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—Original Article—

Mitochondrial-related consequences of heat stress exposure during bovine oocyte maturation persist in early embryo development

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Abstract. Hyperthermia during estrus has direct consequences on the maturing oocyte that carries over to the resultant embryo to compromise its ability to continue in development. Because early embryonic development is reliant upon maternal transcripts and other ooplasmic components, we examined impact of heat stress on bovine oocyte transcripts using microarray. Oocytes were matured at 38.5ºC for 24 h or 41.0ºC for the first 12 h of *in vitro* maturation; 38.5ºC thereafter. Transcriptome profile was performed on total (adenylated + deadenylated) RNA and polyadenylated mRNA populations. Heat stress exposure altered the abundance of several transcripts important for mitochondrial function. The extent to which transcript differences are coincident with functional changes was evaluated by examining reactive oxygen species, ATP content, and glutathione levels. Mitochondrial reactive oxygen species levels were increased by 6 h exposure to 41.0ºC while cytoplasmic levels were reduced compared to controls ($P < 0.0001$). Exposure to 41.0ºC for 12 h increased total and reduced glutathione levels in oocytes at 12 h but reduced them by 24 h (time × temperature $P < 0.001$). ATP content was higher in heat-stressed oocytes at 24 h ($P < 0.0001$). Heat-induced increases in ATP content of matured oocytes persisted in early cleavage-stage embryos (8- to 16-cell embryos; $P < 0.05$) but were no longer apparent in blastocysts ($P > 0.05$). Collectively, results indicate that direct exposure of maturing oocytes to heat stress may alter oocyte mitochondrial processes/function, which is inherited by the early embryo after fertilization.

Key words: ATP content, Glutathione, Heat stress, Oxidative phosphorylation, Transcriptome

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Although maturation constitutes a "short" time period in the life of an oocyte, marked reductions in development to the blastocyst stage after fertilization [1], increased incidence of embryonic losses soon after placental attachment [2, 3], along with data to show that even morphologically "normal" appearing embryos from heat-stressed oocytes are more susceptible to heat stress exposure [4], attest to the enormity of problem(s) related to heat stress-induced hyperthermia during the time period of estrus. Despite the widely accepted magnitude of this problem, little progress has been made towards understanding the mechanistic basis of hyperthermia-induced infertility at the cellular and molecular level of the oocyte, especially as it relates to consequences that carry over to the resultant embryo after fertilization to affect ability to continue in development.

Early embryonic development is reliant on maternal pools of RNA and other cytoplasmic components inherited from the matured oocyte [5]. Maternally-derived RNAs not only drive early cleavage divisions, but also contribute to cell fate and patterning [6]. More than half of the maternal transcripts are deadenylated or translationally-silenced during oocyte maturation [7, 8]. Thus, de novo protein synthesis during maturation and/or during early embryo development, to some extent, is reliant on activation (i.e., cytoplasmic polyadenylation) of stored transcripts [9].

Interestingly, heat stress exposure during oocyte maturation reduces de novo protein synthesis [10] without affecting the total amount of polyadenylated mRNA [11]. Efforts of others using oligo d(T) primed cDNA to examine polyadenylated mRNA populations are beginning to reveal a number of heat-induced differences at the level of specific transcripts. Ticianelli *et al*. [12] recently reported heat-induced differences in the relative abundance of 9 maternal transcripts in Holstein and Nelore oocytes involved in important biological functions such as tissue morphology, embryo, cellular and organism development, and cell death-survival. When severity of heat shock conditions is sufficient to impair ability of Bubaline oocytes to cleave, increases in the relative abundance of transcripts involved in oxidative stress and apoptosis were noted [13]. Regarding embryos from heat-stressed oocytes, Pavani *et al*. [14] reported differential abundance in *Cx43, DNMT1, HSPA14.*

Because transcription is virtually inactive during oocyte maturation, it is unclear if heat-induced differences in the relative abundance of certain transcripts are the result of changes in adenylation status or degradation of mRNA. In our first experiment, we used global transcriptome profiling to examine the consequences of heat-stress exposure on the maternal transcriptome. To capture transcripts, if any, whose adenylation status and/or overall abundance may be altered

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Table 1. Sequences of primers and annealing conditions used for quantitative PCR analyses

Gene symbol	Entrez number	Amplicon location (bp)	Primer sets	Primer conc. (nM)	Annealing temp. (ºC)
ATP5O	281640	46–146	5'-TTGACCTACAGCTGCCAGGAG	600	57
			3'-TTGGCAAATGGCCTGACCACAG		
		340–452	5'-GCCTAAGTGACATGACGGCA	600	61
			3'-AATGCGGAAATCACCGCAGG		
COQ3	540298	114–244	5'-AACTACGTCGGCGGTTTATCC	200	57
			3'-TCATCCAATTCAGGCAGGCA		
		664–761	5'-ACGTTTGATGCTGTCGTAGC	200	56
			3'-GAACCATCAGGCTTTAACAC		
NDUFC2	338046	145–329	5'-CGTCACAATGATGACTGGACGGCA	200	60
			3'-GATGCAAACCGGCCAACAGCACC		
		397–518	5'-GTATGCTGTGAGGGACCACG	200	57
			3'-TACTTCAACGCACTGGATGG		
GFP [1]	–	–	5'-CAACTTCAAGACCCGCCACA	800	–
			3'-TCTGGTAAAAGGACAGGGCCA		

[1] Referenced primer set from [11].

by heat stress exposure, microarray profiling was performed on both total (adenylated + deadenylated) and polyadenylated RNA populations. To that end, several transcripts associated with mitochondrial function or processes were identified to be differentially abundant in heat-stressed versus non-stressed oocytes. Subsequently, a second experiment was performed to determine the effects of heat-stress exposure on mitochondrial and cytoplasmic processes focusing on possible perturbations in oxidative phosphorylation and antioxidant processes during oocyte maturation. In the third experiment, we evaluated whether the consequences of heat-stress during oocyte maturation to alter ATP content carryover to resultant embryos.

