—Original Article—

Impact of Heat Stress Exposure During Meiotic Maturation on Oocyte, Surrounding Cumulus Cell, and Embryo RNA Populations

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Abstract. To determine if reductions in developmental competence related to heat stress exposure were correlated with perturbations in certain RNA populations, poly(A) RNA, total RNA, RNA size distribution, and the abundance of transcripts (cyclin B1, GDF9, BMP15, poly(A) polymerase, HSP70, 18S & 28S rRNA) were examined in oocytes matured at 38.5 or 41 C. Performing *in vitro* fertilization resulted in embryos for examining RNA. Relative to germinal vesicle-stage oocytes, total amount of poly(A) RNA decreased similarly in oocytes matured at 38.5 or 41 C. Total RNA did not change during meiotic maturation or up through the 4 to 8-cell stage of embryonic development. Blastocyst-stage embryos had more total RNA; those originating from heat-stressed oocytes had more than those from nonheat-stressed oocytes. Oocytes and 4 to 8-cell embryos had similar RIN values and ratios for rRNA, 18S/fast region, and 18S/inter region. Values obtained for blastocyst-stage embryos were similar to those obtained for cumulus cell RNA, which did not change during maturation. Culture at 41 C for the first 12 h of meiotic maturation had no impact on RNA size distribution or transcripts examined from oocytes, surrounding cumulus or resultant 4 to 8-cell embryos. Interestingly, however, RNA from blastocysts originating from heat-stressed oocytes had lower 18S/fast region and 18S/inter region ratios compared to other developmental stages and cumulus cells. Although biological significance of these RNA changes is unclear, differences at the molecular level in embryos from heat-stressed oocytes emphasize the importance of minimizing stress exposure during meiotic maturation, if the intent is to obtain developmentally-competent embryos.

Key words: Cumulus, Heat stress, Microcapillary electrophoresis, Oocyte, RNA

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Agriculturally-important females exposed to environmental heat stress experience reduced fertility [1–4]. In cattle, this is largely due to hyperthermia [5–7] whereby rectal temperatures may reach or exceed 41 C [5, 8–12]. Effects of environmental heat stress to reduce female fertility are pronounced during or near estrus [13–15]. This corresponds to the time period when the female is receptive to mating and the oocyte within the Graafian follicle resumes meiosis and progresses to metaphase II (MII; meiotic maturation) in preparation for fertilization. Hyperthermia during this time has been associated with reductions in early embryonic development [16] and increases in embryonic loss near the time of placental attachment [17].

The negative impact of hyperthermia during estrus may be attributable to indirect effects on the maternal environment [reviewed by 18] and/or due to direct effects on the cumulus-oocyte complex. Direct exposure of the cumulus-oocyte complex to 41 C during meiotic maturation reduces early embryo development after fertilization by 30 to 65% [19–25] similar to consequences observed when environmental heat stress elevates rectal temperatures to 41 C [16]. Marked changes occurring in the nucleus and ooplasm may explain why oocytes undergoing meiotic maturation are so susceptible to direct effects of heat stress. Unfortunately,

however, consequences go beyond the obvious of reducing blastocyst development after fertilization, and include yet unidentified heat-induced perturbations that continue to affect otherwise morphologically-normal embryos. For instance, Edwards *et al.* [26] showed that compact morula-stage embryos from heat-stressed oocytes are more susceptible to heat stress than embryos from nonheat-stressed oocytes. This finding is consistent with Rutigliano *et al.* [27] who reported that dairy cows exposed to heat stress during artificial insemination are more likely to lose their pregnancies than nonheat-stressed cows.

Heat-induced reductions in the developmental competence of bovine oocytes have been coincident with a 30 to 50% reduction in *de novo* synthesis of intracellular proteins [19]. In murine oocytes, reductions as high as 80% have been noted [28]. Although the underlying mechanisms remain unclear, results of Payton and Edwards [29] implicate heat-induced reductions in the level of polyadenylated (poly(A)) mRNA in bovine oocytes by 12 h of *in vitro* maturation. Even though differences were no longer detected at 24 h, there is potential for heat stress to impact developmentally-important transcripts that may produce proteins required for proper embryo development [reviewed by 30], as upon resumption of meiosis and germinal vesicle breakdown the oocyte is transcriptionally quiescent [i.e., no longer capable of transcribing RNA; 31–33]. This transcriptional inactivity, or reliance upon maternal RNA stores for protein synthesis, persists through the third cleavage division in the bovine [8 to 16-cell stage; 34].

To determine if reductions in developmental competence of the

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oocyte after heat stress exposure during meiotic maturation are attributable to heat-induced perturbations in certain RNA populations, poly(A) RNA, total RNA, RNA size distribution, and the abundance of specific transcripts (i.e., growth and differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), cyclin B1 (CCNB1), poly(A) polymerase (PAP), heat shock protein 70 (HSP70), 18S & 28S rRNA) were examined. Choice of individual transcripts was based on importance in the oocyte, during maturation, or heat shock response [35–39, reviewed by 40]. Performing *in vitro* fertilization on subsets of oocytes allowed measurement of RNA populations in resultant embryos.

