

RESEARCH ARTICLE | *Genetics of Metabolic Syndrome*

Heat stress negatively affects the transcriptome related to overall metabolism and milk protein synthesis in mammary tissue of midlactating dairy cows

S. T. Gao,^{1*} Lu Ma,^{1*} Z. Zhou,² Z. K. Zhou,¹ L. H. Baumgard,³ D. Jiang,⁵ M. Bionaz,⁴ and D. P. Bu¹

¹State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China; ²Department of Animal Science, Michigan State University, East Lansing, Michigan; ³Department of Animal Science, Iowa State University, Ames, Iowa; ⁴Animal and Rangeland Sciences, Oregon State University, Corvallis, Oregon; and ⁵Statistics, Oregon State University, Corvallis, Oregon

Submitted 26 April 2019; accepted in final form 6 July 2019

Gao ST, Ma L, Zhou Z, Zhou ZK, Baumgard LH, Jiang D, Bionaz M, Bu DP. Heat stress negatively affects the transcriptome related to overall metabolism and milk protein synthesis in mammary tissue of midlactating dairy cows. *Physiol Genomics* 51: 400–409, 2019. First published July 12, 2019; doi:10.1152/physiolgenomics.00039.2019.—Inadequate dry matter intake only partially accounts for the decrease in milk protein synthesis during heat stress (HS) in dairy cows. Our hypothesis is that reduced milk protein synthesis during HS in dairy cows is also caused by biological changes within the mammary gland. The objective of this study was to assess the hypothesis via RNA-Seq analysis of mammary tissue. Herein, four dairy cows were used in a crossover design where HS was induced for 9 days in environmental chambers. There was a 30-day washout between periods. Mammary tissue was collected via biopsy at the end of each environmental period (HS or pair-fed and thermal neutral) for transcriptomic analysis. RNA-Seq analysis revealed HS affected >2,777 genes (false discovery rate-adjusted *P* value < 0.05) in mammary tissue. Expression of main milk protein-encoding genes and several key genes related to regulation of protein synthesis and amino acid and glucose transport were downregulated by HS. Bioinformatics analysis revealed an overall decrease of mammary tissue metabolic activity by HS (especially carbohydrate and lipid metabolism) and an increase in immune activation and inflammation. Network analysis revealed a major role of *TNF*, *IFNG*, *S100A8*, *S100A9*, and *IGF-1* in inducing/controlling the inflammatory response, with a central role of NF- κ B in the process of immunoactivation. The same analysis indicated an overall inhibition of PPAR γ . Collectively, these data suggest HS directly controls milk protein synthesis via reducing the transcription of metabolic-related genes and increasing inflammation-related genes.

heat stress; inflammation response; mammary tissue; metabolism; milk protein

INTRODUCTION

Milk protein is an important economic variable for the dairy industry. Heat stress (HS) decreases milk yield, and this was traditionally believed to be mainly caused by reduced feed intake (18). However, HS studies, using pair-feeding, conducted in dairy cows revealed that the decreased feed intake

only accounts for ~30–50% of the decrease in overall milk yield (3). Prior studies also indicate that HS has an even larger negative effect on milk protein synthesis (19, 23, 46, 57). Similar to overall milk yield, HS-induced hypophagia accounts only for a part of the decrease of milk protein yield (19), indicating other additional mechanisms contribute to reduced milk protein synthesis.

Data from our previous work suggest that the decrease in milk protein is partly caused by a decrease in precursor supply to the mammary gland (19). The decrease in protein synthesis appears to be independently regulated considering the discordant patterns in how acute HS (in environmental chambers) affects the content of other milk solids; HS has only minor effects on milk lactose content but actually increases milk fat percentages (15, 19). Cowley et al. (2015) (15) suggest that the reduction in milk protein from heat-stressed cows is the result of downregulation of mammary protein synthetic activity, especially α - and β -casein synthesis (11). However, it remains to be determined what molecular machinery governs reduced milk protein synthesis during HS. Our hypothesis is that decreased milk protein yield during HS in dairy cows is also directly caused by biological changes within the mammary gland. The objective of the current study was to elucidate the biological changes occurring within mammary tissue in response to HS by using transcriptome analysis via RNA sequencing (RNA-Seq).

METHODS AND MATERIALS

Ethics Statement

This study was approved by the Animal Care and Use Committee of Institute of Animal Science, Chinese Academy of Agricultural Sciences. The use of animals in the present study was in strict accordance with the Directions for Caring of Experimental Animals from the Institute of Animal Science, Chinese Academy of Agricultural Sciences.

Animals and Experimental Design

Data presented herein are results from a larger study (19). All experimental procedures, animals utilized, and production responses to heat stress were previously described. In brief, 2 \times 2 crossover design and four multiparous, lactating Holstein cows (101 \pm 10 of days in milk, 574 \pm 36 kg of body weight, 38 \pm 2 kg of milk/day, 2nd parity, 1–2 mo pregnant)

* S. T. Gao and L. Ma contributed equally to this work.

Address for reprint requests and other correspondence: D. Bu, State Key Laboratory of Animal Nutrition, Inst. of Animal Science, Chinese Academy of Agricultural Sciences, No. 2 Yuanmingyuan W. Rd., Beijing, 100193, China (e-mail: budengpan@126.com).

was used in the present study. The cows were randomly housed in four environmental chambers. The experiment included two periods (*period 1* and *period 2*), with two experimental phases (control phase and trial phase) within each period. During *phase 1* or control phase (9 days), all cows were in thermal neutral conditions [TN; 20°C, 55% humidity; temperature-humidity index (THI) = 65.5] and fed ad libitum. During *phase 2* or treatment phase (9 days), *group 1* ($n = 2$) was exposed to cyclical HS conditions (0600–1800 36°C, 1800–0600 32°C, 40% humidity; THI = 84.0/79.2) and fed ad libitum, while *group 2* remained in TN conditions but was pair-fed (PFTN) to their HS counterparts. Nutrient intake was kept similar between different treatments as previously described (57). A recovery/washout period (30 days) was inserted after *phase 2*, and then the study was repeated (*period 2*) by inverting the environmental treatments of the groups relative to the treatment phase in *period 1*. Cows were fed a total mixed ration formulated to meet or exceed the predicted requirements (38) of energy, protein, minerals, and vitamins (19).

