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DIRECT EFFECTS OF HEAT STRESS DURING MEIOTIC MATURATION ON BOVINE OOCYTE AND CUMULUS RNA

A Dissertation Presented for
the Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Rebecca Ruth Payton
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DEDICATION

This dissertation is dedicated to my best friend and husband, Jason, whose patience and understanding have made the valleys along this journey bearable.

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ABSTRACT

Heat-induced reductions in developmental competence after direct exposure of oocytes to 41ºC have been coincident with reduced protein synthesis. Since heat stress perturbs RNA integrity and polyadenylation in somatic cells, it was hypothesized that heat stress during meiotic maturation may alter RNA within oocytes and/or their surrounding cumulus to account for some of the reductions in development. Initial efforts utilized microcapillary electrophoresis to examine oocyte and cumulus RNA without heat stress as a first step toward transcriptome profile analysis. Size distribution of RNA, rRNA ratio, and other related endpoints differed for oocyte RNA compared to cumulus, and were conserved across other mammalian species. Size distribution of polyadenylated RNA after amplification was similar for oocytes and cumulus.

Effects of heat stress on total and polyadenylated RNA, RNA size distribution, and individual transcripts important for meiotic maturation and response to heat stress were examined in oocytes and cumulus during maturation, and resultant embryos after fertilization. There was no impact of heat stress during the first 12 h of maturation to alter size distribution of RNA, rRNA ratio, and other endpoints in oocytes or cumulus. Heat stress perturbed the abundance of polyadenylated RNA in oocytes in one study. Abundance of eight examined transcripts was not altered after exposure to elevated temperature, suggesting that the impact of heat stress on oocyte RNA, if any, was subtle.

Consequences of elevated temperature exposure during maturation on transcriptomes of oocytes and their surrounding cumulus vestment were investigated using microarray technology. Thousands of transcripts changed in oocytes and surrounding cumulus over meiotic maturation, some in a stage-specific and amplification-dependent manner. Culture at elevated temperature

for the first 12 h of meiotic maturation impacted a small proportion of transcripts in matured oocytes and cumulus at 24 h. Alterations suggested perturbations in oocyte mitochondrial function, and intracellular signaling and extracellular matrix production in cumulus. Heat-induced alterations in oocyte mitochondria and cumulus expansion are supported by existing literature. The findings discussed here are informative of heat-induced molecular alterations in oocytes and cumulus and may prove useful for development of strategies to mitigate negative impacts of heat stress on fertility.

TABLE OF CONTENTS

Page

LIST OF TABLES x

LIST OF FIGURES xii

CHAPTER 1:GENERAL INTRODUCTION 1

CHAPTER 2:LITERATURE REVIEW 7

THE LIFE OF AN OOCYTE 7
Oogenesis and Folliculogenesis 7
Growth Phase 8
Oocyte Maturation 14
EFFECTS OF ELEVATED TEMPERATURE ON THE MATURING OOCYTE 21
Consequences of Maternal Hyperthermia Occurring at the Time of Estrus 21
Direct Effects of Elevated Temperature on Maturing Oocytes 23
EFFECTS OF HEAT STRESS ON MATURED OOCYTES 30
Consequences of Maternal Hyperthermia Occurring Near the Time of Breeding 30
Direct Effects of Elevated Temperature on Matured Oocytes 32
SUMMARY: A TENTATIVE THESIS REGARDING THE MOLECULAR EFFECTS OF HEAT STRESS DURING OOCYTE MATURATION 34

CHAPTER 3: GENERAL FEATURES OF RNA FROM GAMETES VERSUS CUMULUS CELLS 35

ABSTRACT 35
INTRODUCTION 35
MATERIALS AND METHODS 37
General methods for extraction, quantification, and generation of electropherograms of total RNA 37
Features of total RNA from bovine oocytes and associated cumulus cells 40
Features of total RNA from GV-stage oocytes in other mammalian species 42
Features of total RNA from in vivo matured murine oocytes of prepubescent/superovulated or sexually mature females 42
Total RNA from bovine, ovine, porcine, and human sperm 43
Data Analyses 44
RESULTS 44
Summary of experimental endpoints 44
Features of total RNA from bovine oocytes and associated cumulus 45
Features of total RNA from germinal vesicle (GV)-stage oocytes in other mammalian species 52
Features of total RNA from in vivo matured murine oocytes of prepubescent/superovulated or sexually mature females 55
Overall comparison of total RNA from oocytes versus cumulus 55
Features of total RNA from bovine, ovine, porcine, and human sperm 57

DISCUSSION 57

CHAPTER 4: EFFECTS OF DIRECT EXPOSURE TO A PHYSIOLOGICALLY-RELEVANT ELEVATED TEMPERATURE DURING MEIOTIC MATURATION ON POLYADENYLATED RNA, RNA SIZES, AND SPECIFIC TRANSCRIPTS IN BOVINE OOCYTES AND SURROUNDING CUMULUS CELLS 66

ABSTRACT 66
INTRODUCTION 67
MATERIALS AND METHODS 70
Oocyte collection and selection criteria 70
Experiment 2: Abundance of poly(A) RNA in control and heat-stressed oocytes at GV-stage, 12, 16, 20, and 24 hIVM 73
Experiment 3: Size distribution of RNA molecules and relative abundance of developmentally-important transcripts in control and heat-stressed oocytes and their surrounding cumulus cells 75
Statistical Analyses 81
RESULTS 84
Abundance of total RNA and poly(A) RNA in GV-stage or control and heat-stressed oocytes at 12 and 24 hIVM 84
Abundance of poly(A) RNA in GV-stage or control and heat-stressed oocytes at 12, 16, 20, and 24 hIVM 87
Size distribution of RNA molecules and relative abundance of developmentally-important transcripts in control and heat-stressed oocytes and their surrounding cumulus cells 90
DISCUSSION 108

CHAPTER 5:.. TRANSCRIPTOME PROFILES OF OOCYTES AND CUMULUS CELLS AFTER DIRECT EXPOSURE TO A PHYSIOLOGICALLY-RELEVANT ELEVATED TEMPERATURE DURING MEIOTIC MATURATION 119

ABSTRACT 119
INTRODUCTION 120
MATERIALS AND METHODS 124
Collection and treatment of oocyte and cumulus samples 124
RNA isolation, amplification, and microarray analysis 126
Statistical analyses 128
RESULTS 133
Effects of heat stress during meiotic maturation on embryo development 133
Effects of heat stress during meiotic maturation on general features of RNA 133
Relative abundance of oocyte transcripts after IVM and exposure to elevated temperature 136
Functional profiling of differentially abundant oocyte transcripts 142
Heat-induced alterations in polyadenylated cumulus transcripts during oocyte maturation 152
Functional profiling of differentially abundant polyadenylated cumulus transcripts 154
DISCUSSION 160

CHAPTER 6: GENERAL DISCUSSION & CONCLUSIONS 178
LIST OF REFERENCES 183
APPENDIX 211
VITA 222

LIST OF TABLES

Page

Table 3-1. Features of total RNA in bovine oocytes and cumulus cells 46

Table 3-2. Features of total RNA in GV-stage oocytes and surrounding cumulus 54

Table 3-3. Characteristics of total RNA from murine oocytes and surrounding cumulus 56

Table 4-1. Sequences of primers used for qPCR amplification of genes of interest 79

Table 4-2. Embryo development for developmentally-competent oocytes with heat-induced blastocyst reductions of ≥ 20% 91

Table 4-3. Characteristics of total RNA from developmentally-competent oocytes having heat-induced reductions in blastocyst development ≥ 20% 93

Table 4-4. Embryo development for developmentally-competent vs. challenged oocyte 102

Table 4-5. Characteristics of total RNA from developmentally-competent versus challenged GV-stage oocytes 103

Table 5-1. Transcripts called present for at least 6 arrays in different cell types 130

Table 5-2. Embryo development for oocytes matured at a physiologically-relevant elevated temperature 134

Table 5-3. Characteristics of total RNA from oocytes and surrounding cumulus cells 135

Table 5-4. Overlap between heat-induced differences in oocyte transcripts after poly(A) or whole transcriptome amplification 143

Table 5-5. Top molecular function GO terms from comparison of GV-stage to 24 hIVM oocytes 146

Table 5-6 Top biological process GO terms from comparison of GV-stage to 24 hIVM oocytes

.....147

Table 5-7. Top cellular component GO terms from comparison of GV-stage to 24 hIVM oocytes

.....149

Table 5-8. Significant biological pathways during IVM for polyadenylated oocyte transcripts

.....150

Table 5-9. Heat-induced reductions in polyadenylated oocyte transcripts (C-HS) related to mitochondrial function and spindle stability 151

Table 5-10. Top molecular function GO terms from comparison of cumulus cells from GV-stage or 24 hIVM oocytes 156

Table 5-11. Top biological process GO terms from comparison of cumulus cells from GV-stage or 24 hIVM oocytes 157

Table 5-12. Top cellular component GO terms from comparison of cumulus cells from GV-stage or 24 hIVM oocytes 158

Table 5-13. KEGG pathways over-represented in cumulus cells before or after IVM 159