Materials and Methods

Majority of chemicals were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA), exceptions are indicated.

Experiment One: Examine the consequences of heat-stress exposure on the maternal transcriptome

Bovine cumulus-oocyte complexes (COC) were collected and matured as previously described [15] at 38.5ºC for 24 h (control) or 41.0ºC for 12 h followed by 38.5ºC for 12 h (heat stress). After 24 h of *in vitro* maturation (hIVM), a subset of control and heat-stressed COC was denuded of associated cumulus by vortexing in 0.3% hyaluronidase. Cumulus-free oocytes were lysed in RNA extraction buffer (Arcturus/Thermo Fisher Scientific, Waltham, MA, USA), and then stored at –80ºC (n = 50 oocytes per each treatment). Remaining COC were fertilized *in vitro* and embryo culture performed as previously described [16]. Blastocyst development was assessed 8 days later (~201 h post-IVF). Transcriptome analysis was performed on 8 replicate RNA pools where heat stress reduced blastocyst development > 25% (range 27.1 to 74.9%) compared to control after performing IVF.

Total oocyte RNA was isolated using the PicoPure RNA Isolation kit (Arcturus/Thermo Fisher Scientific) with on-column DNA digestion (32 units of TURBO DNase, Ambion/ Thermo Fisher Scientific) and quantified (NanoDrop ND-1000; Nanodrop Technologies, Wilmington, DE, USA). For each sample, total RNA was amplified using Ovation RNA Amplification System V2 (3'-poly(A) primed; 25 ng input RNA; NuGEN Technologies, San Carlos, CA, USA) and also with the WT-Ovation Pico RNA Amplification System (amplified by 3'-poly(A) and internal random priming; 10 ng input RNA; NuGEN). Resulting cDNA was hybridized to GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA, USA) and scanned with the GeneChip Scanner 3000 7G with AutoLoader and the Affymetrix GeneChip Command Console (Affymetrix).

The relative abundance of certain transcripts identified by microarray to differ between control and heat-stressed oocytes at 24 hIVM was assessed by quantitative polymerase chain reaction (qPCR). Mitochondrial ATP Synthase Subunit O (*ATP50*), Methyltransferase Coenzyme Q3 (*COQ3*) and NADH:Ubiquinone Oxidoreductase Subunit C2 (*NDUFC2*) were evaluated using oocyte RNA before it was amplified for microarray analysis. To that end, samples was reverse transcribed with oligo $d(T)_{15}$-primers (Promega; Madison, WI, USA) after spiking with GFP cRNA as described by Payton *et al*. [11]. Gene-specific primers were designed using the FastPCR software (ver 4.0.27; [17]) for amplicons at both 5' and 3' ends of transcripts (Table 1). Conditions for each primer set were optimized to be 90 to 110% efficient. To ensure specificity, PCR products were sequenced. The equivalent of 0.1 oocytes (similar to reported previously [18–22]) was analyzed in either duplicate or triplicate in a 7300 ABI Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific) with cycling conditions of 50ºC for 2 min, 95ºC for 10 min, and 40 cycles of 95ºC for 15 sec, annealing for 30 sec (temperature indicated in Table 1), and 72ºC extension for 30 sec, followed by generation of a dissociation curve. Results were calibrated to 24 hIVM oocytes matured at 38.5ºC and analyzed using the ΔΔCt method with correction with GFP as an exogenous control for loading (calculated with RQ Study Application of 7300 System SDS software; Applied Biosystems/Thermo Fisher Scientific).

Experiment Two: Determine the effects of heat-stress exposure on mitochondrial and cytoplasmic processes

Reactive oxygen species measurement: Levels of reactive oxygen species (ROS) were measured after culture of germinal vesicle (GV)-stage cumulus-oocyte complexes for 6 h at 38.5, or 41.0ºC in polystyrene tubes. After 6 hIVM, oocytes were denuded of associated cumulus cells by vortexing in 0.3% hyaluronidase. Zona pellucida was removed using 0.5% pronase. Oocytes were then stained for mitochondrial and cytoplasmic-localized ROS. Mitochondrial-localized ROS [23] was examined using 60 μM dihydrofluorescein diacetate (DHF; Fluka/Sigma-Aldrich) in HEPES-TL [24] with 1% polyvinylpyrrolidone (HEPES-PVP). To assess cytoplasmic ROS [23], cumulus-free oocytes were incubated in 37 μM 6-carboxy-2'7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) [DCDHF; Invitrogen/Thermo Fisher Scientific] in HEPES-PVP. Hydrogen peroxide (200 μM; certified ACS; Thermo Fisher Scientific) and tert-butyl hydroperoxide (100 μM tertBOOH; Fluka/Sigma-Aldrich) in HEPES-PVP were used a positive controls. After 15 min of staining, oocytes were washed thoroughly in HEPES-PVP and imaged with a Nikon Eclipse TE300 (Nikon Instruments; Melville, NY, USA; 4',6'-diamidino-2-phenylindole filter: excites at 330–380 nm). Fluorescent intensity was determined on each individual oocyte using NIS-Elements BR imaging software (version 3.0; Nikon). Background correction was made from a vacant region of captured images. A fluorescent ratio was calculated by averaging the values for 38.5ºC samples within a given day and dividing the fluorescent value for all individual oocytes by that value. Values obtained from oocytes treated with a 1% DMSO solution (equivalent to final concentration of DMSO in DCDHF and DHF) for both cytoplasmic and mitochondrial stains were negligible. This experiment was replicated on 5 (DCDHF) and 7 (DHF) different oocyte collection days.