Tissue Sample Collection and RNA Isolation

Mammary tissue samples were obtained on *day 10* of each environmental period (i.e., at the end of the experimental period). Biopsy procedure was conducted as previously described (14). In brief, the biopsies were performed just after milking at 0700 and were done in the right or left rear quarter of the mammary gland. Before surgery, 3–4 mL of lidocaine-hydrochloride (2% solution) was subcutaneously injected as local anesthetic. After removal of the biopsy instrument, pressure was applied immediately with sterile gauze to stop bleeding. Michel clips (11 mm; Henry Schein, Melville, NY) were used to close the skin incision. Rectal temperature, milk yield, and feed intake were recorded daily for 7 days to monitor the health of the cows. Tissue samples were frozen immediately in liquid nitrogen until further analysis.

Total RNA of each sample was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). The purity, concentration, and integrity of RNA were checked with the NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA), the Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 System (Agilent Technologies, Santa Clara, CA), respectively. The optical density (OD)₂₆₀/OD₂₈₀ values were ≥ 1.9 , and the RNA integrity number values were ≥ 8.0 .

Library Preparation and Sequencing

The library preparation and sequencing were performed as previously described (43). In brief, we utilized 3 μg RNA per sample as input, for complementary DNA (cDNA) libraries construction. Ribosomal RNA was removed using Epicentre Ribo-zero rRNA Removal Kit (Epicentre) and then further cleaned by ethanol precipitation. Sequencing libraries was generated by using the rRNA-depleted RNA following manufacturer's recommendations, with NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB). The libraries were sequenced on an Illumina HiSeq 4000 platform at the Novogene Bioinformatics Institute (Beijing, China) according to the manufacturer's instructions. The sequencing raw data of this study were deposited in the National Center for Biotechnology Information's Sequence Read Archive (SRP174357).

Quality Analysis, Mapping, and Transcriptome Assembly

We obtained the clean reads by removing reads containing adapter, reads containing poly-N, and low-quality reads in the raw data. Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Reference bovine genome (ftp://ftp.ensembl.org/pub/release-89/fasta/bos_taurus/dna/) and gene model annotation files (ftp://ftp.ensembl.org/pub/release-89/gtf/bos_taurus) were downloaded. Index of the reference genome was built with bowtie2 v2.2.8, and paired-end clean reads were aligned to the reference genome using HISAT2 (v2.0.4) (33). HISAT2 was run with “-rna-strandness RF”; other parameters were set as default. The String-Tie (v1.3.1) was used to assemble the mapped reads of each sample in a reference-based approach (42).

Statistical Analysis

A quasinegative binomial model was used to analyze the normalized data and assess differential expression (35). The model included as covariates the cow effects to account for potential baseline differences between the cows and the order in which the treatment and control conditions were implemented to account for the period effect. The effects of HS on gene expressions were tested by quasilielihood F tests. The analysis was performed with the edgeR package in R.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis was performed using Dynamic Impact Approach (DIA) (10). Entrez Gene ID of detected transcripts were used as background. The data set including Entrez Gene ID, false discovery rate (FDR)-adjusted P value, expression ratio, and P value was uploaded. An FDR-adjusted P value ≤ 0.05 and a P value ≤ 0.001 between the two treatments were used as cut-off. The enrichment analysis of various database including KEGG pathways, Gene Ontology (GO) Biological Process and Cellular Components was run by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (28). For this analysis, all the annotated transcripts that were detected (Entrez Gene ID) were used as background, and three data sets were analyzed: 1) upregulated differently expressed genes (DEG) by HS; 2) downregulated DEG by HS; and 3) both up- and downregulated DEG. Results were downloaded with the Functional Annotation Chart.

Transcription regulators and gene network analysis. Ingenuity Pathway Analysis (IPA) software (Qiagen) was used to uncover the upstream regulators and their downstream genes among DEG. For this purpose, a list of DEG with the same thresholds used for DIA analysis was uploaded into IPA. The data set of DEG (Suppl. File 1), DIA result (Suppl. File 2), and upstream regulators networks (Suppl. File 3) was deposited in figshare (DOI: 10.6084/m9.figshare.7496366).

RESULTS AND DISCUSSION

Originally, the decrease in milk yield caused by HS was attributed to reduced dry matter intake (DMI) (5, 56). The utilization of a PFTN model as a control in the present study demonstrates that reduced DMI only partially (~65%) explains the decrease in milk yield (19), confirming that hyperthermia

itself directly affects milk production (1, 15, 57). Environmentally induced hyperthermia affects not only overall milk production, but also milk composition. In the present study, milk protein concentration was decreased (despite large differences in milk yield, which would normally favor the “concentrating” of milk components) in HS cows compared with PFTN controls (19). The mechanisms regulating milk protein synthesis during HS are largely unknown but likely involve changes in several biological systems. Reasons for reduced milk protein production might be nutritional repartitioning resulting in the shortage of milk protein precursors (3, 19), decrease of energy available for activation of the mammalian target of rapamycin (mTOR)-insulin signaling pathway (8), and/or intrinsic changes happening in the mammary gland (11, 15).

HS Affects Mammary Tissue Transcriptome

To detect the intrinsic changes in the heat-stressed cows' mammary tissue and to discover additional reasons for the declined milk protein yield, we collected mammary tissue, extracted the total RNA, and detected the transcript profiles of HS and PFTN cows by using RNA-Seq. There were 2,777 DEG with $FDR \leq 0.05$ with 1,534 upregulated and 1,243 downregulated DEG in HS versus PFTN (Fig. 1A). The number of DEG indicates a marked effect of HS on the mammary gland. The complete data set is available in Suppl. File 1.