LIST OF FIGURES

Page

Figure 1-1. Model depicting embryonic and fetal losses associated with heat stress of dairy cattle 3

Figure 2-1. Interactions between the oocyte and surrounding cumulus 9

Figure 2-2. Masked and unmasked states of maternal mRNA via protein interactions with the poly(A) tail 13

Figure 3-1. Regions of an electropherogram 39

Figure 3-2. Representative gel images and electropherograms of total RNA from 24 hIVM bovine oocytes and their surrounding cumulus loaded at equivalent amounts 48

Figure 3-3. Representative electropherograms of total RNA from ~4.5 , 2, 1, and 0.5 bovine oocyte equivalents originating from one oocyte pool 51

Figure 3-4. Representative electropherograms and gel images of cDNA from amplified polyadenylated RNA (Nano LabChip) originating from bovine oocytes and surrounding cumulus 53

Figure 3-5. Representative electropherograms of total RNA from sperm 59

Figure 4-1. Schematic of design for experiment 1 72

Figure 4-2. Schematic of design for experiment 2 74

Figure 4-3. Schematic of design for experiment 3 76

Figure 4-4. Total and poly(A) RNA in oocytes matured at 38.5º or 41.0ºC for 0 (GV-stage oocytes), 12 or 24 hIVM 86

Figure 4-5. Poly(A) RNA within oocytes matured at 38.5º or 41.0ºC for up to 24 hIVM 89

Figure 4-6. Relative abundance of transcripts per oocyte or embryo for replicates where culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development after fertilization 95

Figure 4-7. Patterns of relative transcript abundance over development as assessed by regression analysis for replicates whereby culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development (≥ 20%) after fertilization 98

Figure 4-8. Relative abundance of transcripts for surrounding cumulus cells of oocytes whereby culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development (≥ 20%) after fertilization 100

Figure 4-9. Relative abundance of transcripts in developmentally-competent or -challenged GV-stage oocytes and surrounding cumulus 105

Figure 4-10. Patterns of relative transcript abundance over development of developmentally-competent versus developmentally-challenged oocytes and resultant embryos as determined by regression analysis 107

Figure 5-1. Experimental schematic for analysis of oocyte and cumulus transcriptomes 125

Figure 5-2. Amplified cDNA from total RNA of oocytes and cumulus cells during in vitro maturation and after heat stress exposure 138

Figure 5-3. Venn diagrams of differentially abundant oocyte transcripts 139

Figure 5-4. Biological themes (level 2 GO terms for molecular functions, biological processes, and cellular components) for polyadenylated transcript changes occurring in oocytes from GV-stage to 24 hIVM after culture at 38.5ºC (GV-C) 144

Figure 5-5. Venn diagram of differentially abundant polyadenylated cumulus transcripts 153

Figure 5-6. Biological themes (level 2 GO terms for molecular functions, biological processes, and cellular components) for polyadenylated transcript changes occurring in cumulus cells from GV-stage (unexpanded) versus matured oocytes (expanded) after culture at 38.5ºC (GV-C) ...155

Figure 5-7. Log_2 intensity plots to illustrate differences in transcript abundance depending upon amplification method 164

Figure 5-8. Model of heat-induced alterations in mitochondrial-related transcripts and spindle formation 167

Figure 5-9. Potential heat-induced alterations in pathways within cumulus cells at 24 hIVM .173

Figure 5-10. Model summarizing some of the heat-induced alterations in oocytes and cumulus cells during meiotic maturation 177

Figure 6-1. Model summarizing the effects direct heat stress application during meiotic maturation on the oocyte and its surrounding cumulus cells 180

Figure A1. Pseudo-images of the 8 arrays of RNA from surrounding cumulus of GV-stage oocytes 213

Figure A2. Relative log expression and normalized unscaled standard error plots for WT-amplified oocyte RNA at 24 hIVM 215

Figure A3. Histogram showing the frequency of p-values for comparison of GV-stage and control 24 hIVM polyadenylated oocyte transcripts 217

Figure A4. Histogram showing the frequency of p-values for comparison of 24 hIVM polyadenylated oocyte transcripts from control and heat-stressed oocytes 219

Figure A5. Histogram showing the frequency of p-values for comparison of 24 hIVM WT-amplified oocyte transcripts from control and heat-stressed oocytes 221

CHAPTER 1: General Introduction

Females of a variety of species (mice, rats, and rabbits, Austin and Braden 1954a; sheep, Dutt 1963; pigs, Tompkins et al., 1967; cattle, Cavestany et al., 1985) experience reduced pregnancy rates when exposed to elevated ambient temperatures (i.e., heat stress) such as those occurring during the summer months. Reduced fertility during heat stress conditions is largely because of hyperthermia. Heat stress is especially problematic in dairy cattle (Stott 1961) which can be hyperthermic more than half the year in sub-tropical regions (St-Pierre *et al.,* 2003). Rectal temperatures of hyperthermic dairy cattle are capable of meeting or exceeding 41ºC (Gaalaas 1945; Seath and Miller 1946; Monty and Wolff 1974; Roman-Ponce *et al.,* 1981; Turner 1982; Elvinger *et al.,* 1991; Elvinger *et al.,* 1992; Ealy *et al.,* 1993; Wolfenson *et al.,* 1993). Vaginal and uterine temperatures, which parallel rectal temperatures (Gwazdauskas *et al.,* 1973), are also increased in heat-stressed dairy cattle (Roman-Ponce *et al.,* 1981). Rectal temperatures are negatively correlated with pregnancy rates (Ulberg and Burfening 1967). Reduced fertility associated with heat stress costs the dairy industry $1.5 billion annually (St-Pierre *et al.,* 2003) and is not only problematic in southeastern (Rocha *et al.,* 1998; Al-Katanani *et al.,* 2002; Smith *et al.,* 2006; Huang *et al.,* 2008) and southwestern (Monty and Wolff 1974; Chebel *et al.,* 2004) areas of the US but also in cooler states such as Wisconsin (Sartori *et al.,* 2002) and Minnesota (Udomprasert and Williamson 1987). This emphasizes the need to identify causes of heat-induced infertility for development of better management strategies as a large proportion of dairies are located in warmer states such as Arizona and California (Jesse and Schuelke 2001).

Exposure to elevated ambient temperatures negatively impacts several stages of development to reduce pregnancy rates (Figure 1-1). These stages include the germinal vesicle (GV)-stage oocyte contained within antral ovarian follicles (Rocha *et al.,* 1998; Al-Katanani *et al.,* 2002), the ovulated oocyte near the time of fertilization (Fischberg and Beatty 1952; Braden and Austin 1954a; Stott and Williams 1962), the zygote just after fertilization (Ju *et al.*, 1999; Ju and Tseng 2004), and early cleavage-stage embryos (Ealy *et al.,* 1993). The majority of early embryonic losses in lactating dairy cattle, in the presence (reviewed by Hansen and Arechiga 1999) or absence (reviewed by Santos *et al.,* 2004; Diskin and Morris 2008) of environmental heat stress, occur before day 20 of gestation (Figure 1-1). During periods of heat stress, losses are more pronounced and may continue up through day 40 to 50 of gestation (Cartmill *et al.,* 2001). After the embryo becomes a fetus (day 42), pregnancy losses are small (Jousan *et al.,* 2005). Effects of heat stress are particularly prominent during estrus (day -1) when the oocyte is undergoing meiotic maturation in preparation for fertilization (Stott and Williams 1962; Gwazdauskas *et al.,* 1973; Cavestany *et al.,* 1985).

While negative effects of heat stress exposure during meiotic maturation on fertility could result from effects on the maternal environment such as the follicle (Roth *et al.,* 2000) and/or hormonal profiles (reviewed by Rensis and Scaramuzzi 2003), direct (*in vitro*) exposure of bovine oocytes to elevated temperature during maturation reduced embryo development (Baumgartner and Chrisman 1981a, b; Edwards and Hansen 1996, 1997; Lawrence *et al.,* 2004; Edwards *et al.,* 2005; Schrock *et al.,* 2007) to a similar degree as that seen *in vivo* (Stott and Williams 1962; Cavestany *et al.,* 1985; Putney *et al.,* 1989b). Reduced blastocyst development of bovine oocytes after direct exposure to elevated temperature has been associated with