Materials and Methods

Oocyte collection and selection criteria

Bovine ovaries were purchased from a commercial abattoir (Brown Packing, Gaffney, SC, USA) originating predominantly from Angus, Holstein, Hereford, and Charlois cows. Oocytes were collected as previously described [24]. Only cumulus-oocyte complexes having multiple layers of tightly compacted cumulus and dark, homogenous ooplasm were selected for *in vitro* maturation (IVM). An experimental unit was a group (pool) of cumulus-oocyte complexes cultured together within one well of a 4-well Nunclon culture dish (Fisher Scientific, Pittsburgh, PA, USA).

General methods for sample processing and RNA extraction

In vitro maturation, fertilization (IVF), and culture of resultant embryos were performed as previously described [23, 25, 26]. Briefly, cumulus-oocyte complexes were matured in Medium-199 containing Earle's salts, 10% fetal bovine serum, 50.0 µg/ml gentamicin, 5.0 µg/ml FSH, 0.2 mM Na pyruvate, and 2 mM L-glutamine in a 5.5% CO₂ humidified environment. After ~24 h of *in vitro* maturation (hIVM), IVF was performed with Percoll-prepared frozen-thawed semen (500,000 motile sperm/ml) generously provided by Ultimate Genetics (Wheelock, TX, USA) and Harrogate Genetics International (Harrogate, TN, USA). Putative zygotes were denuded of associated cumulus at 18 to 20 h post-IVF and cultured at 38.5 C in a humidified environment of 5.5% CO₂, 7.0% O₂, and 87.5% N₂ in potassium simplex optimized medium as per Schrock *et al.* [25]. Ability to undergo cleavage and blastocyst development was assessed on days 3 and 8 post-IVF, respectively. Blastocysts were assigned stage and quality scores as per Schrock *et al.* [25] according to guidelines set by the International Embryo Transfer Society [41].

For sample processing at the germinal vesicle (GV)-stage, oocytes soon after follicle removal were denuded of surrounding cumulus by vortexing [4.5 min in HEPES-PVA (HEPES-TL [42] supplemented with 0.3% polyvinyl alcohol (Sigma-Aldrich, St. Louis, MO, USA), 0.20 mM sodium pyruvate (Sigma-Aldrich), and 0.5X penicillin/streptomycin (Millipore, Billerica, MA, USA)]. Oocytes at subsequent stages of IVM were also denuded by vortexing, but in 0.3% hyaluronidase (Sigma-Aldrich). The number of oocytes recovered and visibly lysed after denudement was recorded. Each oocyte was manipulated individually under a stereomicroscope to ensure complete removal of cumulus cells before transferring the group to a microcentrifuge tube in 3.5 µl HEPES-

PVA. Samples were lysed and extracted according to manufacturer's specifications for either Absolutely RNA Microprep kit (Stratagene, La Jolla, CA, USA) or PicoPure RNA Isolation kit (MDS Analytical Technologies, Sunnyvale, CA, USA), as indicated in the following sections describing experimental details. Lysates were stored at –80 C until RNA extraction.

Experiment 1: Abundance of the total amount of poly(A) RNA in GV-stage or control and heat-stressed oocytes at 12, 16, 20, and 24 hIVM

Previous efforts had shown that the total amount of poly(A) mRNA decreased throughout meiotic maturation [29]. While heat stress exposure reduced levels observed at 12 hIVM, heat-induced differences in levels of poly(A) mRNA were not apparent at 24 hIVM. To determine when during meiotic maturation poly(A) RNA levels in heat-stressed oocytes return to control levels, cumulus-oocyte complexes were randomly assigned to a total of 9 treatments involving culture at 38.5 or 41.0 C as per Fig. 1. Prior to placement in maturation medium (i.e., 0 hIVM), a group of GV-stage cumulus-oocyte complexes (Fig. 1, treatment 1) was denuded; cumulus-free GV-stage oocytes were frozen at –80 C as pools of 60 in RNA lysis buffer (Stratagene). Control groups were placed in culture at 38.5 C for 12 (Fig. 1, treatment 2), 16 (treatment 4), 20 (treatment 6), or 24 hIVM (treatment 8). Remaining cumulus-oocyte complexes were heat-stressed at 41 C for the first 12 hIVM and denuded (Fig. 1, treatment 3) or placed at 38.5 C until 16 (treatment 5), 20 (treatment 7), or 24 hIVM (treatment 9). Pools of cumulus-free oocytes (n=60/pool) were frozen at –80 C in RNA lysis buffer. Total RNA, isolated using Absolutely RNA Microprep kit (Stratagene), was eluted in 30 µl. Total amount of poly(A) RNA was quantified using the Poly(A) mRNA Detection System (Promega, Madison, WI, USA) as per manufacturer's instructions using a six-point standard curve ranging from 0 to 200 pg/µl (inter-assay CV of 7.8%).

On five different occasions, subsets of control and heat-stressed oocytes underwent IVF and were allowed to develop to the blastocyst stage to document the extent to which heat stress reduced developmental potential. Cleavage and blastocyst development were assessed at approximately 73 h post-IVF (hpi) and 192 hpi, respectively. This experiment was replicated on 13 different occasions (n=60 oocytes/pool; 780 total per treatment) between January and middle of June using a total of 7,020 oocytes.