Transcription of Main Milk Protein Genes is Downregulated by HS

Consistent with the decrease both in milk protein concentration and yield in the present study (19), the expression of milk protein-encoding genes in mammary tissue was downregulated by HS (Fig. 1). Furthermore, transcripts coding for major regulators of transcription and translation of milk protein genes were also downregulated (Fig. 1). Among them, *STAT5A* and *STAT5B* are known to play an important role in controlling transcription of various caseins and lactalbumin (8). Activated STAT5 binds to DNA sequence known as GAS (interferon- γ activated sequence) elements and upregulates target genes (8). *LAMTOR2* was also downregulated by HS (Fig. 1). *LAMTOR2*, as member of the Regulator/LAMTOR complex, is crucial for mTOR activation (50). The Regulator/LAMTOR complex, including *LAMTOR2*, was shown to be essential for recruiting the Rag proteins to the lysosome, where those, in response to amino-acid stimuli, interact with and activate mTORC1 (48). Phosphorylated mTORC1 activates protein synthesis by promoting the formation of the 43S preinitiation complex (31) and, through the phosphorylation of the RPS6-p70-protein kinase (p70S6K), indirectly prevents the inhibition of the eukaryotic translation elongation factor 2 (eEF2) (9), thus allowing translation to occur. The E74-like factor 5 (*ELF5*) is

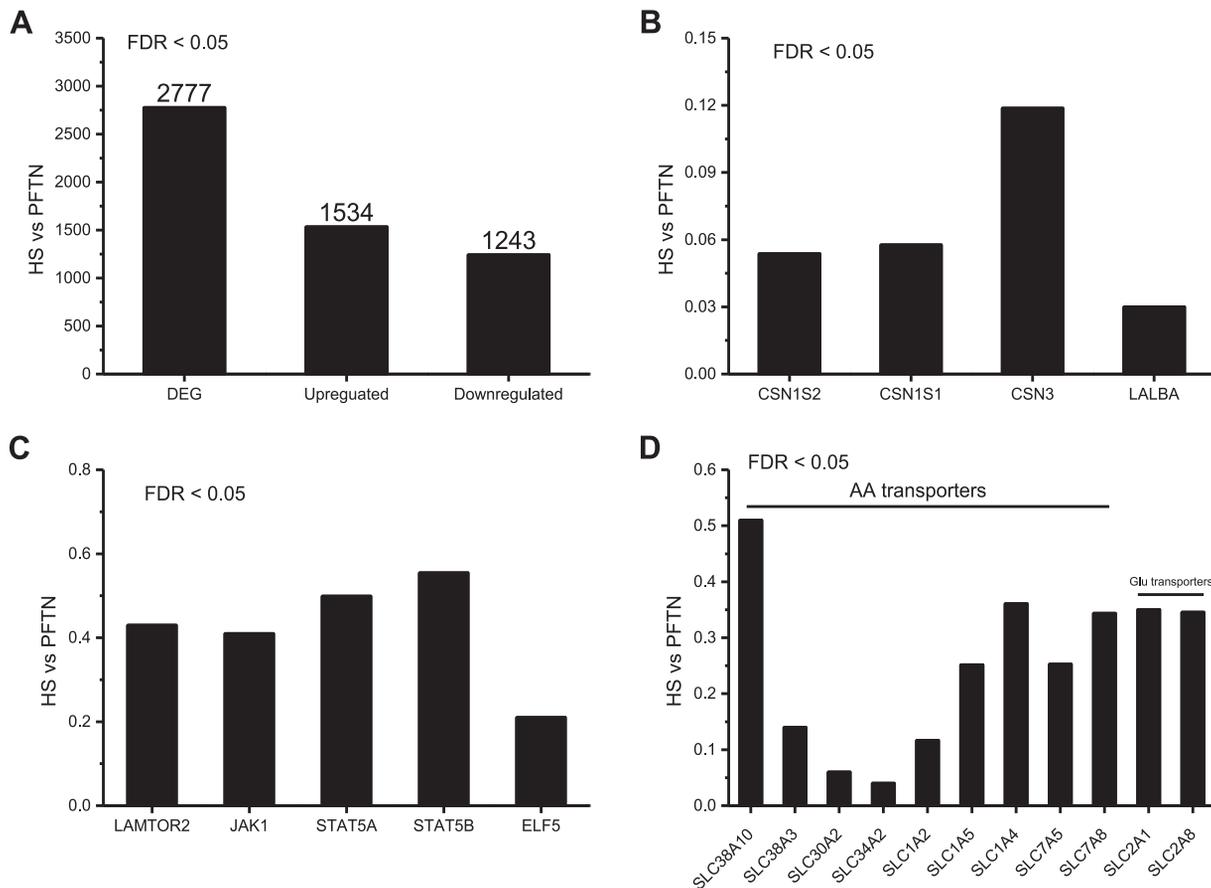


Fig. 1. Genes differentially expressed (DEG) in bovine mammary tissue in cows subjected to heat stress (HS) vs. cows pair-fed and kept in thermo-neutral conditions (PFTN) A: total DEG and DEG up- or downregulated. B: expression ratio (HS vs. PFTN) of transcripts coding for various caseins and lactalbumin. C: expression ratio of key genes related to protein synthesis regulation. D: expression ratio of transcripts coding for various amino acid and glucose transporters. AA, amino acid; FDR, false discovery rate.

also known to play an important role in activating STAT5 in mammary tissue (8, 58). Therefore, downregulation of the transcripts of milk protein-encoding genes and their upstream regulators suggests an intrinsic transcriptomic regulation of milk protein synthesis in the mammary tissue in response to HS. It is not possible to conclude if the downregulation of the above genes is the main cause of reduced milk protein synthesis, but it likely contributes with reduced availability of precursors [amino acid (AA) and glucose] that reduce mTOR activation (19).