Adenosine triphosphate and glutathione measurements: Adenosine triphosphate (ATP) and glutathione levels were determined according to the manufacturer using the ATP Determination kit (standard curve ranging from 0.078 to 10 pmol; Life Technologies/Thermo Fisher Scientific) and the GSH-Glo Glutathione assay (standard curve ranging from 0 to 25 pmol; Promega, Madison, WI, USA). Intact cumulus-oocyte complexes were matured for 24 h at 38.5 or 41.0°C (first 12 h only, 38.5°C thereafter) and subsets of complexes were removed from maturation at 0, 12 or 24 h. For the majority of observations, a sample consisted of three zona-free oocytes frozen at –80ºC in Milli-Q water. The equivalent of one oocyte was assayed for amount of reduced glutathione (GSH), total glutathione (reduced + oxidized forms) and ATP content. Total glutathione was determined by adding TCEP (tris(2-carboxyethyl)phosphine) to a final concentration of 887 mM. Levels of oxidized glutathione (GSSG) were calculated and used to determine ratio of reduced to oxidized glutathione as measure of cellular health. This was replicated on 6 different oocyte collection days.

Experiment Three: Evaluate whether the consequences of heat-stress during oocyte maturation to alter ATP content carryover to resultant embryos

Cumulus-oocyte complexes were matured for 24 h at 38.5 or 41.0°C (first 12 h only, 38.5°C thereafter). After a total of 24 h, cumulus cells and zona pellucida were removed from a subset of oocytes using the same methods as described for experiment two (0.3% hyaluronidase and 0.5% pronase, respectively). Denuded oocytes were frozen at –80ºC for later analysis of ATP. Remaining COC underwent IVF. Using different subsets of presumptive zygotes, cleavage stage embryos (2-, 4-, and 8-to-16-cells) were collected from culture at 70 to 72 h post-IVF, whereas blastocyst stage embryos were collected from culture and evaluated for stage and quality at 204 to 216 h post-IVF as previously described [16]. In brief, numeric scores were assigned to blastocysts for stage (early = 5, normal = 6, expanding = 7, or hatched = 8) and for quality (excellent/good = 1, fair = 2, poor = 3 or degenerate = 4). ATP content was assessed in individual embryos using ATP Determination kit (Life Technologies/Thermo Fisher Scientific). Experiment 3 was replicated on 14 different oocyte collection days.

Statistical analyses

Differential transcript abundance was determined for each amplification method (polyadenylated – PA and whole transcriptome – WT) as described in Rispoli *et al*. [25] with minor modifications. Standard quality measures identified two arrays as outliers, a 24 hIVM control WT-amplified and a 24 hIVM heat stress total-amplified, and these were not included in the analysis. Three normalization methods were applied to probe level data ($\log_2$ scale) to estimate transcript intensities: variance stabilization normalization (VSN) [26], robust multi-array average (RMA) [27], and GCRMA [28]. After removal of marginal or absent probes, differential expression was tested for each normalization method using Limma (ver 3.14.1) [29]. Only probes having a multiple testing adjusted concordance P value < 0.01 across all three summarization methods and called present for at least half of the arrays were considered significant. This approach increases the stringency of the study, improves robustness to experimental noise, and reduces the false discovery rate (FDR). Thus, no FDR correction method for multiple testing was performed. To limit the lists of differentially abundant transcripts, a raw fold change of 1.3 ($\log_2$ fold = 0.38) from each summarization method was set based on the increased statistical power provided by number of biological replicates performed (n = 8) [30, 31]. Furthermore, fold changes of a similar magnitude have been reported to have functional significance in mature oocytes [32], human cancer cells [31] and yeast [31]. The intersection of the probe lists produced by each normalization method was used to generate the list of differentially expressed transcripts. Identified list of differentially abundant transcripts was then analyzed for functional enrichment using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8, Laboratory of Human Retrovirology and Immunoinformatics, Federick, MD, USA) [33, 34]. Functional categories and pathways were considered overrepresented when P < 0.05 and the enrichment score was ≥ 1.3. For ease of presentation and readability, gene lists for each amplification method were combined.

