression level of each gene across all samples in the dataset				

Symbol	Gene Name	Mean ¹	log ² FC	FDR
LTBP2	Latent Transforming Growth Factor Beta Binding Protein 2	2887.5	1.6	0.0073
RNF112	Ring Finger Protein 112	149.1	1.9	0.0063
SLC4A11	Solute Carrier Family 4 Member 11	137.1	1.9	0.0034
PLD2	Phospholipase D2	91.8	2.2	0.0083
UBAP2	Ubiquitin Associated Protein 2	51.2	9.1	0.0009
IP6K2	Inositol Hexakisphosphate Kinase 2	65.5	9.4	0.0004
MIER2	MIER Transcriptional Regulator 2	99.9	10.0	7.19E-05
NUP85	Nucleoporin 85	100.3	10.0	8.34E-05
PRKACA	Protein Kinase cAMP-Activated Catalytic Subunit Alpha	46.7	23.2	1.72E-11
AKAP13	A-Kinase Anchoring Protein 13	66.8	23.7	5.03E-12
GNB1	G Protein Subunit Beta 1	144.0	24.7	6.75E-13
¹ Mean represents the average normalized expression level of each gene across all samples in the dataset				

Pathways and upstream regulators impacted by in utero heat stress

A total of 662 NSG (unadjusted $P \leq 0.05$, $|\log_2 \text{fold-change}| \geq 1.5$) were recognized by IPA and used for pathway and upstream regulator analysis. Analysis using IPA revealed enrichment of 60 pathways ($P \leq 0.05$, $|z\text{-score}| \geq 2$) due to *in utero* heat stress. Among these, 58 pathways were predicted to be inhibited and 2 were predicted to be activated (heme signaling and PTEN signaling). The inhibited pathways included fatty acid metabolism (carnitine metabolism, fatty acid biosynthesis), cell communication (gap junction regulation, Rho GTPase signaling), and intracellular signaling cascades (PI3K/AKT and MAPK). Forty pathways most relevant to adipose biology and mammary development, organized by function, can be found in Fig. 2. The full list of enriched pathways can be found in **Supplementary Table S2**.

Using the same NSG, upstream regulator analysis by IPA predicted 23 regulators in the mFP ($|z\text{-score}| \geq 2$; $P \leq 0.05$), with 8 activated and 15 inhibited in the mFP of iuHS heifers. Inhibited regulators included ESR1 (estrogen receptor α), EGF (epidermal growth factor), and FOXO1 (forkhead box O1), while micro RNA regulating metabolism (mir-802, mir-145-5p) were predicted to be activated. Top candidates most directly related to adipose physiology and mammary development are presented in Fig. 3 and were

ranked by activation Z-score, with positive values (> 2) indicating predicted activation and negative values indicating predicted inhibition (< -2). The full list of upstream regulators is reported in **Supplementary Table S3**.

DISCUSSION

The mFP is a specialized adipose depot that interacts closely with the developing epithelial structures of the mammary gland. Beyond providing structural integrity for the developing epithelium, the mFP is a source of fatty acids and paracrine signals essential for epithelial proliferation, ductal elongation, and morphogenesis. The long-standing view that this depot is metabolically quiescent during early life has been challenged by rodent explant and *in vitro* studies, which demonstrate bidirectional biochemical communication between adipocytes and epithelial cells [26]. In these models, adipocyte-derived factors modulate epithelial branching and differentiation, while epithelial cues influence adipocyte maturation. This experiment provides evidence that *in utero* heat stress is associated with altered expression of genes involved in structural organization, metabolic support, and paracrine-related processes in the mammary fat pad at birth.