HS Decreases Transcription of Genes Coding for Main Glucose and AA Transporters

Research on mammary nutrient extraction (AA and glucose) in livestock during HS is scarce and inconsistent. The efficiency of nutrient extraction from circulation is different between monogastric and ruminant animals under HS conditions. In monogastric animals the mammary gland of lactating sows exposed to HS has greater extraction rates of glucose and triglycerides and tend to have greater extraction of α -amino acids compared with thermal neutral controls, leading to an increase in mammary nutrient uptake (44). However, in mid-lactating goats acute HS for 4 days has a negative effect on the mammary uptake of glucose, also partly due to a reduced mammary blood flow (49). Somewhat similar to that study, transcripts coding for key glucose transporters (9) were downregulated in mammary tissue by HS in our study (Fig. 1). Prior data from our experiment suggested an overall decrease availability of AA for the mammary gland (19), and this is coupled with the downregulation of transcription of most genes encoding for AA transporters in HS compared with PFTN cows (Fig. 1). The above data suggest a decreased uptake of AA and glucose by the mammary tissue in response to HS. The decrease in glucose uptake in HS compared with PFTN cows would also reduce the energy availability in the mammary tissue, reducing the activation of mTOR and, thus, milk protein synthesis (8).

Overall, the above data indicate that the decrease in milk protein yield during HS is partly explained by a decrease in uptake of precursors that is regulated at the transcriptomic level. In the present study, the concentration of the substrates for milk protein synthesis (amino acids, glucose, and NEFA) in heat-stressed cows were decreased compared with PFTN cows (19). In addition to the decrease in substrate blood concentrations, blood flow to the mammary gland might also have decreased (34). Lough et al. (1990) (34), utilizing an experimental design similar to our study, observed that both HS and feed restriction decreased mammary blood flow compared with thermal neutral ad libitum-fed cows. Additionally, portal flow also tended to decrease during HS, and it was better correlated with HS-induced hypophagia (36), indicating that nutrient absorption may also be inhibited by the reduced splanchnic blood flow.

HS Depresses Metabolic-related Pathways in Mammary Tissue

The few protein-coding genes discussed above are only a small fraction of all protein-coding genes in the bovine genome, and a more holistic view should be used to determine the overall biological effect of HS in the mammary gland tran-

scriptome. Furthermore, it is important to investigate in more detail the causation of the observed effect. The mammary gland is not autonomous but, rather, participates in the biological adaptation of the organism. This was indicated in a prior

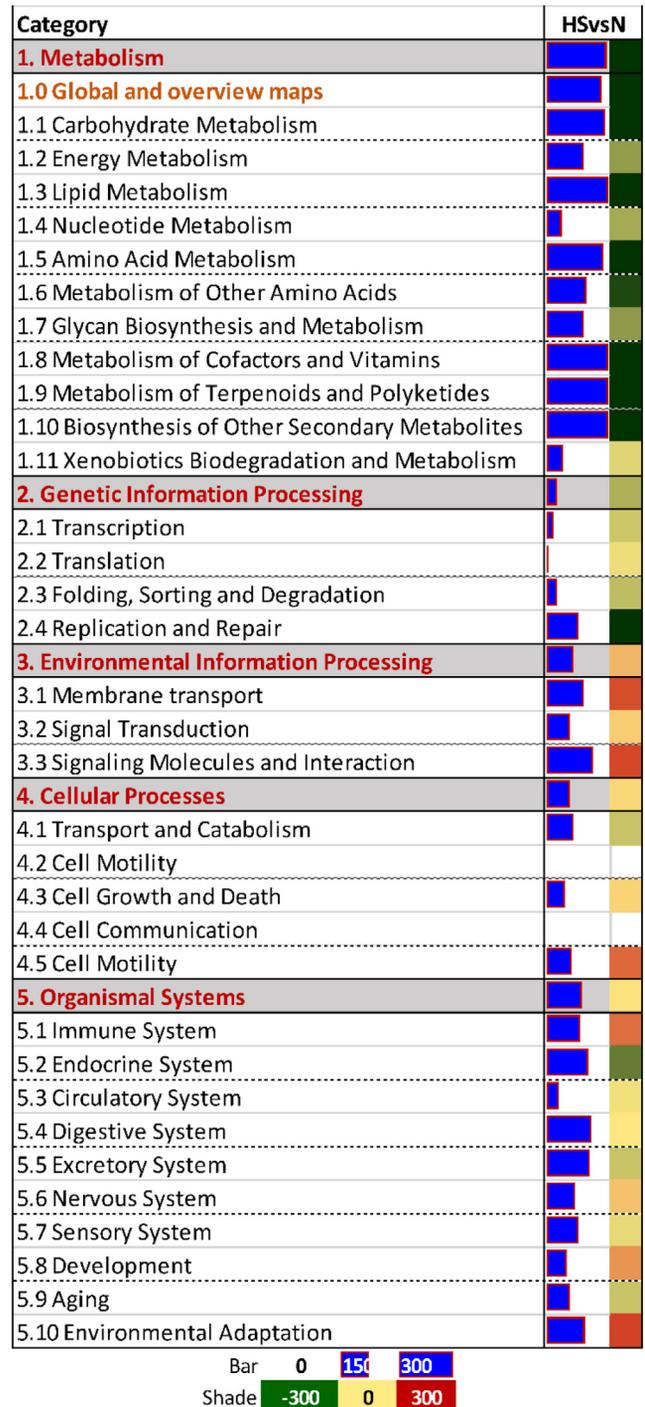


Fig. 2. Summary of the main categories and subcategories of KEGG pathways as results of the transcriptomic effect on mammary tissue of heat stress (HS) compared with pair-fed cows in neutral ambient conditions (N) as analyzed by the Dynamic Impact Approach. At right: bar denoting the overall impact (in blue) and the shade denoting the effect on the pathway [from green (inhibited) to red (activated)]. The darker the color the greater the activation (if red) or inhibition (if green) of the pathway.

study where a strong cross talk between the mammary gland and liver was revealed (14).