Experiment 2: RNA size distribution and abundance of certain transcripts in control and heat-stressed oocytes, their surrounding cumulus cells, and resultant embryos

To determine if reductions in developmental competence are attributable to heat-induced perturbations in certain RNA populations, total RNA, RNA size distribution, and the abundance of specific transcripts (i.e., CCNB1, GDF9, BMP15, PAP, HSP70, 18S & 28S rRNA) were examined in control and heat-stressed oocytes. Performing IVF on subsets tested the extent to which perturbations in various RNA populations affected resultant embryos.

Immediately after removal from ovarian follicles but before placement in maturation medium (i.e., 0 hIVM when oocytes were at GV-stage), or after IVM at 38.5 or 41.0 C for 12 or 24 h, subsets

Fig. 1. Schematic of design for experiment 1. Cumulus-oocyte complexes were collected and randomly allotted to one of 9 treatments. Soon after removal from ovarian follicles, but before placement in *in vitro* maturation medium (i.e., 0 hIVM), oocytes (treatment 1) were denuded of surrounding cumulus and frozen in lysis buffer at -80°C . Additional cumulus-oocyte complexes were cultured at 38.5°C (indicated by white circles) for 12 (treatment 2), 16 (treatment 4), 20 (treatment 6), or 24 hIVM (treatment 8). Additional groups of oocytes were matured at 41.0°C for the first 12 hIVM and denuded immediately (treatment 3; indicated by black circle) or cultured at 38.5°C thereafter (indicated by gray circles) for a total of 16 (treatment 5), 20 (treatment 7), or 24 h (treatment 9). All cultured oocytes were denuded of cumulus at indicated time and stored in lysis buffer at -80°C until RNA extraction and analysis of poly(A) RNA. For 5 of the 13 replicates, a subset of cumulus oocyte complexes underwent *in vitro* fertilization (IVF) at ~24 hIVM (designated as day 0 of embryo development); cleavage and blastocyst development were assessed on days 3 and 8, respectively.

of oocytes were denuded, lysed in groups of 50 in $25\ \mu\text{l}$ RNA extraction buffer (MDS Analytical) before storage at -80°C (Fig. 2, treatments 1 through 5). Cumulus cells from each group of oocytes were kept separate according to treatment origin, washed in HEPES-PVA, and pelleted for lysis in $200\ \mu\text{l}$ of RNA extraction buffer as per Fig. 2. Additional subsets of cumulus-oocyte complexes underwent IVF at 24 hIVM. At approximately 40.5 hpi, a subset of 4 and 8-cell embryos from control and heat-stressed oocytes ($n=32$ to $117/\text{pool}/\text{replicate}$, $\text{mean}=65.6$) were removed from culture and lysed in $25\ \mu\text{l}$ of RNA extraction buffer (Fig. 2, treatments 6 and 7). Ability of remaining embryos to undergo cleavage and blastocyst development was assessed at approximately 72 and 192 hpi, respectively. Blastocyst-stage embryos from control and heat-stressed oocytes (Fig. 2, treatments 8 and 9) were lysed in $25\ \mu\text{l}$ of RNA extraction buffer ($n=6$ to $35/\text{pool}/\text{replicate}$, $\text{mean}=14.4$).

RNA was extracted using PicoPure kit as per manufacturer instructions and was DNase-treated on the column using TURBO DNase [4 (oocytes and embryos) or 16 units (cumulus), Applied Biosystems/Ambion; Austin, TX, USA] before elution. DNA-free RNA was eluted in an 11 or $20\ \mu\text{l}$ volume, for oocytes/embryos and cumulus, respectively. Total RNA was quantified using Quant-iT RiboGreen RNA Reagent and kit (Invitrogen, Carlsbad, CA, USA) by analyzing $1\ \mu\text{l}$ of eluted RNA (diluted $200\times$) in triplicate using a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA, USA). Amount of total RNA per oocyte was calculated from simple linear regression of a standard curve (1 to $400\ \text{ng}/\text{ml}$; interassay CV=9.1% and 2.5% for 2000- and 200-fold dilution assays, respectively).

Microcapillary-electrophoretic separation and detection via laser-induced fluorescence using RNA 6000 Pico Lab Chips

allowed for assessing size distribution of RNA [43] in oocytes, surrounding cumulus and resultant embryos as previously described by Payton *et al.* [44]. For each sample ($1\ \mu\text{l}$ of eluted RNA), the Bioanalyzer software generated a rRNA ratio (area under 28S peak divided by area under 18S peak) and an RNA integrity number (RIN) which is an algorithm-based value taking into account all the regions of the electropherogram [43]. This value was derived to provide an unbiased metric of RNA integrity using all RNA sizes present within a sample. In addition, relative amounts of RNA sizes in the fast, 18S, inter, and 28S regions were estimated with the smear analysis for examining the proportion of 18S rRNA to smaller RNA sizes (i.e., those between ~200 to $1500\ \text{nt}$; 18S/fast region ratio). This was done to estimate the abundance of smaller RNA sizes in a given sample relative to 18S rRNA. Since 28S rRNA is typically degraded before 18S and is generally coincident with increased abundance of RNA sizes in the inter region [45, 46], an 18S/inter region ratio was also calculated.