Studies in dairy heifers have shown that the mFP is metabolically sensitive and contributes to mammary development during early life. For example, a higher plane of nutrition during the preweaning period increases mFP mass and increases lipid deposition, which is paralleled by mPAR expansion within the mFP [27, 28]. These effects may be attributable to enhanced lipid availability from the mFP to the developing epithelium [11]. In response to *in utero* heat stress, the mFP is reduced in size at birth and has been previously noted to have a smaller average adipocyte size [3]. The present study further demonstrates that exposure to *in utero* heat stress alters the mFP adipocyte size distribution, with an increased number of smaller adipocytes and fewer large adipocytes, suggesting shift in the distribution to smaller adipocyte sizes. It is important to note that histological analysis measures the two-dimensional size of adipocytes which has the potential to underestimate the true three-dimensional adipocyte size.

The limited mFP size and smaller adipocytes may indicate a limited energy reserve, which could explain the reduced mPAR growth in response to heat stress *in utero* [3]. In further support of this concept, the mFP of heifers exposed to maternal heat stress exhibited coordinated downregulation of key genes involved in lipogenesis (*ACACA*, *GPAM*, *GK*), mitochondrial oxidative metabolism (*PPARGC1A*), and amino acid transport (*SLC7A8*), as well as predicted inhibition of pathways which govern carnitine metabolism and insulin signaling (e.g., GLUT4 translocation and PI3K/AKT signaling). Further, upstream analyses alluded to inhibition of triglyceride production (e.g., *ARID3A*) and adipocyte differentiation (e.g., *FOXO1*), as well as predicted upstream inhibition of glucose, suggesting limited energy substrate availability in the mFP of iuHS heifers. While not measured in this study, maternal energy status can affect the development of adipose tissue within the offspring [29, 30], which could contribute to the differences in adipocyte sizes and gene expression of this study.

In contrast to preweaning effects, enhanced mFP growth and adipocyte deposition within the mPAR postweaning due to increased energy intake are instead associated with impaired mPAR growth [31, 32]. These divergent effects of mFP energy reserve on driving mPAR growth could be due to other locally secreted factors, a dysregulated mFP, or simply a consequence of the timing of normal development. Our results indicate that there are changes in the development or functional capacity of mFP adipocytes in response to heat stress. For example, the downregulation of the *PPARGC1A* gene and predicted inhibition of the adipose tissue browning pathway observed could indicate that the mFP is immature or in a dysregulated state [33]. These molecular changes parallel the observed shift in adipocyte morphology, suggesting heat stress *in utero* may impair lipid synthesis and storage capacity at the cellular level. Taken together, these findings suggest that environmental changes *in utero* may alter adipogenesis and lipogenesis within the mFP of neonates, which could limit the energy available for future epithelial invasion and, later, influence structural and functional development of the mammary gland.

The stiffness and composition of mFP tissue can influence the growth and development of mPAR. This concept is supported by cancer and fibrotic studies linking stromal stiffness (e.g., collagen and other extracellular matrix protein deposition or viscoelasticity properties) to impaired epithelial dynamics [8]. In addition to the previously described tissue level changes, there are also transcriptional changes in cell-cell physical signaling mechanisms within the mFP of iuHS heifers. The present study reveals that expression of the *TNS3* gene as well as integrin signaling, gap junction trafficking and regulation, post-translational modifications of tubulin, LICAM interactions, and ILK signaling pathways are downregulated in response to *in utero* heat stress. While the physical interactions were not directly measured, this suggests that signaling induced by these cell-cell interactions may be decreased, which could impair future mPAR and mFP development.

The mFP is responsive to systemic hormones and can serve as a local source of paracrine signaling for developing epithelial structures. Important hormones which drive mFP development are thought to be growth hormone, epidermal growth factor, progesterone, and estrogen [1]. In the present study, the growth hormone signaling pathway was downregulated and upstream analysis indicated that both epidermal growth factor and estrogen receptor alpha were predicted to be inhibited in iuHS heifers. Perhaps *in utero* heat stress restricts growth and maturation of the mFP by reducing the tissue's sensitivity to endocrine signals; potentially through decreased receptor expression or impaired downstream signaling, despite the presence of systemic hormonal cues. While data in preweaned heifers is scarce, heat stress reduces circulating estradiol in peripubertal heifers [34] as well as decreases circulating estradiol and estrone sulfate in lactating cows [35, 36], compounding impaired signaling pathways. In further support of this concept, there are fewer epithelial cells expressing estrogen receptor alpha within the mPAR, and there a decrease in circulating estradiol in pubertal heifers exposed to heat stress *in utero* [17].