Unless otherwise indicated, all other data were analyzed as a randomized block design with fixed effects of maturation temperature, blocking on replicate, using PROC MIXED (SAS 9.2; SAS Institute, Cary, NC, USA) and transformed when necessary to correct normality. Glutathione analysis employed a factorial treatment design. For oocyte ATP data, main effect of treatment was analyzed and contrasts were performed to determine the equivalent of main effect and

Table 2. Top enriched functional categories and associated transcripts altered at 24 hIVM in oocytes by heat stress exposure for the first 12 hIVM [1]

Annotation cluster & members [2]	Enrichment score	Gene count	P-value	Genes [3]
Cluster 1	1.93			*SNRPB2*$^{\downarrow}$, *LSM6*$^{\downarrow}$, *PPIH*$^{\downarrow}$, *SNROC*$^{\downarrow}$, *TXNL4A*$^{\downarrow}$, *SRSF5*$^{\downarrow}$
Spliceosome (KEGG)		6	< 0.01	
U4/U6 x U5 tri-snRNP complex (CC)		3	< 0.01	
Spliceosomal complex (CC)		3	< 0.05	
Cluster 2	1.89			*RNF144B*$^{\downarrow}$, *ATP5O*$^{\downarrow}$, *ADHFE1*$^{\downarrow}$, *COQ3*$^{\downarrow}$, *COQ5*$^{\downarrow}$, *MRPS17*$^{\downarrow}$, *PPIF*$^{\downarrow}$, *MRPL2*$^{\downarrow}$, *ABCG2*$^{\downarrow}$, *MRPL1*$^{\downarrow}$, *NDUFC2*$^{\downarrow}$, *NDUFC1*$^{\downarrow}$, *NIT1*$^{\downarrow}$
Mitochondrion (UP)		13	< 0.01	
Transit peptide (UP)		8	< 0.01	
Transit peptide:mitochondrion (UP)		8	< 0.05	
Mitochondrion inner membrane (UP)		5	< 0.05	
Cluster 3	1.48			*RNF144B*$^{\downarrow}$, *VPS41*$^{\downarrow}$, *RAD18*$^{\downarrow}$, *RNF170*$^{\downarrow}$, *RNF166*$^{\downarrow}$, *MNAT1*$^{\downarrow}$, *RNF139*$^{\downarrow}$, *BHMT*$^{\downarrow}$, *WT1P*$^{\downarrow}$, *DDX58*$^{\downarrow}$, *ANF593*$^{\downarrow}$, *SNRPC*$^{\downarrow}$
Zinc finger, RING-type (IP)		7	< 0.01	
RING (SM)		6	< 0.05	
Zinc finger, RING-type, conserved site (IP)		5	< 0.05	
Zinc ion binding (MF)		12	< 0.05	
Zinc finger, RING/FYVE/PHD-type (IP)		7	< 0.05	
Ubiquitin protein ligase activity (MF)		4	< 0.10	
Protein polyubiquitination (BP)		3	< 0.10	
Cluster 4	1.28			*ATP5O*$^{\downarrow}$, *ATP5L*$^{\downarrow}$, *ATP6V0D1*$^{\downarrow}$, *NDUFC2*$^{\downarrow}$, *COX6A1*$^{\uparrow}$, *NDUFC1*$^{\downarrow}$
Oxidative phosphorylation (KEGG)		6	< 0.01	
Parkinson's disease (KEGG)		5	< 0.05	
Mitochondrion inner membrane		5	< 0.05	
Huntington's disease (KEGG)		5	< 0.10	
Alzheimer's disease (KEGG)		4	> 0.05	
Non-alcoholic fatty liver disease (KEGG)		3	> 0.05	

[1] Heat stress (41.0°C) applied only during first 12 h of *in vitro* maturation (hIVM), followed by incubation at control (38.5°C) temperature for remaining 12 hIVM. [2] DAVID category terms: BP, biological function; CC, cellular component; IP, Interpro; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; MF, molecular function; UP, UniProt. [3] $^{\uparrow}$ Levels increased by heat stress, $^{\downarrow}$ Levels decreased by heat stress.

interaction P-values to allow inclusion of GV-stage. Least squares means ± standard error of the mean (SEM) are presented on an individual oocyte or embryo basis. For the experiment examining the potential for heat stress during oocyte maturation to carryover to resultant embryos, ATP data were analyzed with main effect of treatment comparing within each stage (matured oocyte, cleavage, 2-, 4-, 8-, 16-cell, or blastocyst).

Results

Experiment One: Examine the consequences of heat-stress exposure on the maternal transcriptome

Relevant for oocytes collected on the same dates for which our 8 different groups of control and heat-stressed oocytes were utilized for transcriptome analysis, exposure to 41.0ºC during the first 12 h of maturation resulted in a 46.3% reduction in blastocyst development (28.3 *vs*. 15.2% for 38.5º and 41.0ºC, respectively; SEM = 2.0%; P < 0.01). Coincident with these negative effects on development, we identified changes in the relative abundance of 52 transcripts by at least 1.3-fold in the heat-stressed maternal transcriptome (polyadenylated and deadenylated mRNA) at 24 hIVM (P < 0.01; Supplementary Table 1: online only). Using the same parameters and profiling only the portion of the total mRNA with polyadenylation, 107 transcripts were differentially abundant in heat-stressed oocytes (Supplementary Table 1). Only 5 transcripts were identified to be common to both profiles (Supplementary Table 1).