Quantitative PCR analysis (qPCR) was utilized to assess the relative abundance of developmentally-important transcripts (Table 1). Primers were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) or FastPCR (version 4.0.27; Institute of Biotechnology; University of Helsinki, Finland) from bovine sequences in GenBank. Before reverse transcription, oocyte and embryo RNA samples were spiked with 12 pg of green fluorescent protein (GFP) RNA per oocyte or embryo as an exogenous control for normalization of transcript abundance [47, 48]; cumulus samples were spiked with 500 pg per 100 ng total RNA. Green fluorescent protein construct with T7 promoter was linearized with EcoRI (Fisher), *in vitro* transcribed (4 h at 37°C) using the MEGA-script kit (Ambion), and purified (MEGAclean kit; Ambion). Reverse transcription was performed with 500 ng random hexam-

Fig. 2. Schematic of design for experiment 2. Cumulus-oocyte complexes were randomly allotted to culture at 38.5 for 0 (soon after removal from ovarian follicles but before placement in *in vitro* maturation medium), 12 or 24 hIVM (indicated by white circles). Others were matured at 41.0 C for 12 h and removed for analysis (indicated by black circle) or cultured for another 12 h at 38.5 C (indicated by gray circles). At 0 and 24 hIVM, cumulus-free oocytes and their surrounding cumulus were lysed separately in extraction buffer and stored at -80°C until RNA extraction and analysis of total RNA, RNA integrity, and individual transcripts. Other remaining subsets of cumulus-oocyte complexes were subjected to *in vitro* fertilization (IVF; designated as day 0 of embryo development). Presumptive zygotes were denuded 18–20 h post insemination (hpi). A subset of 4 to 8-cell embryos was collected at 40.5 hpi and processed for RNA analysis. Cleavage and blastocyst rates were assessed on remaining embryos on days 3 and 8, respectively. Blastocysts were processed for RNA analysis as indicated.

Table 1. Sequences of primers used for qPCR amplification of genes of interest

Gene	GenBank accession number	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Final primer concentration (nM)
18S rRNA	AY779625	AAGACGGACCAGAGCGAAAG	GGTCGGAACCTACGACGGTATCT	100	500
28S rRNA	AY639443.1	TCTGGTGGAGGTCCGTAGCGGT	TGCGTTTGGGAGAGCGCCAGCT	139	500
BMP15	NM_001031752	TGGAACATGTTGGGCAAAAG	AACTCACGAACCTCACTACCTCTTG	100	500
CCNB1	L26548	TCAGATTACTGCAGGAGACCATGT	ACTCCAACCACTGCAGCAT	100	750
GDF9	NM_174681	TTGCCTGGCTCTGTTTCTCT	CAAGTCTCAGCCTCAGATTCCA	100	750
HSP70 ^a	NM_203322	ACGTCGTTGATCCTGTGGCCGT	ACCTTGCCGTGCTGGAACACC	147	750
PAP	X63436	AGCAGCCTCGACTTGTCTATGG	GAGAGCTGCCAGAACTGTTCAA	100	250
GFP	---	CAACTTCAAGACCCGCCACA	TCTGGTAAAAGGACAGGGCCA	102	750

^a Primers specific for bovine HSP70-1 (HSPA1A) and HSP70-2 (HSPA1B) located in tandem on chromosome 23 [84].

ers (Promega), 200 units MMLV-RT (Promega), 500 nM dNTP mix (Fisher Scientific), and 25 units Superase-in (Ambion) in 1X MMLV buffer to a final volume of 25 μL . Reverse transcription reactions (37 C for 1 h, 94 C for 10 min) were cooled to 4 C before storage at -20°C . A subset of RNA from each sample was reverse transcribed in the absence of enzyme and tested with each primer set to assess genomic contamination (RT-minus).

Efficiency for each primer set was between 90 and 110% (where efficiency = $10^{(-1/\text{slope})} - 1$; slope of $-\log$ concentration of standard

curve versus threshold cycle). To ensure specificity, PCR products were sequenced. Relative quantification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in a 25 μL volume. The equivalent of 0.1 oocytes or embryos [similar to that reported by 49, 50–53] or 100 pg cumulus total RNA were analyzed in either duplicate or triplicate in a 7300 ABI Real-Time PCR System (Applied Biosystems) for 35 cycles at either 60 C or 63 C (28S and HSP70) for 30 sec and 72 C for 30 sec followed by a dissociation curve. The calibrator sample was GV-stage oocyte or

cumulus from GV-stage oocytes within each replicate. Normalized and calibrated data were analyzed using the $\Delta\Delta C_t$ method (calculated with RQ Study Application of 7300 System SDS software as per manufacturer's specifications; Applied Biosystems).

This experiment was replicated on 23 different occasions between the months of October to December and January to May. Heat-induced blastocyst reductions $\geq 20\%$ were observed for seven replicates, the data from which are presented [a total of 4,267 oocytes utilized to obtain oocyte pools of ~ 50 per each of 5 treatments after denuding (total of 2,090 oocytes), 340 and 316 4 to 8-cells per treatment (total of 656 4 to 8-cells), and 122 and 79 blastocysts per treatment (total of 201 blastocysts)].

Statistical analyses

For each experiment, all results are presented as least squares means (LSM) $\pm$ standard error of the mean (SEM) unless otherwise noted. Data obtained in the first experiment were analyzed as a randomized block design (RBD) using PROC GLIMMIX of SAS (SAS 9.1, SAS Institute, Cary, NC, USA) with main effect of treatment and random effect of replicate for normally-distributed data. *A priori* contrasts were performed to examine changes in poly(A) RNA over maturation. In addition, contrasts were performed to assess the effect of heat stress exposure on poly(A) at each time point, and the interaction of maturation time and temperature (factorial treatment design). Replicates with corresponding development data were analyzed separately to assess effects of poly(A) RNA levels during oocyte maturation on subsequent ability to develop into blastocyst stage embryos.