DIA estimates the perturbation of a biological pathway by the “impact,” while the overall direction of the perturbation is represented by the “flux” (or Direction of the Impact) (10). A summary of the perturbation by HS on the main categories of the KEGG pathways in mammary tissue as estimated by the DIA is available in Fig. 2. The category “Metabolism” was the most impacted and was strongly overall inhibited followed by the category “Organismal System” with most of the pathways overall downregulated but few pathways induced, such as immune system and environmental adaptation-related pathways (Fig. 2, Supp. File 2). The 20 most affected pathways were all part of the two aforementioned categories (Fig. 3). Among those, most of the metabolic-related pathways were associated with glucose and lipid metabolism (Fig. 3) with “Galactose metabolism” (i.e., lactose synthesis) and “Fatty acid biosynthesis” being the most impacted (Fig. 3). Interestingly, among the most inhibited pathways were also the “PAR signaling pathway” and the “Phenylalanine, tyrosine, and tryptophan biosynthesis” (Fig. 3).

The effect of HS on mammary tissue metabolism was also revealed by DAVID analysis, with most of the enriched pathways among downregulated genes related to glucose, lipid metabolism, and amino acid biosynthesis (Fig. 4, Suppl. File 2). Furthermore, activation of the immune system was also revealed by DAVID analysis (Fig. 4).

Due to the extreme high impact and enrichment, a more in-depth discussion of pathways associated with glucose, lipid, and amino acid metabolism is warranted, especially considering prior observations of a decrease in precursors for milk synthesis and an overall decrease of milk components by HS vs. PFTN cows (19).

Lipid metabolism. Lipid metabolism was highly inhibited in this study by HS compared with PFTN (Fig. 2), and all the six pathways among the 20 most impacted pathways were inhibited, including the pathways of “Steroid biosynthesis,” “Fatty acid biosynthesis,” “Synthesis and degradation of ketone bodies,” “Glycerolipid metabolism,” “Linoleic acid metabolism,” and “Arachidonic acid metabolism” (Fig. 3).

Studies investigating the effect of HS on milk fat synthesis are scant. Tao et al. (2013) (53) did not find any effect on milk fat synthesis-related genes in mammary tissue of dry cows experiencing HS compared with cows cooled with sprinklers and fans. The effect of HS on milk fat concentration has been extensively studied. The first report of low summer milk fat was first described in 1926 (25) and observed frequently afterward, as previously reviewed (6, 26, 29). In contrast, other studies did not indicate a seasonal pattern of milk fat (24, 51). In pair-fed experiments, similar inconsistencies were observed. The milk fat percentage of heat-stressed cows was decreased (37), not changed (19) or increased (20) compared with PFTN controls. Although how HS effects milk fat remains ill-defined and inconsistent, HS reduces milk fat yield consistently (19, 46). Our study revealed that the decrease in milk fat yield is regulated at the transcriptomic level (Figs. 2 and 3). PPAR signaling appears to be central in the regulation of milk fat synthesis in ruminants (40, 59). Prior studies suggested that PPAR γ is activated by fatty acids in ruminants (7); thus, it is possible that the decrease in availability of fatty acids from circulation (19) has decreased the activation of PPAR γ reducing the expression of milk fat synthesis-related genes.

Amino acid metabolism. Consistent with prior studies (6, 15, 23), HS cows in the present study had lower milk protein synthesis compared with PFTN cows (19). Transcriptomics data analyzed by DIA and DAVID uncovered an overall

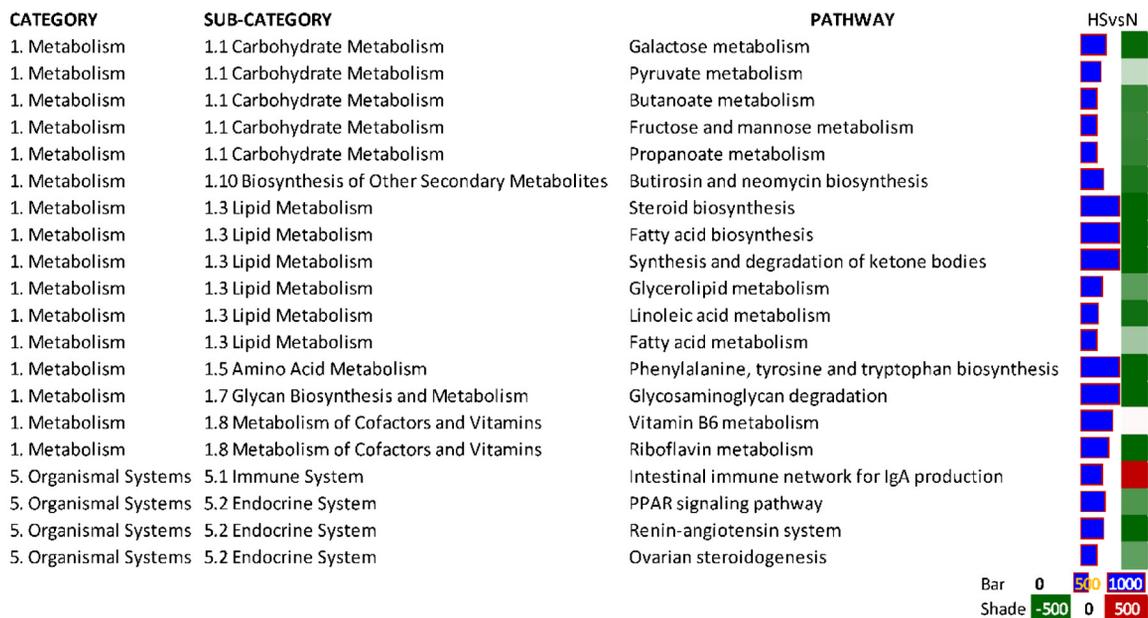


Fig. 3. The 20 most impacted pathways in mammary tissue of heat stress (HS) compared with pair-fed cows in neutral ambient conditions (N) uncovered by the Dynamic Impact Approach. At right: bar denoting the overall impact (in blue) and the shade denoting the effect on the pathway [from green (inhibited) to red (activated)]. The darker the color the greater the activation (if red) or inhibition (if green) of the pathway. Of the most impacted pathways, 80% belong to the “Metabolism” main category of pathways and were all inhibited, especially glucose and lipid metabolism. Remaining pathways are associated with organismal system, with immune system pathway strongly activated, while endocrine system pathways were inhibited.