Lists of differently abundant transcripts from both mRNA populations were combined to examine functional relationships of all of the transcriptomic differences due to heat stress. The majority of these 159 transcripts were annotated (n = 149) and were reduced by heat stress (n = 137; Supplementary Table 1). Functional enrichment analyses revealed over-representation in categories for mitochondria, spliceosomes, zinc finger motifs, and oxidative phosphorylation (Table 2). Based on results of the functional enrichment analyses, we created a model highlighting the possible heat-induced impacts on mitochondrial-related processes (Fig. 1).

We confirmed the relative abundance for three transcripts related to the electron transport chain and oxidative phosphorylation (Fig. 1). Consistent with microarray findings, relative abundance for the transcripts was reduced in polyadenylated mRNA from mature oocytes exposed to heat stress during first 12 of the 24 h maturation period (Table 3).

Experiment Two: Determine the effects of heat-stress exposure on mitochondrial and cytoplasmic processes

Because we detected a number of transcripts related to oxida-

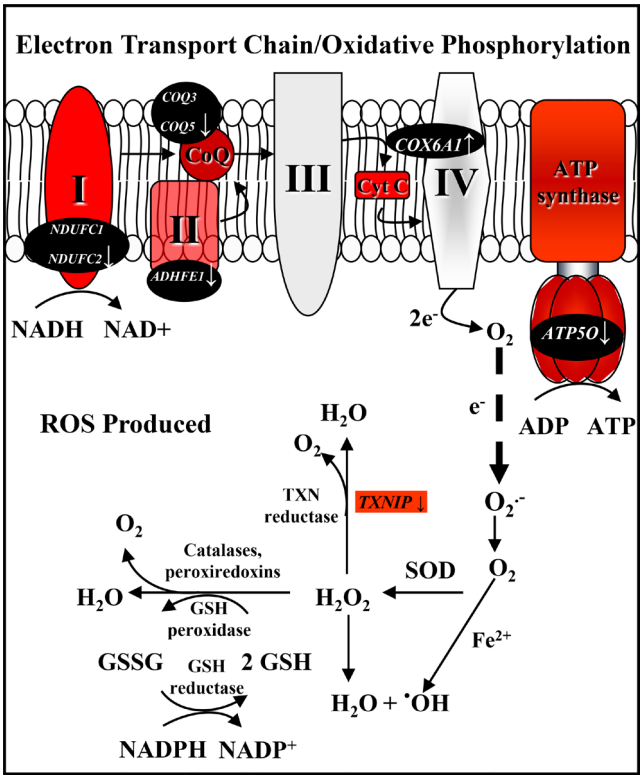

Fig. 1. Schematic highlighting the impact of heat stress on transcripts involved in electron transport and oxidative phosphorylation. Affected transcripts are indicated in each black oval next to the relevant component (shown in red) with the arrow designating the effect of heat stress (up or down). Multiple transcripts whose proteins are critical for electron transport and oxidative phosphorylation were decreased by oocyte exposure to elevated temperature. Only one transcript had higher abundance (*COX6A1*) which is involved in shuttling electrons from reduced cytochrome C to oxygen. While the main product of these reactions is ATP, free radical production is also common. TXNIP protein acts to inhibit the antioxidant action of thioredoxin. Glutathione, synthesized in the cytosol, is imported into the mitochondrion to aid in ROS neutralization.

tive phosphorylation altered by heat stress exposure in oocytes, we evaluated ROS generation localized to the mitochondria and cytoplasm. Culture at 41.0ºC for the first 6 hIVM increased levels of mitochondrial ROS and decreased cytoplasmic ROS levels when compared to non-stressed controls ($P < 0.0001$; Table 4). The levels of mitochondrial ROS after 41.0°C exposure was similar to values obtained after treatment of non-stressed oocytes with hydrogen peroxide or tertBOOH (Table 4).

Heat-induced increases in free radicals in the oocyte mitochondria lead us to examine glutathione, which is important for neutralizing ROS to prevent cellular damage. To that end, heat stress effects on glutathione content differed between 12 and 24 hIVM (IVM temperature × hIVM interaction; Table 5). Specifically, heat stress application for the first 12 hIVM increased the total glutathione content at 12 hIVM, but levels were reduced at 24 hIVM ($P < 0.001$; Table 5). Heat-induced differences were similar for levels of the reduced form (GSH; $P < 0.001$) whereas the amount of oxidized glutathione was not impacted by heat stress (GSSG; $P > 0.05$; Table 5). There was a tendency for heat stress to increase the ratio of reduced to oxidized forms (GSH:GSSG ratios 2.18 ± 0.14 *vs*. 2.42 ± 0.15 for 38.5 and 41.0ºC, respectively; $P < 0.08$). Independent of maturation temperature, ratios of reduced to oxidized forms were higher at 24 hIVM (2.66 ± 0.14) than at 12 hIVM (1.95 ± 0.14; $P < 0.0001$).

Because heat stress exposure altered the relative abundance of ATP synthase (*ATP5O*) and other transcripts playing a role in electron transport/oxidative phosphorylation (Fig. 1), we also evaluated ATP content during oocyte maturation. ATP content was influenced by maturation time and temperature (hIVM × temperature $P < 0.01$). Relevant for non-stressed controls, ATP content remained constant between 0 (GV-stage) and 12 hIVM with values being higher at 24 hIVM. While this was also noted for heat-stressed oocytes, 41.0ºC exposure during the first 12 hIVM resulted in higher amounts of ATP at 24 hIVM than non-stressed controls (Fig. 2).