For the second experiment, embryo developmental data were analyzed as a RBD with factorial treatment design with main effects of hIVM (12 or 24 hIVM) and temperature (38.5 or 41.0 C) and random effect of replicate using PROC GLIMMIX with a binomial distribution. Stage and quality scores for blastocysts were analyzed similarly but with a normal distribution. Oocyte and embryo RNA data were analyzed as a randomized incomplete block (4 to 8-cell embryos not present in every replicate) with factorial treatment arrangement, random effect of replicate, and covariate for amount of RNA per qPCR reaction (since samples were loaded on a per oocyte or embryo basis). Cumulus RNA data were analyzed as a RBD with main effect of treatment and random effect of replicate using PROC GLIMMIX with a normal distribution. Experimental endpoints included total RNA, rRNA ratio, RIN, 18S/fast region ratio, 18S/inter region ratio, and relative abundance of individual transcripts. Possible changes occurring during meiotic maturation were assessed by examining oocytes at GV-stage and those matured at 38.5 C for 12 and 24 hIVM.

To determine if heat stress exposure during meiotic maturation altered the relative abundance of certain transcripts across development (i.e., GV-stage oocytes through blastocyst-stage embryos after fertilization), qPCR data for experimental replicates exhibiting heat-induced reductions in blastocyst development $\geq 20\%$ were analyzed using multisource nonlinear mixed model regression (PROC NL MIXED; SAS). Quadratic (18S and 28S) or exponential decay (BMP15, CCNB1, GDF9, HSP70, and PAP) equations were used to fit transcript abundance curves for control and heat stress treatments.

Results

Experiment 1: Abundance of the total amount of poly(A) RNA in GV-stage or control and heat-stressed oocytes at 12, 16, 20, and 24 hIVM

Recovery (97.2 to 98.6%; SEM=0.7%; $P=0.79$) and lysis (1.7 to 3.1%; SEM=0.5%; $P=0.73$) of oocytes after cumulus denudement by vortexing were similar regardless of hIVM or IVM temperature examined. However, amount of poly(A) RNA per oocyte differed depending upon duration of maturation ($P<0.0001$). Specifically, poly(A) RNA was highest in GV-stage oocytes and was markedly lower in oocytes after 12 hIVM ($P<0.0001$; Fig. 3A). Poly(A) RNA levels per oocyte continued to decline up to 24 hIVM (Fig. 3A). There was no impact of heat stress exposure during the first 12 hIVM to affect poly(A) RNA levels at any of the time points examined ($P>0.25$; Fig. 3A).

To provide a more precise test of the hypothesis (i.e., heat-induced reductions in certain RNA populations during meiotic maturation may explain why a subset of oocytes fail to develop to the

Fig. 3. Poly(A) RNA within oocytes matured at 38.5 or 41.0 C for up to 24 hIVM. White bars indicate culture at 38.5 C for 0, 12, 16, 20, or 24 hIVM. Black bar indicates heat stress for 12 h. Gray bar indicates heat-stress for 12 h and culture at 38.5 C thereafter. Data from all 13 replicates are provided in panel A. Data from the four replicates having heat-induced reductions in blastocyst development of at least 20% are provided in panel B. Least squares means $\pm$ standard error of the mean are shown with treatment differences. Bars with different letters within a panel differ ($P<0.0001$).

Table 2. Embryo development of control and heat-stressed oocytes after direct heat stress exposure reduced blastocyst development $\geq 20\%$ (n=7 experimental replicates)

		~72 hpi [€]			~192 hpi [€]	
IVM temperature	PZ [‡] cultured	% Cleaved (n)	% 8 to 16-cell* (n)	% Blastocysts/PZ (n)	Blastocyst stage	Blastocyst quality
38.5 C	410	80.46 $\pm$ 2.25 (n=329)	81.99 $\pm$ 2.59 (n=270)	29.76 $\pm$ 2.26 ^a (n=122)	6.20 $\pm$ 1.32	1.13 $\pm$ 0.09
41.0 C	413	80.00 $\pm$ 2.28 (n=329)	79.01 $\pm$ 2.82 (n=261)	19.13 $\pm$ 1.94 ^b (n=79)	6.22 $\pm$ 1.32	1.30 $\pm$ 0.09
	P-value	0.87	0.38	0.004	0.92	0.2

[€]hpi = hours post-IVF; [‡] PZ= putative zygotes; * Proportion of cleaved embryos; ^{ab} Means within a column differ.