Term	FDR
Gene Ontology Biological Process	
GO:0006695~cholesterol biosynthetic process	4E-07
GO:0055114~oxidation-reduction process	2E-06
GO:0006633~fatty acid biosynthetic process	6E-03
GO:0008299~isoprenoid biosynthetic process	7E-03
GO:0006631~fatty acid metabolic process	2E-02
GO:0030198~extracellular matrix organization	2E-03
GO:0006954~inflammatory response	6E-03
GO:0035987~endodermal cell differentiation	3E-02
GO:0019221~cytokine-mediated signaling pathway	3E-02
GO:0045669~positive regulation of osteoblast differentiation	4E-02
KEGG pathways	
bta01100:Metabolic pathways	3E-23
bta01130:Biosynthesis of antibiotics	2E-12
bta00100:Steroid biosynthesis	2E-07
bta00190:Oxidative phosphorylation	1E-05
bta01200:Carbon metabolism	2E-04
bta01230:Biosynthesis of amino acids	5E-04
bta00280:Valine, leucine and isoleucine degradation	2E-03
bta00620:Pyruvate metabolism	2E-03
bta00640:Propanoate metabolism	5E-03
bta00020:Citrate cycle (TCA cycle)	2E-02
bta04146:Peroxisome	2E-02
bta00010:Glycolysis / Gluconeogenesis	5E-02
bta04060:Cytokine-cytokine receptor interaction	6E-04
bta04550:Signaling pathways regulating pluripotency of stem cells	1E-03
bta04512:ECM-receptor interaction	1E-03
bta04145:Phagosome	4E-03
bta04380:Osteoclast differentiation	2E-02
bta04510:Focal adhesion	2E-02
bta04610:Complement and coagulation cascades	2E-02
bta04151:PI3K-Akt signaling pathway	7E-02

Fig. 4. The 30 most enriched gene ontology Biological Process terms and KEGG pathways revealed by DAVID analysis of the transcripts up (in dark gray)- or down (in light gray)-regulated in mammary tissue of cows experiencing heat stress compared with pair-fed cows in neutral ambient conditions. Reported is the significant of enrichment by Benjamini-Hochberg-corrected *P* value (false discovery rate).

inhibition of the subcategory of pathways “Amino Acid Metabolism,” “Metabolism of Other Amino Acids”, and “Energy Metabolism” in HS cows compared with the PFTN cows (Figs. 2 and 3 and Suppl. File 2). Additionally, among the top 20 impacted pathways, the pathway of “Phenylalanine, tyrosine, and tryptophan biosynthesis” was inhibited (Fig. 3). The above data suggest that HS can affect the synthesis of milk proteins also by inhibiting the metabolism of specific amino acids.

The decrease in milk protein synthesis may have also been driven by decreased activation of the mTOR pathways (8). In present study, neither DIA nor DAVID analysis revealed large effect of the treatment on the expression of genes related to mTOR or JAK-STAT signaling pathways (Suppl. File 2). However, in this study, both the AA concentration in blood (19), the pathway of “Phenylalanine, tyrosine, and tryptophan biosynthesis” (Fig. 3), and expression of genes related to AA transporters (Fig. 1) were all decreased by HS, indicating that the intracellular AA availability was likely reduced. mTORC1 and its regulators resides on the lysosomal surface so as to sense a function of lysosomal that reflects the intracellular pools of AA (48). The mTORC1 complex has sensors that sample the cytosol and the lumen of the lysosome for amino acids (13). When cells are deprived of amino acids, mTORC1 is located in the cytosol and inactive, while upon the addition

of amino acids, mTORC1 rapidly translocates to the lysosomal surface, where it is activated by Rheb (48). Activated mTORC1 could induce translation indirectly (8). The trimeric Ragulator protein complex encompassing *LAMTOR2* is essential for localizing mTORC1 to the lysosomal surface, and is necessary for the activation of the mTORC1 pathway by amino acids (48). In the absence of Ragulator, Rag detaches from the lysosome and fails to localize mTORC1 to the lysosome on where the mTORC1 is activated (2, 48). However, Ablation of *LAMTOR2* in human dendritic cells results in strong mTOR activation (50). Thus, in this study, it cannot be confirmed that whether reduced intracellular availability of amino acids could inactivate mTOR activity through downregulating of *LAMTOR2* expression (Fig. 1). However, it is possible that lower intracellular availability of amino acids in this study can explain partially the decrease of milk protein synthesis by inactivating of the activity of mTOR.