Importantly, under basal conditions and in response to systemic cues, the mFP expresses growth factors, hormones, and adipokines that can orchestrate mPAR morphogenesis. One of the most well

studied local factors is IGF-1, which can have positive effects in driving mPAR growth [1, 5]. The importance of IGF-1 in contributing to mPAR growth likely begins *in utero*, as IGF-1 receptors are located within the developing mammary epithelium of sheep *in utero* [37]. Within rodents, the mFP produces IGF-1 in response to growth hormone which can act synergistically with estrogen to increase production [38]. The growth hormone signaling pathway and upstream regulator ESR1 (estrogen receptor alpha), were predicted to be inhibited within our transcriptomic analysis. This suggests that growth hormone and estrogen signaling within the mFP may be impacted, possibly decreasing the production of mFP derived IGF-1 to stimulate mPAR growth. Future studies are needed to confirm this hypothesis and will require direct phenotypic and hormonal assessments.

Other local factors which are important for driving mPAR growth include fibroblast growth factors, keratinocyte growth factor, hepatocyte growth factor (HGF), vasoactive endothelial growth factor (VEGF), adipokines, and other cytokines [1, 12]. Our transcriptomic dataset indicates that VEGF, HGF, and nerve growth factor (NGF) signaling pathways are all downregulated in mFP of iuHS heifers. In the mFP of nulliparous mice, VEGF plays a role in the vascular development needed to support lactation [39]. Expression of NGF has been detected in the mFP of sheep around puberty and stimulates proliferation and also prevents apoptosis of a breast cancer cell line *in vitro* [40, 41]. *In vitro*, HGF was shown to induce proliferation and branching morphology of a bovine mammary epithelial cell line [42]. Direct measurements of these signaling factors and/or measurements during future developmental timepoints would be beneficial in future studies, yet the coordinated transcriptional downregulation of each of these signaling pathways may indicate a decreased ability to respond to a variety of growth factors.

Resident immune cells within the mFP also contribute to tissue remodeling by releasing cytokines that mediate epithelial–stromal crosstalk. While previously identified factors were not identified in our dataset, downregulation of genes (e.g. *TRIM*, *SMAD2*, *SMURF2*) and pathways (e.g. natural killer cell signaling, lymphotoxin- β receptor signaling, NF- κ B activation by viruses, and HMGB1 signaling) hint at possible dysregulation in immune/inflammatory signaling which could contribute to impaired mammary growth. In addition to the aforementioned signaling molecules, studies comparing mPAR and mFP transcriptomes in heifers during early mammary development predicted candidate paracrine factors and signaling molecules mediating inter-tissue communication (e.g. ANGPTL1, SPP1, IL1-B in mPAR; ADIPOQ, IL-13, FGF-2, LEP in mFP; Piantoni et al., 2010). Future studies on the effects of *in utero* exposure to heat stress should also include how secreted factors from the mPAR act upon the mFP.

While this study reports the effects of *in utero* heat stress on the mFP at birth, the long-term effects of *in utero* heat stress have not been well studied in the mFP. In contrast, the normal growth and development of the mPAR in response to *in utero* heat stress is impacted at least until a heifer's first lactation. *In utero* heat stress alters mPAR microstructure, leading to fewer epithelial ducts, reduced branching, and lower cellular proliferation at birth which are hallmarks of impaired tissue growth and organization [3]. Impairments in mPAR proliferation extend until weaning and into the peripubertal phase of mammary development [3, 17]. Ultimately, these perturbations on early mammary gland growth and development result in a mature gland that produces less milk, has altered alveolar structure, and an increased

proportion of connective tissue [16]. Through this investigation we are beginning to understand the early-life implication of heat stress on mFP development; however, the temporal effect of *in utero* heat stress on mFP development and the specific contribution of adipocyte morphology and molecular programming to these long-term outcomes remain poorly understood and are a needed area for further study.