Table 3. Characteristics of total RNA from developmentally-competent oocytes having heat-induced reductions in blastocyst development of $\geq 20\%$ (n=7 replicates)

Cell type	Stage	IVM temp [†] (C)	Treatment ID [‡]	Total RNA**	rRNA ratio	RIN	18S/fast region ratio	18S/inter region ratio
Oocyte	GV-stage	—	1	1.82 ^c	0.87 ^{bc}	5.37	0.31 ^a	0.69 ^b
	12 hIVM	38.5	2	2.18 ^{bc}	0.65 ^c	5.16	0.28 ^a	0.66 ^b
		41.0	3	2.10 ^{bc}	0.80 ^{bc}	5.18	0.28 ^a	0.64 ^b
	24 hIVM	38.5	4	2.41 ^{bc}	0.75 ^{bc}	5.08	0.29 ^a	0.65 ^b
		41.0	5	2.28 ^{bc}	0.84 ^{bc}	5.08	0.23 ^a	0.63 ^b
Embryo	4 to 8-cell	38.5	6	2.64 ^{bc}	0.93 ^b	4.80	0.28 ^a	0.52 ^{bc}
		41.0	7	3.26 ^{bc}	0.80 ^{bc}	4.22	0.22 ^{ab}	0.51 ^{bc}
	Blastocyst	38.5	8	3.80 ^b	1.78 ^a	6.06	0.22 ^a	0.85 ^a
		41	9	6.43 ^a	1.69 ^a	5.11	0.10 ^b	0.42 ^c
		SEM*		0.81	0.10	0.46	0.04	0.06
		P-value		0.0001	<0.0001	0.46	0.02	0.002
Cumulus	GV-stage	—	1c	17.17	1.62	8.13	0.91	1.19
	12 hIVM	38.5	2c	16.67	1.74	8.54	0.98	1.75
		41.0	3c	22.79	1.79	8.11	0.95	1.47
	24 hIVM	38.5	4c	20.40	1.74	8.31	1.00	1.74
		41.0	5c	28.24	1.60	8.51	1.29	1.68
		SEM*		6.13	0.11	0.36	0.18	0.24
		P-value		0.85	0.60	0.35	0.20	0.24

[†] IVM temperature; [‡] Treatment number from Fig. 2. ** Total RNA is expressed as ng per oocyte or embryo. For cumulus, total RNA is expressed as ng of cumulus RNA per cumulus-oocyte complex denuded. "c" designates cumulus from oocytes within each treatment. * Pooled standard error of the mean (SEM); ^{abc} Different superscripts within a column indicate significant differences (P<0.05) for oocytes & embryos.

blastocyst stage after fertilization), only those experimental replicates where heat stress exposure reduced blastocyst rates by 20% or more compared to controls were included in the analysis (n=4 replicates; 30.6 vs. 20.4% blastocyst development for control and heat-stressed oocytes, respectively; SEM=4.2%; P=0.01). Similar to previous results, poly(A) RNA was highest in GV-stage oocytes and decreased as maturation progressed (Fig. 3B). Even when the effect of heat stress was to reduce development by $\geq 20\%$, there was no impact of 41 C exposure during the first 12 hIVM to alter poly(A) RNA per oocyte (Fig. 3B).

Experiment 2: RNA size distribution and abundance of certain transcripts in control and heat-stressed oocytes, their surrounding cumulus cells, and resultant embryos

Effects of heat stress exposure during meiotic maturation to reduce blastocyst development after IVF ranged from 0 to 55.3%. To provide a more precise test of hypothesis, only those experi-

mental replicates whereby heat stress reduced blastocyst rates by $\geq 20\%$ are reported herein (n=7). Developmental data for these experimental replicates are presented in Table 2. Notably, the proportion undergoing cleavage and progressing to the 8 to 16-cell stage after IVF was similar for control and heat-stressed oocytes. However, heat stress exposure resulted in a 35.7% reduction in blastocyst development.

Analysis of oocytes not undergoing IVF showed that total RNA per oocyte did not change during meiotic maturation, nor was it impacted by exposure to 41 C during the first 12 hIVM (Table 3). Total RNA in 4 to 8-cell embryos was similar to amounts observed in oocytes, whereas blastocyst-stage embryos had significantly more total RNA than all other stages examined. Interestingly, blastocysts from heat-stressed oocytes had higher amounts of total RNA than those from nonheat-stressed oocytes (Table 3). Total RNA in surrounding cumulus cells did not change during meiotic maturation and was not impacted by heat stress exposure (Table 3).

Analysis of RNA size distributions revealed similar RIN values and ratios for rRNA, 18S/fast region, and 18S/inter region for oocytes during meiotic maturation (Table 3). Four to 8-cell embryos had values comparable to those observed for oocytes, whereas values for blastocyst-stage embryos were most similar to those obtained for cumulus cell RNA which did not change during meiotic maturation (Table 3). When imposed during the first 12 h of meiotic maturation, 41 C exposure had no impact on any of the RNA size distribution values reported herein for oocytes, surrounding cumulus or resultant 4 to 8-cell embryos (Table 3). Interestingly however, RNA from blastocyst-stage embryos originating from heat-stressed oocytes had lower 18S/fast region and 18S/inter region ratios compared to other developmental stages examined and cumulus cells (Table 3). Using multiple regression, it was determined that blastocyst development was not explained by total RNA ($R^2=0.13$; $P=0.20$), 18S/fast ($R^2=0.01$; $P=0.73$) or 18S/inter region ($R^2=0.07$; $P=0.42$) ratios independently or collectively ($R^2=0.28$; $P=0.48$). Additional analysis indicated that the total amount of RNA extracted per sample was directly proportional to the number of blastocysts in the lysate ($P=0.1$) and that the number of blastocysts per lysate did not affect total RNA per blastocyst ($R^2=0.14$; $P=0.18$).