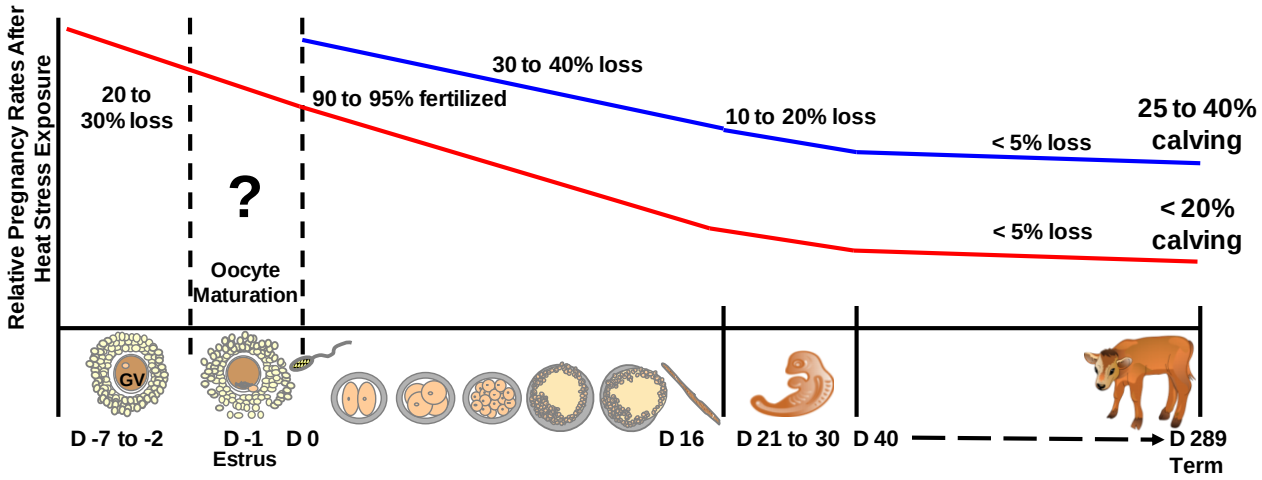


Figure 1-1. Model depicting heat-induced reductions in embryonic and fetal development in dairy cattle exposed to elevated ambient temperatures at different times before and during gestation. The blue line is indicative of normal pregnancy rates and timing of loss for lactating dairy cattle (reviewed by Santos *et al.*, 2004). The red line indicates the relative reductions in pregnancy rate associated with exposure to high ambient temperature during the time periods indicated (Ingraham *et al.*, 1976; Badinga *et al.*, 1985; Al-Katanani *et al.*, 1999). It is unclear what proportion of losses are a direct result of heat stress during estrus (indicated by "?").

accelerated nuclear (i.e., progression to metaphase II (MII) of meiosis) and cytoplasmic (i.e., migration of cortical granules to the oolemma) maturation (Edwards *et al.,* 2005). However, heat stress of maturing oocytes also induces alterations on a molecular level. Specifically, *de novo* protein synthesis was reduced by approximately 30% after exposure to 41ºC for the first 12 h of meiotic maturation, coincident with a 65% reduction in blastocyst development (Edwards and Hansen 1996). Heat stress has also been demonstrated to induce differential abundance of specific (but unknown as of yet) proteins after exposure of bovine oocytes to 41ºC for the first half of meiotic maturation (West-Rispoli *et al.,* 2006). Protein synthesis during the first 12 h of bovine oocyte maturation is critical for proper meiotic maturation (Sirard *et al.,* 1989) and subsequent embryo development after fertilization (Saeki *et al.,* 1997). Thus, heat-induced alterations in protein synthesis after culture of bovine oocytes at 41ºC for the first 12 h of meiotic maturation may be a key factor in explaining the observed reductions in development of heat-stressed oocytes.

Alterations in protein synthesis may be mediated by several mechanisms at the level of translation. Specifically, heat stress of mammalian HeLa cells altered the phosphorylation status of certain translation initiation factors (Duncan and Hershey 1989), reduced the proportion of ribosomes actively involved in protein synthesis (Duncan and Hershey 1989), and may enhance the translation of specific transcripts, namely those belonging to the heat shock protein family, via internal ribosomal entry sites on mRNA molecules (Kim and Jang 2002). Degradation of RNA may occur after exposure to supraphysiological elevated temperature (Coias *et al.,* 1988). However, other factors necessary for translation such as poly(A) binding proteins (reviewed by Gorgoni and Gray 2004), did not change after heat stress of Drosophila cells (Duncan 1995).

Another potential mechanism regards post-transcriptional RNA modification; heat stress has been demonstrated to alter the degree of polyadenylation (Kumar *et al.,* 1995) for transcripts such as heat shock protein 70 (Dellavalle *et al.,* 1994; Mezquita *et al.,* 1998) and ubiquitin (Mezquita *et al.,* 1998). Since transcript polyadenylation can be modified to accommodate the needs of the oocyte or embryo (Paynton *et al.*, 1988) and is a conserved means of regulating translation under non-heat stress conditions (reviewed by Radford *et al.,* 2008), this may be a factor in reduced protein synthesis after heat stress. Any perturbation in oocyte transcripts resulting from heat stress at the onset of meiotic maturation could be problematic as oocytes are transcriptionally inactive soon after resumption of meiosis (Rodman and Bachvarova 1976) and rely upon stored messages in the ooplasm for protein synthesis (Wassarman and Letourneau 1976).

Based on published data, the working hypothesis of this research was that ***physiologically-relevant elevated temperatures may alter maternal pools of oocyte RNA, reducing subsequent embryo development.*** To test this hypothesis, a series of experiments were performed to ***determine effects of heat stress on maternal pools of RNA within maturing bovine oocytes and their surrounding cumulus cells.*** The first research efforts characterized oocyte and cumulus RNA during maturation in the absence of heat stress as a first and necessary step toward analysis of transcriptome profiles (see Chapter 3). As a next step, the effects of heat stress on different populations of RNA including the polyadenylated fraction, rRNA and overall RNA electrophoretic profile, and individual transcripts of known importance for development were examined (see Chapter 4). The final research chapter ascertained the effects of exposure to

elevated temperature during oocyte maturation on the transcriptomes of oocytes and their surrounding cumulus vestment (see Chapter 5).

CHAPTER 2: Literature Review

To begin to decipher the mechanisms whereby heat stress impairs developmental potential of oocytes exposed to elevated temperature during meiotic maturation, it is imperative to understand certain processes necessary for successful embryo development. The following is a review of pertinent literature regarding the interactions and events necessary for proper meiotic maturation, the molecular composition of the oocyte prior to and during meiotic maturation, and the impact of heat stress on these events. Special attention will be placed on the role of RNA in oocyte maturation and subsequent development.

THE LIFE OF AN OOCYTE

Oogenesis and Folliculogenesis

Oogenesis is the process by which the female germ cells or oocytes are established in the ovary, enter meiosis, and grow in preparation for ovulation. The events of oogenesis occur concomitantly with folliculogenesis, the formation and growth of ovarian follicles to separate oocytes from each other and the ovarian stroma. These processes are initiated during gestation and are completed gradually over the reproductive lifespan of the female.

During fetal development, primordial germ cells, originating from the extraembryonic mesoderm (Lawson and Hage 1994), migrate to the ovary of the female. These cells then undergo a series of mitotic division to colonize the ovary and become known as oogonia (reviewed by McLaren 2003). After proliferating to form egg nests (i.e., groups of oogonia), oogonia enter meiosis and arrest at the dictyate stage of prophase I at which point they are termed primary oocytes (reviewed by De Felici *et al.*, 2005). A single layer of squamous pre-granulosa cells develops around individual primary oocytes (25 to 30 µm diameter in cattle),

forming the primordial follicle (30 to 40 µm, reviewed by Hyttel *et al.*, 1997). The signals governing primordial follicle formation are still being elucidated but the transcription factor Figla appears to be critical (reviewed by McLaughlin and McIver 2009), as mice null for this factor have persistent egg nests and are infertile (Soyal *et al.*, 2000).

Growth Phase

Primordial follicles and their enclosed oocytes remain in a relatively quiescent state until recruited to enter the growth phase. A rudimentary understanding of the mechanism(s) underlying initiation of the growth phase has emerged only recently and data is somewhat conflicting (reviewed by McLaughlin and McIver 2009). In mice, this transition is coincident with major changes in oocyte gene expression associated with protein synthesis, cell cycle, DNA repair, and chromatin structure (Pan *et al.*, 2005). Regardless, recruitment of primordial follicles commences a period of growth by primary oocytes that may last 60 to 80 days in cattle (Britt 1992). During this time period, the nucleolus of the oocyte nucleus becomes active, enabling transcription (reviewed by Hyttel *et al.*, 1997), the driving force behind oocyte growth and subsequent development (reviewed by Mermillod *et al.*, 2008).