Carbohydrate metabolism. Heat-stressed cattle enter a unique physiological state during which they cannot consume enough nutrients to meet maintenance and milk production costs, which causes them to enter a state of negative energy balance, and this is uniquely characterized without the expected increase in circulating NEFA (3, 4). This paradoxical effect was also observed in our study where only PFTN cows had increased NEFA (19). We also observed a tendency for a lower circulating glucose concentration, but, considering a possible hampered blood flow in mammary by HS (34), it is likely that the mammary gland had less overall glucose availability. In this study, the HS evidently decreased the transcription of genes associated with carbohydrate metabolism in mammary gland as indicated by the bioinformatics analyses (Figs. 2–4 and Suppl. File 2). In addition, among the top 20 most impacted pathways in DIA, the process of “Riboflavin Metabolism” was also downregulated (Fig. 3). Riboflavin, also known as vitamin B2, is the coenzyme of flavoenzyme, and it is involved in oxidation and ATP production (55). Accordingly, our data suggest a reduced use of glucose to produce energy in mammary gland (also pathways related to glycolysis and TCA cycle were estimated to be inhibited by DIA, see Suppl. File 2). Thus, besides the apparent decrease in glucose availability, the mammary tissue may have reduced its capacity to extract energy from glucose oxidation. Interestingly, decreased ATP concentrations are associated with decreased activation of mTOR via AMPK (8). Not surprisingly, the “AMPK signaling pathway” was the most impacted and activated among the pathways related to signal transduction during HS (Suppl. File 2).

Our data also suggest a decreased channeling of glucose toward lactose synthesis, considering the large inhibition of “Galactose Metabolism” (Fig. 3), mostly driven by the decreased expression of *LALBA* (Fig. 1). Lactose is composed of a molecule of galactose and a molecule of glucose (55). Thus, decreased galactose production would reduce the yield of lactose as well as overall milk yield, because lactose is the primary osmotic regulator of milk yield and the major determinant of milk volume (27), suggesting that HS can decrease milk yield by downregulating the expression of the genes involved in galactose metabolism.

In summary, the data from the present study suggest that HS not only induces changes in systemic bioenergetics (3, 47) but can also markedly alter mammary macronutrient metabolism.

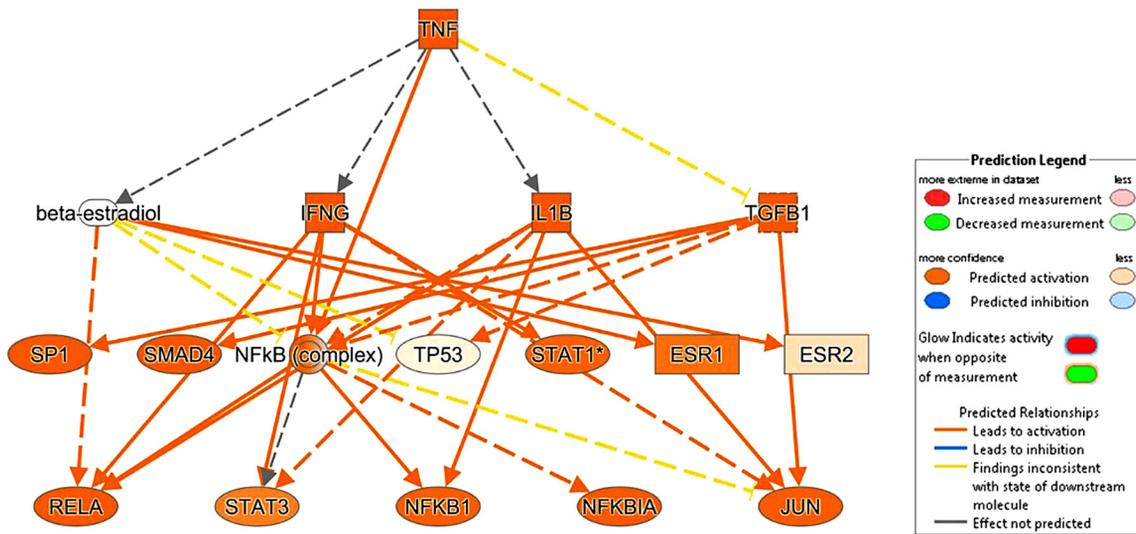


Fig. 5. Upstream regulators mechanistic network of tumor necrosis factor (TNF) in mammary tissue of cows during heat stress vs. pair-fed thermal neutral conditions cows. The arrows represent the direction of the effect on target molecules. The orange color represents predicted induction of activity. The network was constructed with Ingenuity Pathway Analysis.

For lactose and fat, the data clearly indicated a strong role of the transcriptome in reducing their production; for the protein synthesis, although the overall analysis does not support a major role of expression of key genes related to mTOR and JAK-STAT pathways in the decrease of milk protein synthesis, the change of the availability of AA in the mammary tissues may partially explain the decrease in milk protein synthesis during HS.

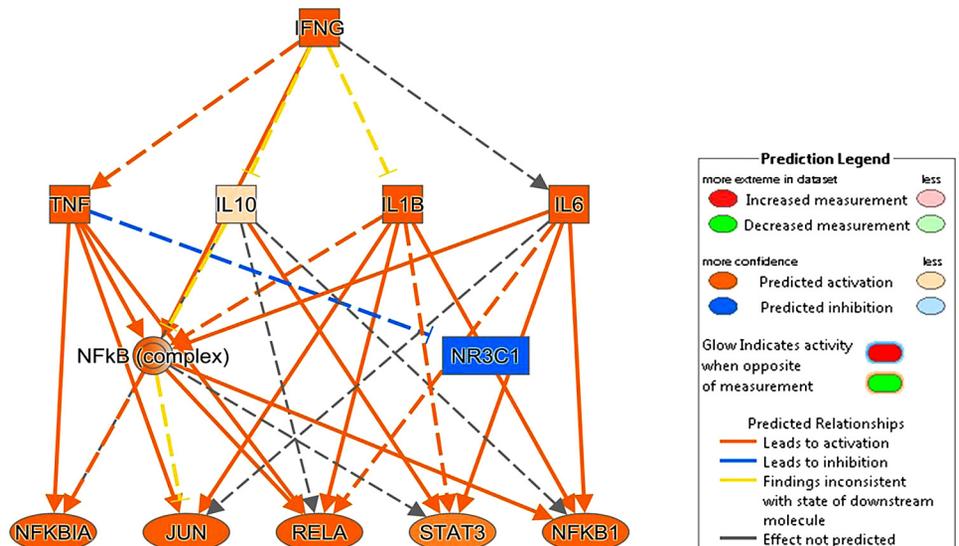
HS Increases Inflammation Response

DIA and DAVID analysis both indicated intense immune activation and inflammation during HS (Figs. 2–4 and Suppl. File 2). To understand the transcription mechanisms occurring within the mammary gland and further explore the potential mechanism for the metabolic perturbation under HS, we analyzed upstream regulators and their associated networks with IPA. The analysis revealed the importance of six upstream