Overall, the relative abundance of 18S rRNA, 28S rRNA, HSP70, CCNB1, BMP15, PAP, and GDF9 transcripts was similar in GV-stage and matured oocytes but lower in 4 to 8-cell and blastocyst-stage embryos (Fig. 4). Exposure to 41 C for the first 12 hIVM did not alter the abundance of any transcripts examined in oocytes or in resultant embryos after fertilization (Fig. 4). A few transcripts including CCNB1, BMP15, PAP, and GDF9 had a lower relative abundance in blastocysts than 4 to 8-cell embryos (Figs. 4D, E, F, G). Regression analysis was performed to better clarify changes in transcript abundance from onset of meiotic maturation to blastocyst-stage embryos. Doing so revealed quadratic behavior of 18S and 28S rRNA and exponential decay of HSP70, CCNB1, BMP15, PAP, and GDF9 transcripts (data not shown). Exposure of maturing oocytes to 41 C during the first 12 hIVM did not alter the pattern of transcript abundance for the stages of development examined (data not shown).

In surrounding cumulus cells, relative quantity of 18S rRNA did not change as oocyte maturation progressed (Fig. 5A). However, 28S rRNA was lower in cumulus from GV-stage oocytes compared to those matured for 12 hIVM (Fig. 5B). Quantity of CCNB1 transcripts decreased in step-wise fashion as maturation progressed

Fig. 4. Relative abundance of transcripts per oocyte or embryo for replicates ($n=7$) where culture at 41.0 C during the first 12 h of maturation reduced blastocyst development ($\geq 20\%$) after fertilization. Data are least squares means $\pm$ standard error of the mean with treatment differences indicated by letter designation at significance level indicated for each panel. White bars indicate culture at 38.5 C for 0, 12, or 24 hIVM or embryos derived from oocytes cultured at 38.5 C. Black bar indicates heat stress for 12 h. Gray bar indicates heat-stress for first 12 hIVM and culture at 38.5 C thereafter. A) 18S rRNA, B) 28S rRNA, C) heat shock protein 70 (HSP70), D) cyclin B1 (CCNB1), E) bone morphogenetic protein 15 (BMP15), F) poly(A) polymerase (PAP), and G) growth and differentiation factor 9 (GDF9).

Fig. 5. Relative abundance of transcripts for surrounding cumulus cells of oocytes in replicates where culture at 41.0 C during the first 12 h of maturation reduced blastocyst development ($\geq 20\%$) after fertilization. A) 18S rRNA, B) 28S rRNA, C) heat shock protein 70 (HSP70; significant interaction between culture temperature and time of maturation), D), cyclin B1 (CCNB1), and E) poly(A) polymerase (PAP). White bars indicate culture at 38.5 C for 0, 12, or 24 hIVM. Black bar indicates heat stress for first 12 hIVM. Gray bar indicates heat-stress for first 12 hIVM and culture at 38.5 C for the remaining 12 hIVM. Least squares means $\pm$ standard error of the mean are shown with treatment differences indicated by letter designation at significance level indicated for each panel.

(Fig. 5D). Transcripts for PAP in cumulus cells were significantly lower at the onset of maturation than at 12 or 24 hIVM (Fig. 5E). There was no effect of heat stress during the first 12 hIVM on 18S rRNA, 28S rRNA, CCNB1, or PAP transcript abundance. In contrast, there was a significant interaction between maturation time and temperature for HSP70 transcripts ($P=0.005$). Specifically, relative abundance of HSP70 was higher in cumulus from heat-stressed oocytes at 12 hIVM compared to controls but difference was no longer detected at 24 hIVM (Fig. 5C). No transcripts for BMP15 or GDF9 were detected in cumulus cell RNA.

Discussion

A major aim of these studies was to relate changes in developmental competence after heat stress exposure during meiotic maturation to changes, or lack thereof, in certain oocyte and cumulus cell RNA populations (sizes >200 nucleotides (nt)). RNA populations were also examined after IVF to evaluate the extent to which heat-induced perturbations occurring during meiotic maturation were inherited by resultant embryos. To this end, it was shown that heat stress exposure during the first 12 h of meiotic maturation had minimal impact on the abundance of RNA populations and individual transcripts examined in oocytes, their surrounding cumulus cells, or in resultant 4 to 8-cell embryos. In contrast, blastocyst-stage embryos from heat-stressed oocytes had more total RNA which exhibited lower 18S/fast region and 18S/inter region ratios when compared to that of blastocysts from nonheat-stressed oocytes, suggesting a higher abundance of RNA sizes less than 3200 nt.

These results are significant as they document the extent to which heat-induced perturbations occurring at the molecular level during meiotic maturation are inherited by later stage embryos after fertilization. This is not likely without consequence as previous efforts of Edwards *et al.* [26] showed that later stage embryos from heat-stressed oocytes are not as developmentally-competent as those from nonheat-stressed oocytes. While the underlying mechanisms remain unclear, increased amount of total RNA in embryos from heat-stressed oocytes has been coincident with heat-induced perturbations in metabolism inherited by the embryo. To this end, recent efforts have shown that heat stress exposure during meiotic maturation increases metabolic activity (i.e., increases intracellular ATP content) which may persist in resultant embryos after fertilization up through the blastocyst stage (Payton *et al.*, unpublished). Interestingly, Nilsson *et al.* [54] reported that higher total RNA content in cold-starved *Helicobacter pylori* was coincident with increased ATP content.