The formation of bovine primary follicles (40 to 50 µm) involves the proliferation of cuboidal-shaped granulosa cells and an increase in oocyte diameter to approximately 35 µm (reviewed by Hyttel *et al.*, 1997). The oocyte initiates intimate connections with the surrounding granulosa cells (reviewed by Senbon *et al.*, 2003), mediated by granulosa processes extending into the ooplasm and gap junctions (Figure 2-1). These gap junctional contacts (reviewed by Gershon *et al.*, 2008) mediate bi-directional communication that benefits both the oocyte and its

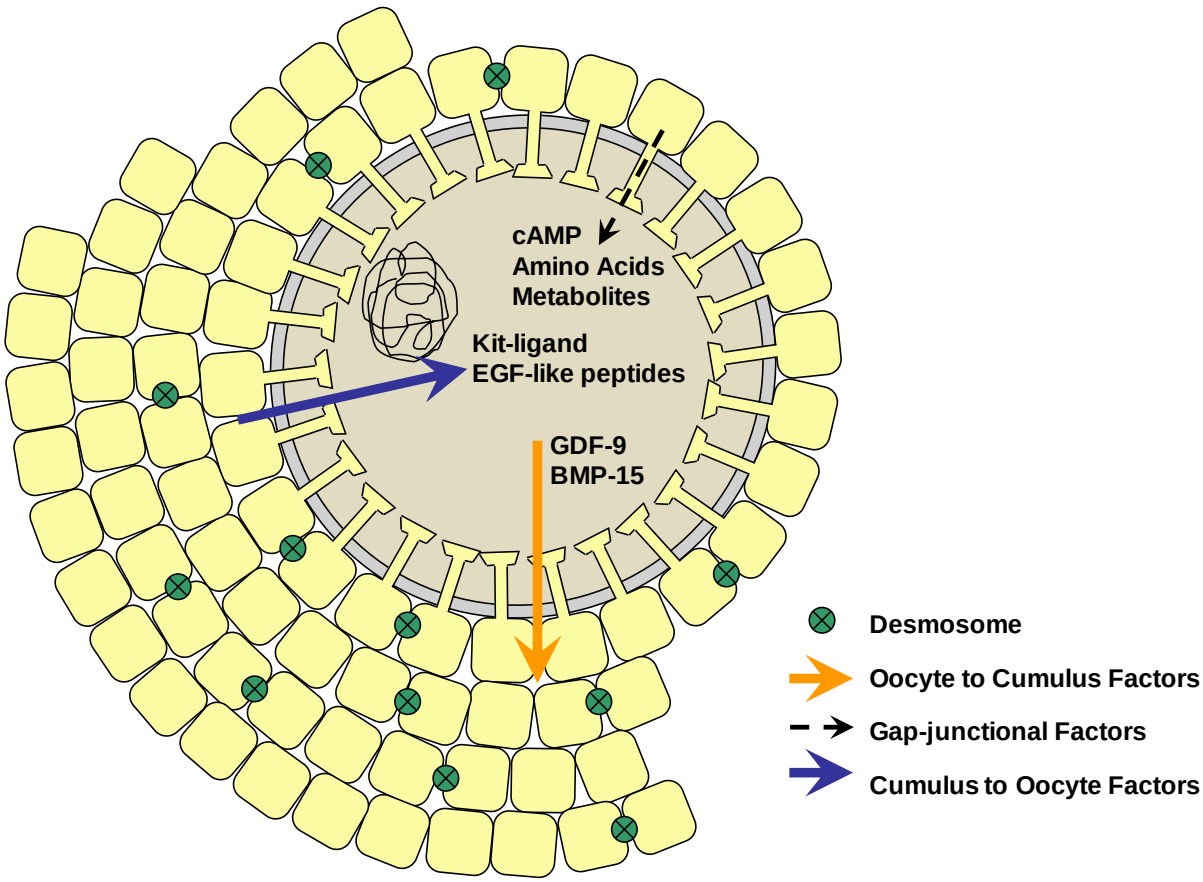


Figure 2-1. Interactions between the oocyte and surrounding cumulus cells (adapted from Gilchrist et al., 2004). Cumulus processes project through the zona pellucida and into the ooplasm to maximize bidirectional communication between the two cell types. Cumulus also communicate with each other via desmosomes. The oocyte directly influences its microenvironment via secretion of paracrine factors, namely GDF-9 and BMP-15, which promote proliferation and metabolic activity in cumulus cells. Cumulus cells secrete various factors that serve to nourish the oocyte and maintain meiotic arrest until initiation of oocyte maturation.

granulosa vestment. Specifically, oocytes secrete various factors that enable surrounding granulosa cells to "feed" the oocyte during growth and by metabolizing energy substrates otherwise unusable by the oocyte (e.g., oxidation of glucose to pyruvate, reviewed by Senbon *et al.*, 2003). The oocyte-secreted factors that are best studied are within the transforming growth factor superfamily. Two of these are growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). Secretion of GDF9 stimulates granulosa cell proliferation while BMP15 promotes kit-ligand expression in cumulus granulosa cells. In turn, cumulus-derived kit-ligand binds kit on the oocyte resulting in oocyte growth and survival (reviewed by Thomas and Vanderhyden 2006).

As the growth phase of the oocyte within the follicle progresses, granulosa cells continue to multiply to form secondary follicles (< 150 µm; reviewed by Hyttel et al., 1997). Development of secondary follicles is coincident with secretion of the zona pellucida by the oocyte (reviewed by Jovine *et al.*, 2007), a protective layer analogous to the calcified shell of a chicken egg surrounding the oocyte which increases in size to 30 to 60 µm in diameter. When the oocyte reaches < 80 µm in diameter, the surrounding follicle is considered tertiary and is distinguished by formation of an antrum or fluid filled pocket (reviewed by Hyttel *et al.*, 1997). Subsequent increases in antrum size result in two distinct populations of granulosa cells: the cumulus granulosa (those closest to the oocyte) and mural granulosa cells (those lining the follicular wall). Growth of bovine oocytes within antral follicles slows and those with a diameter of at least 110 µm are considered fully-grown and are competent to resume meiosis (Fair *et al.*, 1995). However, gap junctional communication between the oocyte and cumulus serves to maintain meiotic arrest in the oocyte (reviewed by Edry *et al.*, 2006), largely through high cAMP

levels (Figure 2-1), and to coordinate nuclear and cytoplasmic maturational competence (Carabatsos *et al.,* 2000).

Increased oocyte size during the growth phase is largely due to the accumulation of water, ions, and lipids within the ooplasm (Gosden and Bownes 1995). However, synthesis and storage of RNA and protein during oocyte growth is crucial for subsequent survival (reviewed by Bettegowda and Smith 2007). These stores of maternal transcripts and proteins are necessary after GV breakdown (GVBD) and fertilization until embryonic genome activation (i.e., when the embryo initiates transcription of its own genome for further survival, reviewed by Bettegowda and Smith 2007). Transcriptional activity, as assessed by [^{3}H] uridine uptake by the nucleolus and nucleus of murine oocytes within ovarian follicles is rapid early in oocyte growth and declines in conjunction with the formation of the follicular antrum in tertiary follicles (Oakberg 1968; reviewed by Mermillod *et al.,* 2008). Additional studies of growing mouse oocytes found that the majority of maternal RNA stores are present before ovulation (Bachvarova 1974) with approximately 95% of total RNA present prior to the oocyte becoming fully grown, at only 65% of final volume (Sternlicht and Schultz 1981). Growing bovine oocytes also undergo significant RNA synthesis (Fair *et al.,* 1997) that declines as the oocyte attains full growth. Little detectable transcription occurs once oocyte diameter reaches 110 µm (Fair *et al.,* 1995), paralleling changes in nucleolar morphology (Crozet *et al.,* 1986; Fair *et al.,* 1996). Once GVBD occurs and meiosis proceeds, transcriptional activity becomes undetectable (Rodman and Bachvarova 1976; Memili *et al.,* 1998; Lodde *et al.,* 2008). These findings are congruent with high mRNA polymerase activity (assessed by radiolabeled RNA precursor incorporation in the presence of α-amanitin, an

RNA polymerase II inhibitor) in growing oocytes that wanes in fully-grown oocytes contained within antral follicles (Moore and Lintern-Moore 1978).

While a portion of the transcripts produced within growing murine oocytes are translated and utilized immediately, the majority (~ 60%) are stable with half-lives of at least 48 h (Kaplan *et al.*, 1982). This is due to storage mechanisms mediated by the 3' untranslated region (UTR) of the transcript (reviewed by Gandolfi and Gandolfi 2001). During post-transcriptional modification prior to intron splicing, a polyadenylated region (poly(A) tail) is synthesized on the 3' end of mRNA that is subsequently shortened (reviewed by Bachvarova 1992). Numerous proteins then interact with the tail (Stutz *et al.*, 1998) and the cap located at the 5' end of the transcript to form a loop structure (Figure 2-2, reviewed by Piccioni *et al.*, 2005) that reversibly suppresses the ability of the transcript to be translated. These proteins include translation initiation factors, cytoplasmic polyadenylation element binding protein (CPEB), and maskin (Figure 2-2). Processing of RNA in this manner is referred to as "masking" and depends upon the presence of a cytoplasmic polyadenylation element (CPE, Paynton and Bachvarova 1994). Masking enables maternal transcripts to have long half-lives, ranging from 8 days (Brower *et al.*, 1981) to greater than 2 weeks in murine oocytes (Jahn *et al.*, 1976) in stark contrast to typical RNA with half-lives of minutes up to one day (Berger and Cooper 1975). The stability offered by masking of maternal RNA is also reflected in the low rates of transcript degradation, as amount of radiolabeled RNA was similar in murine oocytes pulse-chased for 3 or 6 days (Brower and Schultz 1982; Kaplan *et al.*, 1982). When masked transcripts are required for protein synthesis, the poly(A) tail is lengthened via poly(A) polymerase and bound by binding proteins,