Experiment Three: Evaluate whether the consequences of heat-stress during oocyte maturation to alter ATP content carryover to resultant embryos

Relevant for this study, exposure to 41.0ºC during the first 12 hIVM did not affect the ability of oocytes to cleave ($P > 0.05$) after IVF, however the proportion of cleaved embryos progressing to the 8- to 16-cell stage was less than that observed from non-stressed counterparts ($P < 0.05$; Table 6). Ability of heat-stressed oocytes to develop to the blastocyst stage was reduced by > 30% ($P < 0.001$; Table 6). Of the blastocyst embryos that did develop, there was no effect of IVM temperature on stage or quality ($P > 0.05$; Table 6).

ATP content was examined in subsets of oocytes and early embryos along this developmental continuum. Consistent with results of the

Table 3. Effects of heat-stress exposure during first 12 hIVM on abundance of certain transcripts involved in electron chain transport & oxidative phosphorylation in 24 hIVM [1] oocytes

Gene	Microarray [2]		qPCR 5'end [3]		qPCR 3'end [3]	
	Fold change	P-value	Fold change	P-value	Fold change	P-value
ATP5O	–1.39	< 0.01	–1.33	< 0.05	–1.02	> 0.05
COQ3	–1.54	< 0.01	–1.05	> 0.05	–1.64	< 0.05
NDUFC2	–1.49	< 0.01	–2.30	< 0.0001	–1.49	< 0.01

[1] Heat stress (41.0°C) applied only during first 12 h of *in vitro* maturation (hIVM), followed by incubation at control (38.5°C) temperature for remaining 12 hIVM. [2] Values for fold change and P-value are averaged from three normalization methods. [3] Gene specific primers for quantitative PCR (qPCR) were designed to amplify amplicons for the 5' or 3' ends of transcripts.

Table 4. Reactive oxygen species levels in heat-stressed oocytes at 6 hIVM

Treatment	Mitochondrial ROS		Cytoplasmic ROS	
	n [1]	Fluorescent ratio [2]	n [1]	Fluorescent ratio [2]
38.5ºC	320	0.96 [b]	138	1.04 [b]
41.0ºC	268	1.40 [a]	138	0.86 [c]
H_2O_2	118	1.37 [a]	137	2.99 [a]
tertBOOH	199	1.34 [a]		n.d.
Pooled SEM		0.14		0.18
P-value		< 0.0001		< 0.0001

hIVM, hours of *in vitro* maturation; H_2O_2, hydrogen peroxide; n.d., not determined; ROS, reactive oxygen species; tertBOOH, tert-butyl hydroperoxide. [1] Total number of oocytes examined per treatment. [2] Relative ROS levels per oocyte expressed as a ratio of average fluorescence in 38.5ºC controls. [abc] Means differ within a location for reactive oxygen species (P < 0.0001).

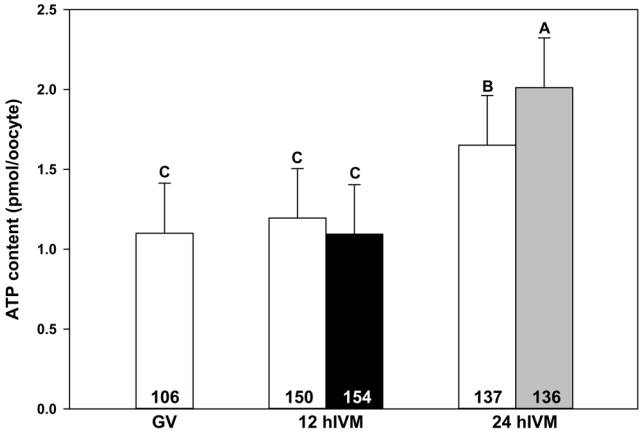

Fig. 2. Heat-induced alterations in oocyte ATP content after culture for 12 or 24 h at 38.5 or 41.0ºC (first 12 h only, 38.5°C thereafter). All controls (38.5ºC), regardless of hour of *in vitro* maturation (hIVM), are denoted in white. Oocytes immediately after 12 h of heat stress are denoted in black. Heat-stressed oocytes given a recovery period from 12 to 24 h (i.e., heat stressed for 12 h at 41.0ºC and then moved to 38.5ºC for the remaining 12 h of maturation) are denoted in gray. Numbers within bars indicate the number of oocytes analyzed. [ABC] Means differ (P < 0.0001).

preceding study, oocytes matured at 41.0°C for the first 12 of 24 hIVM (38.5°C thereafter) contained more ATP than non-stressed counterparts (P < 0.05; Fig. 3). When examined at ~72 h post-IVF, ATP content was higher in cleaved embryos from heat-stressed oocytes (P < 0.05; Fig. 3); specifically in 2-cell (P < 0.05) and 8- to 16-cell (P < 0.05) embryos (Fig. 3 inset). Heat-stress exposure during first half of oocyte maturation had no effect on ATP content in resulting blastocysts at ~204 h post-IVF (P > 0.05; Fig. 3).