CONCLUSIONS

In utero heat stress appears to disrupt mFP development at the microstructural and transcriptomic levels. The coordinated differential expression of transcription factors and upstream regulators suggests potential shifts in pathways linked to adipocyte differentiation, lipid handling, and metabolic regulation. The reported findings are based on gene expression data and do not reflect direct measurements of cellular function or signaling activity, yet they provide a transcriptional context that may help explain previously reported alterations in mammary tissue development following prenatal heat stress. Future studies integrating mFP and mPAR co-culture systems, functional assays, or spatial transcriptomic approaches will be necessary to directly evaluate the epithelial-stromal cross-talk and elucidate the mechanisms by which prenatal heat stress may influence mammary gland development and subsequent lactational performance in dairy cattle.

Declarations

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AUTHOR CONTRIBUTIONS

J.L. and G.E.D. were responsible for the conception, design, and funding of research project; J.L., G.E.D., and B.D.S. performed experiments and acquired tissue samples; M.N.M. and G.A.C. conducted histological analysis; J.R.D. and Z.Z. conducted bioinformatic analysis for gene expression data; J.L. and

M.C.G. interpreted results of experiments; M.C.G. prepared figures and drafted manuscript; all authors edited and revised manuscript drafts and approved final version of the manuscript.

DATA AVAILABILITY

RNA-sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE316291. Additional data provided within this manuscript is available upon reasonable request.

COMPETING INTERESTS

The authors declare no competing interests.