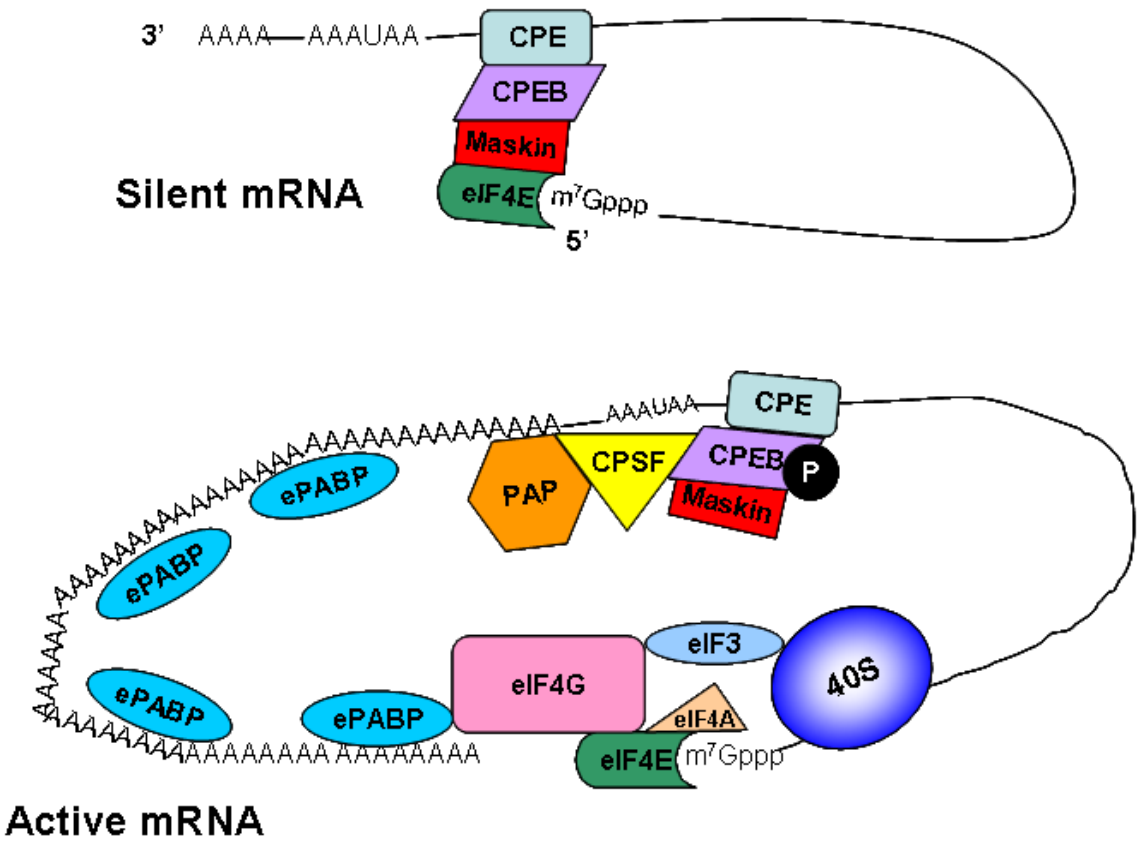


Figure 2-2. Masked and unmasked states of maternal mRNA via protein interactions with the poly(A) tail (adapted from Piccioni et al., 2005). Translationally-silent mRNA has a short poly(A) tail and physical interaction between the 5' cap (m^7Gppp) bound to eukaryotic initiation factor 4E (eIF4E) and 3' elements including the cytoplasmic polyadenylation element (CPE) and its binding protein (CPEB) via maskin. When needed for translation, the poly(A) tail is lengthened by poly(A) polymerase (PAP) and bound by various poly(A) binding proteins (ePABP). CPEB is phosphorylated, terminating the interaction with the 5' mRNA end. The translation initiation complex forms at the 5' end in preparation for protein synthesis.

CPEB is phosphorylated, and the tail-cap interaction is released to allow other initiation factors and ribosomal subunits to assemble the initiation complex (Figure 2-2).

Oocyte Maturation

The events of oogenesis, folliculogenesis, and oocyte growth, including accumulation of maternal RNA, are necessary for resumption of meiosis or oocyte maturation. Oocyte maturation is the intricate process by which the oocyte prepares at the nuclear, cytoplasmic, and molecular levels for fertilization by reductive division of the maternal DNA and dynamic changes in intracellular organization (reviewed by Barnes and Sirard 2000). The oocyte within the ovulatory follicle resumes meiosis in response to the preovulatory surge of luteinizing hormone (reviewed by Eppig *et al.,* 1996) or in culture after removal from the follicular environment (Pincus and Enzmann 1935). Meiotic resumption is facilitated by cumulus cells. Specifically, gap junctional communication allows for Ca^{2+} transients in cumulus to pass to the oocyte, resulting in non-hormone mediated meiotic induction via ATP and/or UTP (Webb *et al.,* 2002). Gap junctions begin to breakdown after meiotic resumption, however, and were reduced by > 50% within 5 hours of initiation of meiosis and completely absent by 9 h in bovine cumulus-oocyte complexes (Thomas *et al.,* 2004). As maturation progresses, the cumulus undergo expansion by production of extracellular matrix proteins (reviewed by Russell and Salustri 2006) and express numerous genes, some of which are involved in neuron and immune function (Hernandez-Gonzalez *et al.,* 2006) and may also serve as markers of oocyte developmental potential after fertilization (van Montfoort *et al.,* 2008).

Nuclear Maturation

The purpose of nuclear maturation is to reduce the number of copies of maternal chromatin from 4 to 2. This requires progression from prophase I of meiosis through metaphase I, anaphase I, telophase I and rearrest at metaphase II (MII) with extrusion of the first polar body containing two copies of DNA. The accumulation of maturation promoting factor (MPF) components, cyclin B1 and $p34^{cdc2}$ kinase, is necessary for resumption of meiosis (Levesque and Sirard 1996). Specifically, MPF migrates from the ooplasm to the nucleus to initiate breakdown of the germinal vesicle (i.e., oocyte nucleus) after the signal (luteinizing hormone or removal from follicle) to resume meiosis is received (Ookata *et al.,* 1992). Breakdown of the germinal vesicle (GVBD) is followed by condensation of maternal chromatin into bivalent chromosomes which align on the meiotic spindle. Progression from metaphase I to MII is mediated, in part, by phosphatidylinositol 3-kinase as evidenced by inability of oocytes cultured with an inhibitor of this kinase to develop beyond metaphase I (Anas *et al.,* 1998). Continuation from metaphase I to anaphase I was coincident with reduced $p34^{cdc2}$ kinase activity in both bovine (Anas *et al.,* 2000) and murine oocytes (Hampl and Eppig 1995). Cyclin B1 is degraded during the transition from metaphase I to MII (Hampl and Eppig 1995) suggesting that reduced MPF activity is necessary for the second half of meiotic maturation. In this regard, MPF displays a cyclic pattern in bovine oocytes, increasing after GVBD until approximately 15 h of maturation at which point activity declines until 19 h but increases to similar levels seen at 15 h by 24 h (Fissore *et al.,* 1996; Wehrend and Meinecke 2001).

Cytoplasmic Maturation

Concurrent with dynamic changes in nuclear material are alterations in cytoplasmic components (reviewed by Ferreira *et al.,* 2009). In general, the majority of organelles migrate from the inner regions of the oocyte to a cortical position mediated by the cytoskeletal network (reviewed by Hyttel *et al.,* 1997). The microtubular cytoskeleton is particularly important as chemical stabilization of this network in rat oocytes prevented meiotic maturation (Albertini 1987). As maturation progresses, the endoplasmic reticulum becomes concentrated opposite the MII plate at the oocyte cortex (Mehlmann *et al.,* 1995) which is important for localized calcium release in conjunction with exocytosis of cortical granules lining the oolemma at fertilization (reviewed by Wessel *et al.,* 2001). Mitochondria also cluster near other organelles and nuclear material in association with increased ATP production as maturation progresses (Stojkovic *et al.,* 2001), which is necessary for protein synthesis during maturation (Krisher and Bavister 1998).

Molecular Maturation

Molecular maturation is "a legacy of the instructions accumulated during the GV-stage that controls both the nuclear and the cytoplasmic progression" (Sirard 2001). In other words, molecular maturation refers to the accumulation and storage of RNA and proteins during oocyte growth for use during and after meiotic maturation. Protein synthesis after resumption of meiosis is critically dependent upon the maternal stores of RNA acquired during oocyte growth. During the period between initial stages of meiotic resumption and GVBD, limited RNA synthesis occurs (Bloom and Mukherjee 1972) and is necessary for resumption of meiosis, as transcriptional inhibitors prevent oocyte maturation (Hunter and Moor 1987; Farin and Yang 1994). In murine cumulus-oocyte complexes, transcription occurs within the first 15 minutes of

FSH-induced maturation, as assessed by addition of a transcriptional inhibitor (5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole; DRB) at 5 min intervals from time of placement in maturation medium and determining when inhibitor became ineffective to block GVBD (Rodriguez *et al.,* 2006). This early burst of transcription is also required for protein synthesis after GVBD in bovine oocytes, determined by culture with the protein synthesis inhibitor, cycloheximide (Kastrop *et al.,* 1991).