Different mRNA sizes in mammals range from approximately 300 to 6500 nt, with an average length of 1400 nt [55]. In our study, heat-induced increases in total RNA content in blastocyst-stage embryos from heat-stressed oocytes were related to increases in RNA sizes ≤ 3200 nt. Whether RNA sizes in this range represent some level of degradation or increased abundance of certain mRNAs warrants further study. In support of the latter, Mortensen *et al.* [56] showed that equine blastocysts originating from oocytes experiencing heat stress at the end of IVM had higher HSP70 levels relative to 18S rRNA than blastocysts from nonheat-stressed

oocytes.

Assessments regarding nuclear stage of oocytes or enumeration of embryonic nuclei were not performed before placement into RNA lysis buffer as fixation with formalin-derivatives had been shown to not only to reduce total RNA yield and alter RNA integrity [57, 58] but also to chemically modify RNA such that it can no longer be utilized in qPCR studies [59]. Rather, blastocysts were assigned a numeric stage and quality score. In our study, there was no difference in the average stage or quality of blastocysts from heat-stressed or control oocytes, in agreement with previous findings [25, 26]. Within a given culture system, blastocyst stage scores are highly correlated with total cell number [e.g., expanded blastocysts assigned a stage score of 7 will have more total cells on average than normal blastocysts assigned a stage score of 6; 60]. Thus, obtaining similar stage and quality scores is consistent with numerous other studies reporting equivalent cell numbers in embryos from control and heat-stressed oocytes [22, 23, 61–66]. This reduces concerns related to marring results interpretation due to possible differences in morphology or total cell number. Moreover, *a posteriori* analysis using multisource regression revealed that results were not influenced by embryo number per RNA lysate.

Although without a discernible effect of heat stress, results described herein provide additional insight regarding certain features of oocyte RNA and how they compare to early embryo and cumulus cell RNA. To this end, total RNA did not change during meiotic maturation which is consistent with previous findings for bovine oocytes [44, 67]. The amount of RNA obtained for GV-stage and matured oocytes was in agreement with others [~2 ng/oocyte; 67, 68]. Finding similar amounts of total RNA in oocytes and 4 to 8-cell embryos but higher levels in blastocyst-stage embryos was also consistent with a recent report by Gilbert *et al.* [69]. Considering that the cytoplasm and its components are almost entirely from the oocyte [70] and major embryonic genome activation has not yet occurred [71], it is not surprising that certain features of RNA from 4 to 8-cell embryos are similar to oocyte RNA. The blastocyst-stage embryo being largely comprised of trophoblast cells (i.e., the first epithelium) provides some explanation as to why this RNA is similar to that obtained from the somatic-like cumulus cells.

Depending on the species and stage, poly(A) RNA comprises approximately 10 to 20% of total RNA in oocytes [67, 72] and declines by 30 to 50% during meiotic maturation [this study, 67, 72–74]. In the present study, heat stress exposure had no impact on the total amount of poly(A) RNA. Disparity with an earlier observation [29], while difficult to explain, may be related to inherent differences among individual batches of oocytes related to genotype [75], individual donors [76], physiological status of donor cows (disease [77] or nutrition [78]), subtle differences in oocyte collection, ovary transport, and oocyte evaluation, and/or other yet unidentified factors. Whatever the reason, absence of heat-induced reductions in poly(A) RNA, even when blastocyst development was reduced by >30%, minimizes concerns related to reduced developmental potential of oocytes after exposure to a physiologically-relevant elevated temperature. This is consistent with Lequarre *et al.* [67] reporting similar amounts of total poly(A) RNA in cow versus calf oocytes previously shown to differ in

developmental competence [79]. Rather, developmental differences are more often related to changes occurring at the transcript level. For example, Patel *et al.* [47] showed a positive association of follistatin mRNA abundance with oocyte competence.

Relative abundance of transcripts examined in this study remained unchanged during meiotic maturation which is consistent with results reported by others [GDF9 and BMP15: 51, PAP: 52, CCNB1: 80, 81]. Poly(A) polymerase (PAP) transcripts were examined to provide insight into heat-induced reductions in poly(A) RNA content previously noted by Payton and Edwards [29]. While abundance of PAP was not altered in oocytes, this transcript increased in the intimately associated [reviewed by 82] and transcriptionally-active [83] cumulus cells by 12 hIVM. Interestingly, the only noted consequence of exposure of cumulus-oocyte complexes to elevated temperature was increased cumulus cell HSP70 mRNA, effectively serving as a reminder that some of the negative heat stress effects on the oocyte may be mediated through the surrounding cumulus cells.

In summary, results described herein document the extent to which heat-induced perturbations occurring at the molecular level during meiotic maturation affect later stage embryos after fertilization. Previous efforts of Edwards *et al.* [26] demonstrating that later stage embryos from heat-stressed oocytes are not as developmentally-competent as those from nonheat-stressed oocytes suggests that heat-induced alterations in RNA may not be without consequence. Demonstrated differences occurring at the molecular level in embryos from heat-stressed oocytes emphasizes the importance of minimizing stress exposure during meiotic maturation. This is of particular importance if the intent is to obtain developmentally-competent embryos.

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