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Figures

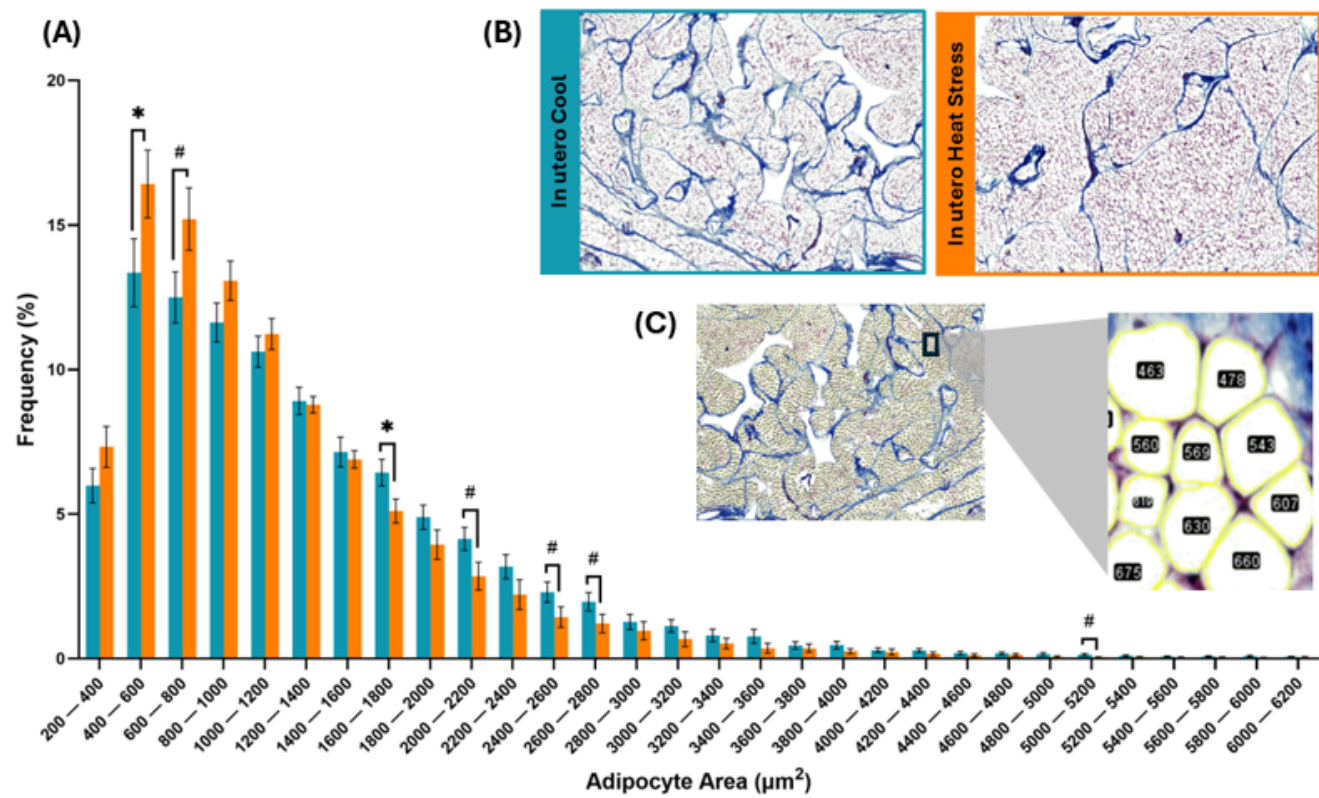


Figure 1

(A) Frequency distribution of adipocyte area sizes in the mammary gland fat pad of Holstein heifers at birth gestated by heat-stressed cows (iuHS, orange bars, n = 8) or gestated by cows with access to an evaporative cooling system (iuCL, teal bars, n = 8) during the last 54 ± 5 d of gestation in a subtropical humid summer. (B) Representative images of Masson's trichrome staining and (C) adipocyte count and size using the Adiposoft plugin in ImageJ Fiji (version 2.0.0). Chi-square analysis indicated a significant alteration in adipocyte distribution between treatments ($P < 0.001$). Adipocytes larger than 6,000 μm^2 are omitted from the graph. Displayed data are treatment means ± SEM for visualization of treatment differences. Percentages of adipocytes within each bin were compared between treatments using a t test. * indicates $P \leq 0.05$ and # indicates tendency at $0.10 \geq P > 0.05$.