The utilization of maternal transcripts for protein synthesis continues throughout meiotic maturation and is increased in maturing oocytes compared to those arrested at GV-stage; heightened translation is first noted in conjunction with GVBD (within the first 6 to 10 h of maturation in bovine oocytes) and increased polyadenylation of mRNA populations (Tomek *et al.,* 2002). Rates of protein synthesis were highest during the first half of bovine meiotic maturation, from before GVBD to metaphase I (Coenen *et al.,* 2004). Inhibition of protein synthesis via cycloheximide prevented both GVBD and cumulus expansion in bovine oocytes (Hunter and Moor 1987). Use of translational inhibitors during the second half of bovine oocyte maturation was also detrimental to meiotic progression (Sirard *et al.,* 1989). However, the overall ability of oocytes to undergo protein synthesis may still be less than that of the embryo or somatic cells as only 20 to 25% of the ribosomes within ovulated murine oocytes are polysomal (i.e., actively involved in protein synthesis, Bachvarova and De Leon 1977). Regardless, dynamic changes in protein synthesis have been reported after GVBD (Kastrop *et al.,* 1991) corresponding to three patterns of protein synthesis during bovine oocyte *in vitro* maturation: the first 4 h, the middle (4 to 16 h) and the end (16 to 28 h, Coenen *et al.,* 2004). Furthermore, dynamic changes in the specific proteins produced occur with roughly half of the proteins

expressed during the first 4 hours of maturation still expressed at the end of maturation (Coenen *et al.,* 2004).

Total amount of RNA in murine oocytes declined by 19% from 430 pg per oocyte at the GV-stage to 350 pg per oocyte at MII as determined by poly(U) sepharose chromatography (Bachvarova *et al.,* 1985), possibly due to reductions in rRNA from 262 pg to 202 pg (Paynton *et al.,* 1988). This is in contrast to observations in bovine oocytes for which the amount total RNA [ranging from ~1 ng (Olszanska and Borgul 1993) to ~2 ng (Lequarre *et al.,* 2004)] and rRNA (Bilodeau-Goeseels and Schultz 1997; Lequarre *et al.,* 2004) remain relatively stable during maturation. While total RNA may be reasonably stable as maturation progresses, dynamic changes occur in the polyadenylated fraction. Specifically, polyadenylated RNA (poly(A) RNA) in murine oocytes is reduced by half during meiotic maturation, from 19% of total to 10%, both *in vitro* (Paynton *et al.,* 1988) and *in vivo* (De Leon *et al.,* 1983), some of which is apparently due to changes in adenylation status of specific transcripts such as β-actin (Bachvarova *et al.,* 1985). The magnitude of the reduction in poly(A) RNA observed for murine oocytes is in agreement with reports for bovine oocytes, in which global levels of polyadenylated RNA dropped from ~50 pg to ~25 pg during maturation (Lequarre *et al.,* 2004). The ability of oocytes to modulate levels of poly(A) RNA is necessary for meiotic maturation as inhibition of polyadenylation during bovine oocyte maturation prevented GVBD (Krischek and Meinecke 2002; Traverso *et al.,* 2005), indicating the importance of active poly(A) polymerase in the maturing oocyte. Furthermore, increased polyadenylation of specific transcripts has been associated with the subsequent appearance of more protein as noted for cyclin B1 (Tremblay *et al.,* 2005). The length of the poly(A) tail of certain transcripts during bovine oocyte maturation

has been correlated to the size of follicle from which the oocyte was derived, with longer tails associated with larger (> 5 mm) follicles (Brevini-Gandolfi *et al.*, 1999). In this regard, oocytes that have not undergone sufficient growth (i.e., contained within follicles < 3 mm in diameter for cattle) are not competent to undergo meiosis and subsequent embryo development after fertilization (reviewed by Hendriksen *et al.,* 2000) and contain different amounts of specific transcripts than oocytes from larger follicles (Donnison and Pfeffer 2004; Pfeffer *et al.,* 2007; Racedo *et al.,* 2008). ***These data suggest that the ability of oocytes to successfully undergo meiotic maturation may be driven by transcripts accumulated during the growth phase.***

The changes taking place in maternal mRNA during meiotic maturation are consistent with the events occurring during this time as described above. Specifically, *in vitro* maturation of bovine oocytes is dependent upon adenylation of transcripts important for chromatin condensation, spindle assembly, and MPF and MAP kinase activation, indicated by inability of oocytes cultured with a polyadenylation inhibitor (cordycepin) to undergo GVBD and chromatin condensation (Krischek and Meinecke 2002). During the transition from GV-stage to MII, polyadenylated transcripts (as assessed by microarray) important for regulation of MAP kinase activity, initiation of translation, mitochondrial inner membrane and ribosome, and protein binding were reduced in bovine oocytes (Fair *et al.,* 2007), similar to microarray findings for murine (Cui *et al.,* 2007) and human oocytes (Wells and Patrizio 2008). Polyadenylated transcripts increasing during murine oocyte maturation were associated with DNA replication, amino acid metabolism, and cell signaling via G protein-coupled receptors (Cui *et al.,* 2007). Furthermore, the relative abundance of polyadenylated transcripts for GDF-9, cyclin B1, and peroxiredoxin 6 declined over bovine maturation but the overall amount of transcript

(determined using random hexamers rather than oligo d(T) primers) remained constant (Lequarre *et al.,* 2004). This highlights an important aspect of oocyte RNA in that not all maternal transcripts have a poly(A) tail and, thus, would not be detected via poly(A)-based amplification. This is the case for the histone family, where most members lack any polyadenylation and rely upon a stem-loop structure at the 3' end of RNA for stability (reviewed by Dominski and Marzluff 2007).

The importance of examining the entire mRNA population rather than only those that are polyadenylated is further demonstrated in an elegant study utilizing a whole transcriptome RNA method in conjunction with microarray analysis in which selective degradation of maternal transcripts in murine oocytes was observed (Su *et al.,* 2007). Transcripts encoding proteins important for meiotic arrest at the GV-stage and progression to MII like those involved in oxidative phosphorylation (energy production) such as NADH-ubiquinone oxidoreductases and cytochrome c oxidases as well as protein synthesis (components of the cytoplasmic ribosomal subunits) were degraded while others necessary for MII-arrest and protein kinase pathways (namely those involved in MAP kinase signaling) were stable (Su *et al.,* 2007). This indicates that the reductions in maternal RNA during oocyte maturation are not a reflection of indiscriminate degradation, but rather targeted for specific functions. A classic example of this phenomenon occurs for c-mos which is transcribed and stored during oocyte growth, polyadenylated and translated during oocyte maturation (reviewed by Gebauer and Richter 1997) but must be degraded after fertilization or development will arrest (Alizadeh *et al.,* 2005).

After fertilization, oocyte mRNA stores continue to decline, with the majority of maternal mRNA no longer present in murine embryos by embryonic genome activation at the late 1-cell

stage (Bachvarova *et al.,* 1985) and 2-cell stage (Piko and Clegg 1982). In murine embryos, most RNA is derived from the embryonic genome by the 2-cell stage (Clegg and Piko 1983). However, some maternal RNA persists and is present in the blastocyst-stage embryo (Bachvarova and De Leon 1980; Fourcray 1982) in both the inner cell mass and trophectoderm (Bachvarova and Moy 1985). ***These findings indicate the importance of maternal transcripts not only in the oocyte and early-cleavage stage embryo, but also in the blastocyst and further emphasize how heat-induced alterations in oocyte RNA stores may carryover to negatively impact blastocyst development.***

EFFECTS OF ELEVATED TEMPERATURE ON THE MATURING OOCYTE

Consequences of Maternal Hyperthermia Occurring at the Time of Estrus

Oocyte maturation *in vivo* occurs in response to the preovulatory surge of luteinizing hormone corresponding to the period when the animal is in estrus and receptive to breeding. As discussed in Chapter 1, females of numerous species experiencing hyperthermia near the time of breeding when the oocyte is undergoing maturation are particularly susceptible to heat-induced infertility (Stott and Williams 1962; Cavestany *et al.,* 1985; Putney *et al.,* 1989b). However, effects on fertility are dependent upon duration and severity of the heat stress imposed (Cavestany *et al.,* 1985). In this regard, exposure of superovulated heifers to 42ºC and 75% relative humidity for 10 h (roughly equivalent to the first half of bovine oocyte maturation) at the onset of estrus raised respiration rates by more than 200% and rectal temperatures from 38.9º to 41.3ºC (Putney *et al.,* 1989b). This maternal heat stress was sufficient to increase the proportion of retarded embryos and reduce embryo quality (graded poor to fair versus good to excellent)

seven days after artificial insemination but did not alter the fertilization rate as assessed by uncleaved putative zygotes (Putney *et al.,* 1989b). Consequences of hyperthermia on fertility are also seen in other species; exposure of sheep to high temperature and humidity (90 to 95ºF and 60 to 65% relative humidity) at the time of breeding increased the proportion of abnormal oocytes and prevented lambing (Dutt 1963).