Table 5. Effect of exposure to 41.0ºC during maturation on oocyte glutathione content (pmol/oocyte)

hIVM	IVM temp (ºC)	n [1]	Total (GSH + GSSG)	n [1]	GSH	GSSG [2]	Ratio [3]
12	38.5	97	4.41[d]	97	2.76[d]	0.13	1.78
	41.0	98	5.19[c]	98	3.50[c]	0.13	2.11
24	38.5	96	6.60[a]	96	4.80[a]	0.14	2.58
	41.0/38.5	90	5.90[b]	90	4.20[b]	0.13	2.73
		Pooled SEM	1.26		0.97	0.02	0.19
		Interaction P-value	< 0.001		< 0.001	> 0.05	> 0.05

hIVM, hours of *in vitro* maturation; GSH, reduced glutathione; GSSG, oxidized glutathione. [1] Total number of oocytes examined per treatment. [2] Values calculated by subtracting GSH from Total glutathione content. [3] Ratio of GSH:GSSG. [abcd] Means differ within type of glutathione (P < 0.05).

Table 6. Embryo development of oocytes matured at 38.5 or 41.0°C [1]

IVM temperature (°C)	Cleavage stage development (72 h post-IVF)				Blastocyst development (204 to 216 h post-IVF)		
	Cleaved (%)	2-Cell [3] (%)	4-Cell [3] (%)	8 to 16-Cell [3] (%)	Percent of PZ	Stage	Quality
38.5	70.05	8.72	22.02 [b]	71.00 [a]	29.73 [a]	6.80	1.51
41.0	69.87	9.22	27.08 [a]	63.50 [b]	19.91 [b]	6.89	1.59
Pooled SEM	2.58	1.44	1.38	2.42	2.26	0.09	0.07
P-value	> 0.05	> 0.05	< 0.05	< 0.05	< 0.001	> 0.05	> 0.05

IVM, *in vitro* maturation; PZ, presumptive zygote. [1] First 12 h of maturation followed by 38.5°C thereafter. [2] Number of presumptive zygotes evaluated for cleavage. [3] Relative to proportion of embryos cleaved. [4] Number of presumptive zygotes evaluated for blastocyst development only (a different subset than those for cleavage). [ab] Means differ within a column (P < 0.05).

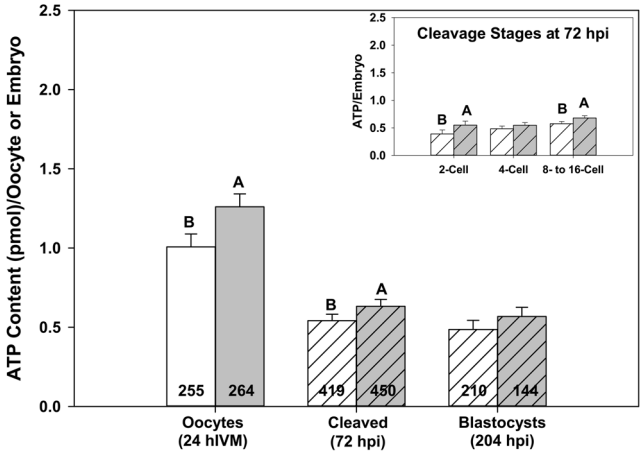

Fig. 3. Pairwise comparisons of ATP content (pmol ± SEM) in matured oocytes, cleaved embryos and blastocyst-stage embryos from control (38.5°C for 24 h of *in vitro* maturation; white bars) and heat-stressed (41.0ºC for the first 12 h of maturation and 38.5ºC thereafter; gray bars) oocytes. Hatched bars indicate embryo stages *vs.* oocytes. Numbers within bars indicate the number of oocytes or embryos analyzed. ^{AB} Means within a stage differ ($P < 0.05$).

Discussion

Exposure of oocytes to heat stress resulted in numerous alterations for maternal transcripts. Of particular interest were the changes for transcripts integral to the electron transport chain and oxidative phosphorylation reactions (Fig. 1), pertaining to the translocation of proteins into mitochondria, and protein synthesis within the mitochondria (Table 2). Collectively, these heat-induced perturbations to the transcriptome could indicate structural and functional changes in oocyte mitochondria. Efforts documenting changes in the reduction-oxidation potential (ROS and glutathione) and energy levels (ATP) substantiate the notion that heat exposure altered mitochondrial functions. Furthermore, ATP content results highlight that functional changes persist during early embryo development after fertilization of heat-stressed oocytes. Alterations in maternally-derived mitochondria may explain to some extent how a heat insult during oocyte maturation results in reduced embryo development [4, 10, 16, 35, 36] and decreased pregnancy success [2, 3] after fertilization.

Heat-stressed oocytes undergo germinal vesicle breakdown and progress to metaphase II sooner than non-stressed counterparts [37, 38]. Mindful that earlier insemination of heat-stressed oocytes improved developmental competence [16] and some of the findings described herein, it is interesting to note that *in vitro* oocyte aging (30 and 40 hIVM) increases ATP content [39] and reduces developmental competence [40] in a manner similar to what we have observed after heat stress exposure. Furthermore, perturbations in mitochondrial function resulting from aging, persist after fertilization. To that end, cleavage-stage embryos display altered localization of highly active mitochondria to the periphery of blastomeres [39]. Reduced development from embryos inheriting altered mitochondria from heat-stressed oocytes having higher ATP content is consistent with the "quiet embryo hypothesis" where embryos with less metabolic activity (largely assessed by nutrient and oxygen consumption) are more developmentally competent than those that are more metabolically active [41–43].