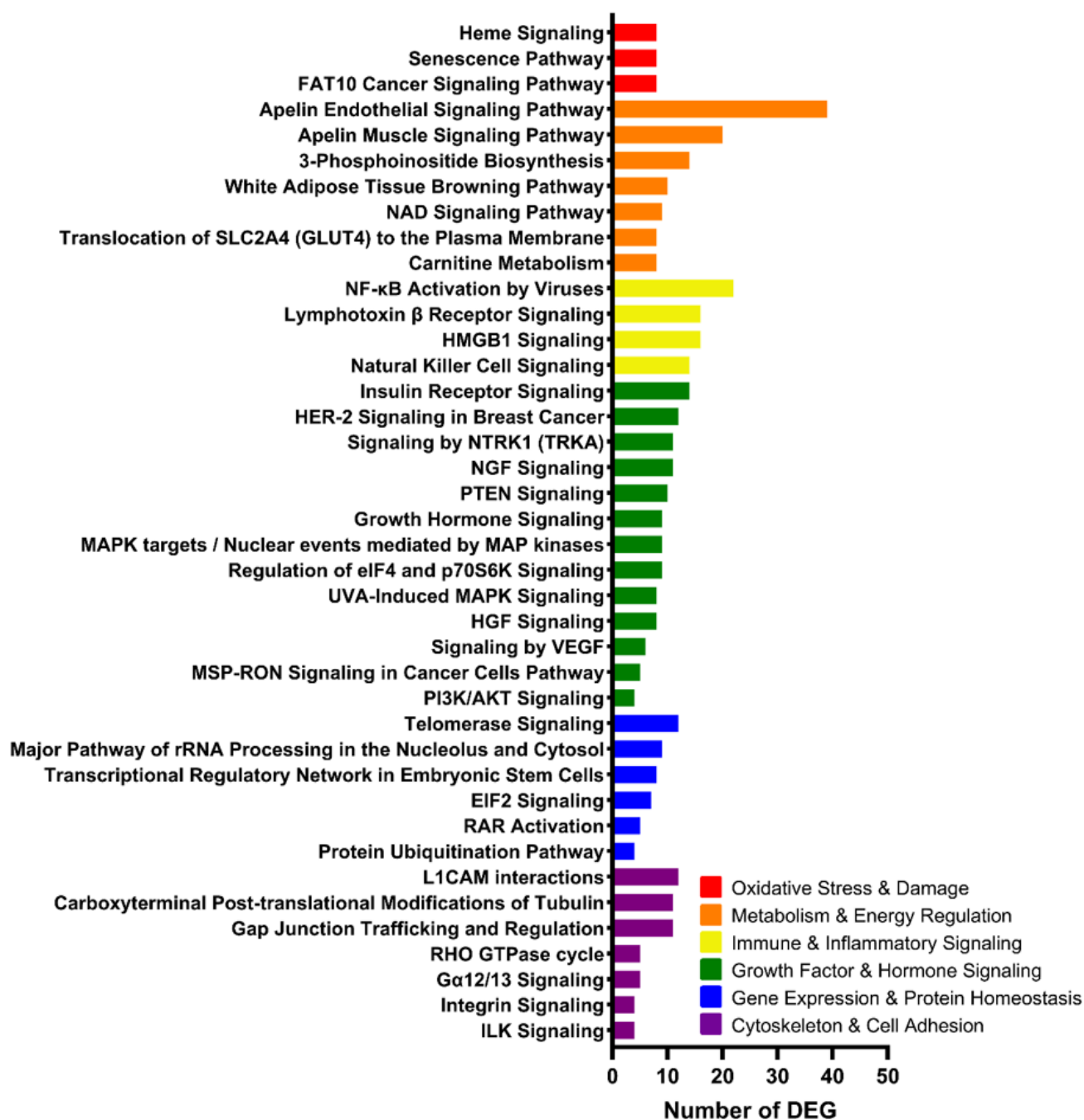


Figure 2

Selected canonical pathways significantly altered in the mammary gland fat pad of Holstein heifers at birth in response to *in utero* heat stress (iuHS) or *in utero* cooling (iuCL; n = 6/treatment). The y-axis displays the pathway while the x-axis displays the number of nominally significant genes (NSG) within the pathway. All pathways have $|z\text{-score}| \geq 2$, $P \leq 0.05$, and at least four NSG present. This is a selection of all significant pathways based on relevance to adipose biology and mammary gland development. All pathways besides Heme Signaling and PTEN Signaling are predicted to be inhibited ($z\text{-score} \leq -2$) using Ingenuity Pathway Analysis software.