As the duration of heat stress increases, the consequences become more pronounced. Specifically, mice exposed to 35ºC and 65% relative humidity for 15.5 h (all of oocyte maturation) at the time of hCG administration (to induce resumption of meiosis) experienced increased arrest of oocytes at metaphase I (MI) and increased oocyte degeneration and fragmentation (Baumgartner and Chrisman 1981a). Similar experimental conditions resulted in heat-induced increases in bicellular and tricellular oocytes (likely oocyte fragmentation) with a concomitant decrease in monocellular oocytes (Baumgartner and Chrisman 1981b) leading the authors to suggest that heat stress induces a phenotype similar to oocyte aging. Exposure of mice to 35ºC and 65% relative humidity for 12.5 h during oocyte maturation altered nuclear maturation by increasing oocytes arrested at MI and those with retained polar bodies; after mating, increased pre- and post-implantation embryo losses were noted along with a tendency for reduced fetal size and rate of development (Baumgartner and Chrisman 1987). A subsequent study with the same heat stress conditions demonstrated that 9 days after mating, mice exposed to heat stress during oocyte maturation had heat-induced delays in embryo development (reduced somites from 18 to 36 h after mating), increased preimplantation losses (higher proportion of empty deciduas compared to corpora lutea), and increased embryonic triploidy (Baumgartner and Chrisman 1988). Exposure to extreme heat stress conditions during oocyte maturation resulted

in maternal hyperthermia sufficient to reduce subsequent embryo development but this effect was alleviated by injection of the antioxidant EGCG prior to exposure to elevated temperature (Roth *et al.,* 2008). Improved embryo development after antioxidant treatment may be indicative of heat-induced increases in reactive oxygen species (ROS) in conjunction with heat stress conditions.

Direct Effects of Elevated Temperature on Maturing Oocytes

Exposure of oocytes to elevated temperature during *in vitro* maturation elicits similar reductions in embryo development as those seen with maternal heat stress, indicating that some of the detrimental effects of heat stress are due to a direct impact on the maturing oocyte. However, as mentioned above regarding maternal hyperthermia, effects are dependent upon severity and duration of the heat stress imposed. In addition, the timing of exposure to elevated temperature during oocyte maturation is important: effects of culture at 41ºC for 12 h on blastocyst development after fertilization were more pronounced during the first half of maturation than the last 12 h (Edwards and Hansen 1996). However, other endpoints such as de novo protein synthesis (Edwards and Hansen 1996), ability to progress to MII, and cortical granule migration were similar in bovine oocytes when heat stress was applied for the first 12 h or last 12 h of maturation (Edwards *et al.,* 2005).

Culture of bovine oocytes at a physiologically-relevant temperature of 41ºC for the first 12 h reduced blastocyst development by 30 to 65% (Edwards and Hansen 1996, 1997; Lawrence *et al.,* 2004; Roth and Hansen 2004a, b; Edwards *et al.,* 2005; Schrock *et al.,* 2007). Recent data indicated that this may be due to a reduced ability of embryos derived from heat-stressed oocytes to undergo compaction (Edwards *et al.,* 2009), a necessary step for blastocoele formation. The

majority of studies indicate that reductions in development are not due to an inability of embryos to undergo early cleavage divisions (Edwards and Hansen 1996; Lawrence *et al.,* 2004; Edwards *et al.,* 2005; Schrock *et al.,* 2007; Edwards *et al.,* 2009). However, exposure of maturing bovine oocytes to 41ºC for the first 12 h of maturation with simultaneous alterations in pH due to increased (7%) CO_2 concentrations reduced the ability of embryos derived from heat-stressed oocytes to cleave (Roth and Hansen 2004a, b). This discrepancy can be reconciled by differences in methodology; maturation of oocytes at an elevated temperature (41ºC) in conjunction with increased atmospheric CO_2 (7%) suggests that simultaneous heat stress and pH alterations are more detrimental than heat stress alone. When examining the different stages of cleaved embryos, some studies found reductions in the proportion of 8 to16-cell stage embryos after fertilization (Lawrence *et al.,* 2004) while others reported no differences in embryos derived from control and heat-stressed oocytes (Edwards and Hansen 1996; Edwards *et al.,* 2005; Schrock *et al.,* 2007). This may be indicative of different responsiveness of individual batches of oocytes to elevated temperature. Regardless, cumulative findings suggest that many of the detrimental effects of heat stress on fertility are due to direct consequences on the oocyte. This may be due to heat-induced perturbations of nuclear, cytoplasmic, and/or molecular maturation, as well as alterations in the relationship between the oocyte and surrounding cumulus cells.

Heat-induced reductions in embryo development do not appear to be due to an inability of the oocyte to mature at the nuclear level. Specifically, exposure of bovine oocytes to 41ºC for 12 or 24 h of maturation did not alter ability of oocytes to progress to metaphase II (MII) by 24 h (Dorado *et al.,* 2001). This is in contrast to earlier findings in which exposure of bovine oocytes

to 41ºC during maturation reduced polar body extrusion (Lenz *et al.,* 1983), suggestive of failure to reach MII. A subsequent study reconciled this apparent discrepancy by finding fewer heat-stressed oocytes had a discernible polar body at 24 h but a similar proportion were at MII, likely indicative of heat-induced polar body degeneration (Edwards *et al.,* 2005). This report also examined the kinetics of bovine oocytes (exposed to 41º for the first 12 h of maturation) to reach MII and revealed heat-induced acceleration of nuclear maturation (Edwards *et al.,* 2005). Others have reported reduced ability of oocytes to progress to MII after heat stress due to increased arrest at MI with misshapen meiotic spindles (Roth and Hansen 2005) but these findings were confounded with alterations in pH due to increased CO_2 concentrations. The use of supraphysiological temperatures induces more severe consequences as exposure of murine oocytes to 41ºC (4ºC above normal) for 17 h completely inhibited meiotic maturation while temperatures of 40ºC or higher reduced normal chromosome spreads and increased abnormal ploidy (Fiorenza and Mangia 1992). A more physiological increase of 1.5ºC above normal (37º vs. 38.5ºC) resulted in similar progession to MII as in control murine oocytes (Fiorenza and Mangia 1992), in agreement with results for bovine oocytes.

While nuclear maturation seems to be minimally impacted by physiological heat stress, several lines of evidence indicate that elevated temperature impacts certain aspects of oocyte cytoplasmic maturation. Exposure of oocytes to physiological heat stress (41ºC) for 12 h during maturation did not alter membrane integrity (Edwards and Hansen 1996, 1997). However, culture of bovine oocytes at 41ºC for the first 12 h of maturation followed by 12 h recovery at thermoneutral temperatures increased the proportion having Type III cortical granules (an indicator of cytoplasmic maturation) at 24 h (Edwards *et al.,* 2005), compared to controls.

Maturation of oocytes at an elevated temperature (41ºC) in conjunction with increased atmospheric CO_2 (7%) altered actin microfilaments (Roth and Hansen 2005). However, in the absence of pH alterations, an elevated temperature of 43ºC was required to perturb the actin cytoskeleton (Payton et al., unpublished). Furthermore, increased incidence of apoptotic oocytes (TUNEL-positive) was observed (Roth and Hansen 2004a), an effect that was alleviated by addition of sphingosine-1-phosphate (a sphingomyelin metabolite involved in cell growth and survival and cytoskeletal rearrangement) to maturation medium (Roth and Hansen 2004b). Culture of maturing oocytes at 41ºC with altered pH (7% CO_2) for the entire period of maturation (21 h) also increased TUNEL-positive oocytes as well as reduced mitochondrial membrane potential (Soto and Smith 2009). However, as noted above for nuclear maturation, confounding effect of pH alterations during elevated temperature exposure precludes further inference.

There is some indication that heat stress may induce elevations in cytoplasmic reactive oxygen species (ROS) to reduce development. When exposure of maturing oocytes to elevated temperature was sufficient to reduce blastocyst yield by at least 20% compared to controls, addition of retinol, an antioxidant, prevented heat-induced reductions in blastocyst development (Lawrence *et al.,* 2004). However, levels of the abundant and ubiquitous intracellular antioxidant, glutathione, were unchanged in heat-stressed *in vivo* matured murine oocytes despite heat-induced reductions in blastocyst development (Matsuzuka *et al.,* 2004). Although these data seem disparate, ROS are present during maturation of non-stressed bovine oocytes concomitant with superoxide dismutase, catalase, and glutathione peroxidase activities (Cetica *et*

al., 2001), indicative of some ability of the oocyte to respond to any heat-induced increases in ROS.