Mitochondrial dysfunction in the oocyte that is inherited by the early embryo [44–47] may be sufficient to alter subsequent fetal and placental development [48, 49]. Persistence of dysfunction and potential to be problematic is not surprising because embryonic mitochondria are predominantly, if not entirely, of maternal origin. In the bovine, some mitochondria may undergo degradation during early cleavage stages [50], which could be especially concerning if the remaining population of mitochondria contained primarily those perturbed by heat-stress exposure. Because maternally-derived mitochondria are essential for providing energy required for early cell divisions as well as calcium homeostasis, reduction-oxidation potential and apoptosis [51], any level of dysfunction during early embryo development in mitochondrial processes are concerning.

Interestingly, ATP content was similar in blastocyst-stage embryos from heat-stressed and non-stressed oocytes. Lack of detectable differences at this embryonic stage may be because embryos having altered ATP content did not progress to this embryonic stage (i.e., blastocyst development was reduced by > 30% as a result of heat stress exposure). It is also plausible that this embryonic stage presents complexities including but not exclusive to differences in transcriptional activity [52] and changes in mitochondrial maturation/function [53, 54] that may mask underlying issue(s). The latter notion is more likely because even morphologically "normal" appearing blastocyst-stage embryos from heat-stressed oocytes are more susceptible to heat stress exposure [4] and experience an increased incidence of embryonic loss soon after placental attachment[2, 3].

Although underlying mechanisms remain unclear, results herein showing impact on the energy levels (ATP content) and reduction-oxidation potential (ROS and glutathione) support the notion that heat exposure alters mitochondrial functions. This finding is consistent with what has been reported by others showing that bovine oocytes exposed to 41.0ºC for either the first half [36] or for the entire maturation period [55] had reduced mitochondrial membrane potential. A lower mitochondrial membrane potential can indicate elevated ATP synthesis or mitochondrial dysfunction [56]. Furthermore, exposure of maturing bovine oocytes to incremental temperature increases from 39.5 to 40.5ºC for a total of 20 h not only reduced mitochondrial membrane potential, but also increased intracellular ROS at the end of maturation [57]. In this study, exposure to elevated temperature during the beginning of maturation (6 hIVM) immediately increased mitochondrial ROS (byproducts of disrupted oxidative phosphorylation; Fig. 1) without increases in cytoplasmic ROS suggesting that the impact heat stress is targeted rather than global. Others have reported heat-induced increases in oocyte ROS content at the end of meiotic maturation [36, 57, 58]. A recent study using the heat stress model of 41.0ºC for the first 12 hIVM reported increased ROS levels in the culture medium at 12 and 24 hIVM compared to thermoneutral controls [59]. Collectively these findings coupled with our data suggests heat-induced increases in ROS begin soon after exposure to elevated temperature and persist until end of maturation, even when allowed recovery time at thermoneutral temperatures [36, 58]. Heat-induced elevations in ROS were not unexpected

because previous efforts had shown that including the antioxidant retinol to the medium while oocytes were matured at an elevated temperature improved blastocyst development [35]. Since oocytes rely upon cumulus cells for glutathione synthesis [60], increased levels at 12 hIVM suggest that the surrounding cumulus may be acting in a thermoprotective manner [10] possibly by providing more glutathione to the oocyte. Reduced levels of total and reduced glutathione in heat-stressed oocytes at 24 hIVM (herein and [58]) may suggest consumption of this intracellular antioxidant, possibly due to increased ROS neutralization. Because protein synthesis is one of the most prominent consumers of cellular energy [61] and heat stress reduces *de novo* protein synthesis in maturing oocytes by 30 to 50% [10], increased ATP content in heat-stressed oocyte may also be a reflection of perturbations in translational efficiency (i.e., lower ATP utilization).

While much of this discussion has focused on possible heat-induced mitochondrial dysfunction, heat stress consequences also extended to numerous transcripts important in other cellular and developmentally important components. The unique translational situation of the maturing oocyte provides an additional level of complexity to interpreting this transcriptome data, especially since the oocyte is transcriptionally quiescent after resuming meiosis and there are marked changes in RNA adenylation status and turnover. Relevant for our initial efforts to profile total (adenylated + deadenylated) versus polyadenylated RNA populations, 47 transcripts from total RNA were differentially affected by heat stress. Transcript number affected by heat stress increased to 102 when only polyadenylated RNA was utilized. Interestingly, however, only 5 of the differentially affected transcripts were common to both profiles (*SH3PXD2A*, *MTURN, FAM114A2, RNF139, LOC100847840*). Isoform-specific events occur after resumption of meiosis as a result of complete degradation, deadenylation, and/or production of stable 5'-fragments [62] which may explain the differential findings in total versus polyadenylated profiles described herein.

Nonetheless, the novel results presented herein document an effect of heat stress to alter the maternal transcriptome. While the functional consequences remain unclear, follow-on studies documenting heat-induced perturbations in oxidative phosphorylation and antioxidant processes suggests possible issues with cytoplasmic and mitochondrial components. The fact that some of the perturbations, occurring in the heat-stressed oocyte, carry over to the early embryo after fertilization likely explains some of the negative impact of heat stress to reduce embryo development thereafter.

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