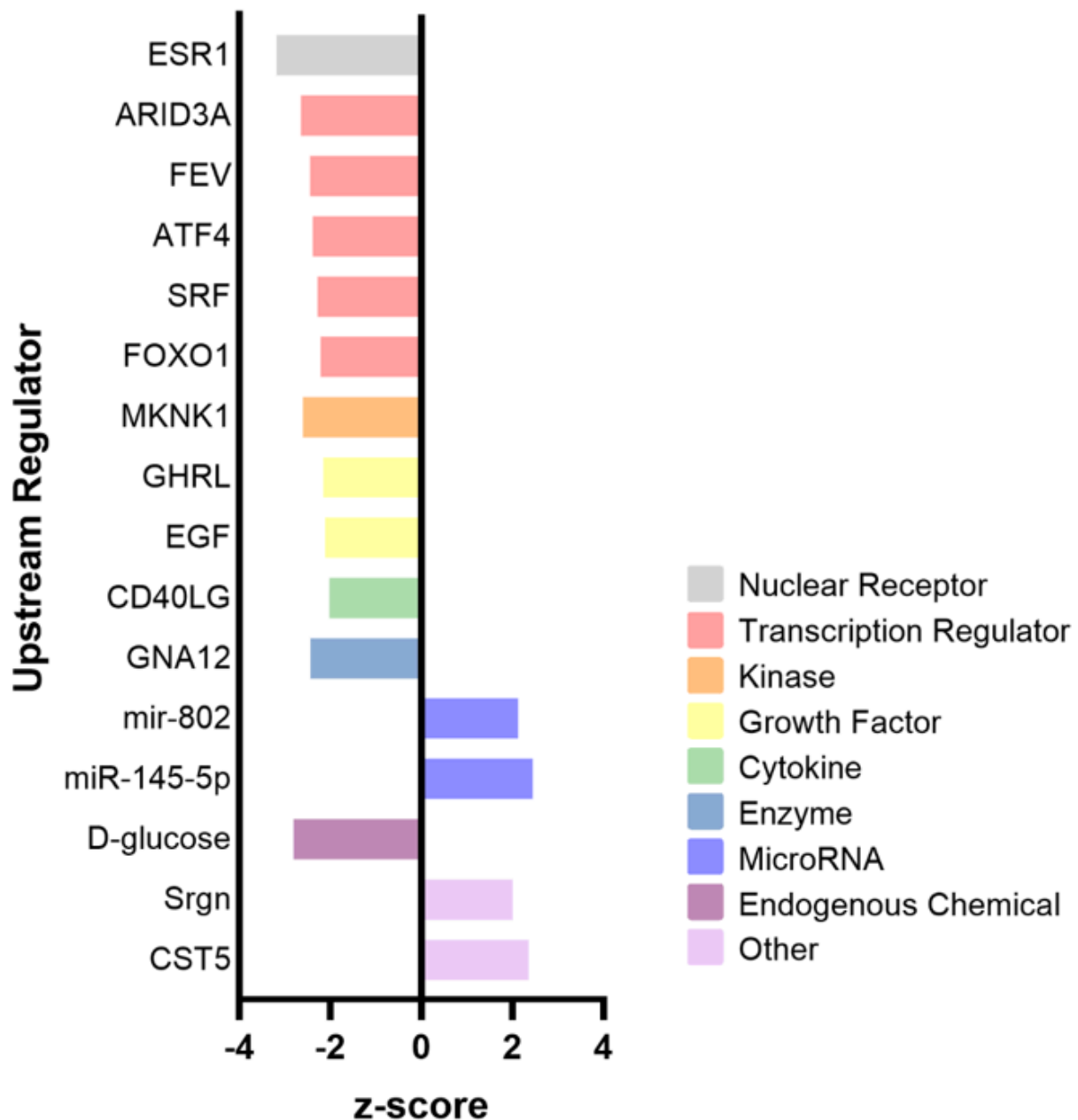


Figure 3

Predicted upstream regulator molecules using gene expression patterns of nominally significant genes in mammary gland fat pad of Holstein heifers at birth that were exposed to *in utero* heat-stress (iuHS) or *in utero* cooling (iuCL; n = 6/treatment). Upstream regulators are predicted to be activated (positive z-score) or inhibited (negative z-score) by Ingenuity Pathway Analysis Software. Selection was based on possible functional relation with adipose tissue physiology and mammary gland development. Only regulators that had a $|z\text{-score}| > 2$, $P \leq 0.05$, and at least four target nominally significant genes were considered.

Supplementary Files

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