regulators (TNF, IFNG, S100A8, S100A9, IGF-1, and PPARγ), which are related to metabolism (IGF-1 and PPARγ) (7, 45) and inflammation (TNF, IFNG, S100A8, and S100A9) (22, 39, 52). We further constructed the networks with downstream target DEG with IPA (Figs. 5 and 6 and Suppl. File 3). Five of the upstream regulators were estimated to be activated (i.e., TNF, IFNG, S100A8, S100A9, and IGF-1), and PPARγ was estimated to be inhibited by IPA in HS compared with PFTN cows. These networks can be considered central for the modulation of the mammary gland transcriptome under HS.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls DNA transcription, cytokine production, and cell survival (21). NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (12, 41). NF-κB plays a key role in

Fig. 6. Upstream regulators mechanistic network of interferon gamma (IFNG) in mammary tissue of cows during heat stress vs. pair-fed thermal neutral conditions cows. The arrow represents the direction of the effect on target molecules. The orange and blue colors represent predicted induction and inhibition of activity, respectively. The network was constructed with Ingenuity Pathway Analysis.



regulating the immune response to infection (12). Incorrect regulation of NF- κ B has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development (12, 21, 41). Interestingly, in IPA analysis NF- κ B was estimated to be induced in all six networks. The above results, together with DIA and DAVID analysis, suggest that HS compared with PFTN cows may have experienced a higher degree of inflammation. Data also support an active participation of the mammary gland to an inflammatory response during HS.

Mammary epithelial cells isolated from water buffalo and exposed to high temperature in vitro had increased expression of inflammation-related genes (30). Increase expression of immune response-associated genes in mammary tissue during HS was also observed very recently in dry cows (16). It is unclear if the inflammatory response in the mammary tissue directly affected its metabolism. A causal relationship between inflammation and systemic (whole body) metabolism has been widely researched. By administering gamma-secretase inhibitor, Kvidera et al. (2017) (32) induced intestinal damage and compromised intestinal permeability causing inflammation and altering the metabolism with consequent reduced feed intake and milk yield. Baumgard and Rhoads (2013) (3) argued that systemic (whole body) inflammatory response induced by LPS in cattle can increase maintenance costs and result in glucose shortage. From a lactation and growth standpoint, this change in the hierarchy of fuel utilization decreases glucose partitioning to the mammary gland and skeletal muscle (3). Similarly, in the mammary gland, inflammatory response induced by HS can also repartition glucose (and other nutrients) toward leukocytes and away from mammary epithelial cells. Because the mammary gland comprises many cell types, including immune cells (54), it is possible that the mammary epithelial cells (forming the parenchyma, the main tissue collected by mammary tissue biopsy) (17) decrease uptake of nutrients as a consequence of the inflammatory response in an attempt to spare nutrients for immune cells. The above speculation is consistent with the results of the bioinformatics analysis indicating an overall inhibited metabolic activity and an activated immune system. Thus, it is possible that the decrease in protein synthesis observed is also partly driven by the inflammatory response within the mammary gland.

Summary and Conclusion

It has long been recognized that the decline of milk protein content and yield during HS is the result of depressed DMI. However, using a pair-fed design we showed that the decline of DMI can only account for 65% of the decrease of milk protein yield indicating a role for intrinsic changes in the mammary gland. Our data indicated a decreased expression of genes coding for main milk proteins and genes coding for key proteins in the regulation of milk protein synthesis. Furthermore, bioinformatics analysis of the whole transcriptome, suggested a strong inhibition of metabolic activity of the mammary tissue, especially glucose and lipid metabolism. Amino acid metabolism was also overall inhibited and data suggested that decreased AA availability may contribute the decreased milk protein synthesis. Furthermore, bioinformatics analysis indicated an increase in inflammatory response or activation of related pathways in mammary tissue of HS versus PFTN cows.

The latter could have played a role in decreasing overall metabolism via reducing uptake of nutrients and, thus, synthesis of milk components, including proteins. Therefore, collectively our data suggest that the decline in milk protein synthesis during HS is mainly the consequence of an overall decrease of metabolic activity partly driven by an increased inflammatory response as consequence of an intrinsic transcriptomic perturbation.

ACKNOWLEDGMENTS

We give special thanks to our former and current students who have contributed the projects and all the staff of State Key Laboratory of Animal Nutrition (Beijing, China) for affording environmental chambers and for sample analyses.

GRANTS

The present study was financially supported by National Natural Science Foundation of China (31872383), National Key Research and Development Program of China (2018YFD0501600), and Agricultural Science and Technology Innovation Program (ASTIP-IAS07; CAAS-XTX2016011-01), Beijing Dairy Industry Innovation Team (BAIC06-2019).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.T.G., L.M., L.H.B., and D.P.B. conceived and designed research; S.T.G., L.M., and D.P.B. performed experiments; S.T.G., Z. Zhou, Z.K. Zhou, D.J., M.B., and D.B. analyzed data; S.T.G., Z. Zhou, M.B., and D.P.B. interpreted results of experiments; S.T.G., M.B., and D.P.B. prepared figures; S.T.G., Z. Zhou, M.B., and D.P.B. drafted manuscript; S.T.G., L.H.B., M.B., and D.P.B. edited and revised manuscript; S.T.G., L.M., and D.B. approved final version of manuscript.

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