Despite several studies examining the effects of elevated temperature on maturing oocytes, very little information is available regarding the effects of heat stress on molecular events occurring during meiotic maturation. Elevated temperature for the first 12 h of maturation reduced *de novo* protein synthesis in bovine oocytes by approximately 40%, as measured by TCA precipitation, compared to controls (Edwards and Hansen 1996). Exposure of GV-stage and matured (MII) murine oocytes to elevated temperature also resulted in reduced protein synthesis (Hahnel *et al.,* 1986; Curci *et al.,* 1987). Furthermore, the abundance of heat shock protein 70 (HSP70), a thermoprotective molecular chaperone, was reduced in heat-stressed bovine oocytes (Edwards and Hansen 1997). Examination of global protein populations in matured bovine oocytes after 12 h exposure to 41ºC at the onset of maturation revealed differential abundance of 4 proteins, some of which should have declined during maturation but failed to do so after heat stress (West-Rispoli *et al.,* 2006). While these proteins are unidentified as of yet, they may be responsible for some of the reductions in development observed after fertilization of heat-stressed oocytes.

The mechanisms underlying heat-induced perturbations in protein synthesis in oocytes are unknown but data from somatic cells studies indicate that it may be due to alterations in factors necessary for translation of maternal transcripts or post-transcriptional alterations in the transcripts themselves. Specifically, exposure of HeLa cells to 41-42ºC up to 2 h reduced the proportion of ribosomes actively involved in protein synthesis by > 50% with concomitant alterations in the phosphorylation status of translation initiation factors (Duncan and Hershey

1989). Heat-stressed cells may also utilize alternative means of protein synthesis such as internal ribosomal entry site-mediated translation, involving specialized areas located within certain transcripts for ribosomes to bind (reviewed by Jackson 2005); this method of protein synthesis was utilized in HeLa cells cultured at 42ºC for 5 to 15 h (Kim and Jang 2002). On the other hand, exposure to supraphysiological elevated temperatures may induce RNA degradation as reported for α- and β- tubulin after heat stress of the protozoan, *Tetrahymena pyriformis* (Coias *et al.,* 1988). Heat stress has also been demonstrated to alter the adenylation status of transcripts. For example, heat shock protein 70 (HSP70) transcripts are preferentially deadenylated in heat-stressed *Drosophila* cell cultures (Dellavalle *et al.,* 1994) while avian testicular cells exposed to elevated temperature have increased polyadenylation of HSP70 and ubiquitin RNA (Mezquita *et al.,* 1998). If heat stress does induce perturbations in oocyte RNA, consequences may be more severe than those seen in somatic cells as oocytes become transcriptionally inactive soon after GVBD (Rodman and Bachvarova 1976) concurrent with exposure to elevated temperature. This renders them unable to respond to heat stress except by utilization of maternal RNA pools previously accumulated during oocyte growth for protein synthesis (Wassarman and Letourneau 1976). Unfortunately***, no information regarding the effects of elevated temperature exposure on RNA from maturing oocytes currently exists***.

Although the literature is lacking on the effects of heat stress on molecular maturation, studies suggest that heat-induced alterations in oocytes may be, in part, mediated by the surrounding cumulus cells. Lenz et al. (1983) found that exposure of oocytes with intact cumulus to 41ºC during maturation prevented full cumulus expansion and reduced hyaluronic acid production as measured by incorporation of [^{3}H]-glucosamine. Incomplete cumulus

expansion could hinder the ability of sperm to reach the oocyte during fertilization. Reductions in fertilization rate after heat stress of maturing oocytes have not been observed *in vitro* (Edwards et al., unpublished) unless also coincident with increased CO_2 concentrations (Roth and Hansen 2005). Other reports indicate that cumulus cells may protect the oocyte from noxious stressors like elevated temperature; denuded oocytes had less protein synthesis overall and reduced abundance of HSP68, HSP70, and HSP71 than cumulus-intact oocytes, regardless of heat stress exposure (Edwards and Hansen 1997). The presence of cumulus did not prevent heat-induced reductions in *de novo* protein synthesis (Edwards *et al.*, 1996) or abundance of HSP70 (Edwards and Hansen 1997).

Recent data suggests that an effect of heat stress during oocyte maturation may be to "age" the oocyte, similar to the effects seen when oocytes are not fertilized in a timely manner both *in vivo* and *in vitro* (post ovulatory and *in vitro* oocyte aging, respectively; review below restricted to only those studies with physiological oocyte aging, excluding those using extreme conditions and those referring to oocytes from older mothers). First, both nuclear and cytoplasmic maturation were accelerated by physiological heat stress during the oocyte maturation (Edwards *et al.*, 2005). Second, fertilizing oocytes earlier (19 vs. 24 h) eliminated heat-induced reductions in blastocyst formation (Edwards *et al.*, 2005). Third, heat stress during maturation shifted the developmental responsiveness of oocytes, with optimum embryo development obtained more than 7.5 h sooner than controls (Schrock *et al.*, 2007).

Several similarities exist between heat-stressed oocytes and aged oocytes. Notably, early embryo development is reduced by heat stress and aging (Ward *et al.*, 2002; Agung *et al.*, 2006). The timing of embryonic loss is also similar after fertilization of heat-stressed or aged oocytes,

occurring prior to or soon after placental attachment to the uterus (Blandau and Young 1939) although later term losses have been reported after postovulatory oocyte aging in women (Wilcox *et al.,* 1998). However, neither heat stress during maturation nor postovulatory aging result in a reduced ability to become fertilized (Barros *et al.,* 1997, Edwards et al., unpublished), indicating the problem is downstream of sperm penetration. Other findings indicate that both aging and heat stress of oocytes enhance their ability to become activated (i.e., undergo changes normally associated with sperm penetration in the absence of sperm, Zackowski and Martin-Deleon 1988, Edwards et al., unpublished)). In this regard, somatic cell nuclear transfer studies utilizing ooplasms from aged oocytes indicates that alterations are occurring in the cytoplasm to reduce subsequent fertility (Liu *et al.,* 2007), possibly due to asynchrony between the cytoplasm and nucleus (Adenot *et al.,* 1997). The predominance of cytoplasmic alterations in oocyte aging are consistent with observations for heat-stressed oocytes (Edwards *et al.,* 2005). Perturbation of maternally-derived cytoplasmic constituents is likely the primary contributor to the phenotype observed for both heat-stressed and aged oocytes. Alterations in protein synthesis have been reported for aged murine oocytes (Xu *et al.,* 1997) as well as heat-stressed bovine oocytes (Edwards and Hansen 1996, 1997).

EFFECTS OF HEAT STRESS ON MATURED OOCYTES

Consequences of Maternal Hyperthermia Occurring Near the Time of Breeding

Ovulated oocytes that have completed meiotic maturation (i.e., arrested at MII) are susceptible to the negative effects of heat stress in a similar manner as those still undergoing maturation. Maternal hyperthermia occurring during the time period when the ovulated oocyte is within the oviduct awaiting fertilization results in reduced fertility (Austin and Braden 1954a, b;

Braden and Austin 1954a, b; Stott and Williams 1962; Cavestany et al., 1985). Braden and Austin (1954b) exposed MII-arrested oocyte within the exteriorized oviduct to 44 to 45ºC water for 5 to 10 minutes 8 to 12 h post-ovulation (i.e., heat stress of an aged and matured oocyte). Doing so resulted in increased degeneration and fragmentation and nuclear reformation resembling pronuclei (Braden and Austin 1954b). Similarly, increased degeneration and fragmentation were observed after exposure of oviduct-enclosed ovulated murine oocytes to 43 to 45.5ºC for 5 to 10 minutes, which was also sufficient to induce parthenogenic activation (i.e., mimicking the effects of sperm without fertilization, Komar 1973).

Other studies have examined effects of heat stress on matured oocytes within the maternal environment after mating but before fertilization. Specifically, exposure of ovulated murine oocytes to 42 to 48ºC for 5 to 10 min within 1.5 to 6 h of mating (via submersion of exteriorized ovaries and oviducts in hot water) prevented embryo cleavage when examined 3.5 days later (Fischberg and Beatty 1952). It should be noted that exposure to elevated temperature after breeding would also involve heat stress of sperm within the reproductive tract. Thus, reductions in embryo cleavage may be due to both effects of elevated temperature on matured oocytes, sperm, and the interaction of the two cell types during fertilization. Nevertheless, a similar study reported reductions in the number of oocytes with a visible second polar body and increased polyspermy after subjection of ovulated murine oocytes within the oviduct to 43.5 to 45ºC for 5 to 10 min at 3 h after mating (Braden and Austin 1954a). Heat stress of ovulated rat oocytes within the oviduct by exposure to 44.5 to 45.5ºC water for 8 to 12 minutes after mating (but before fertilization) prevented extrusion of the second polar body (Austin and Braden 1954b). While the ability of sperm to penetrate oocytes was not affected by heat stress but there

were heat-induced reductions in pronuclear formation after fertilization (Austin and Braden 1954b). The same authors also reported a reduction in discernible second polar bodies and increased polyspermy after exposure of matured murine oocytes within the oviduct to 43.5 to 45ºC water for 5 to 10 minutes 3 h post-mating (Austin and Braden 1954a).

Direct Effects of Elevated Temperature on Matured Oocytes

Removal of the matured oocyte from the maternal environment enables the evaluation of the direct impact of heat stress. In this regard, Chang (1952) exposed ovulated rabbit oocytes (arrested at MII) to 45ºC for 10 to 40 minutes *in vitro* before transferring to a recipient rabbit prior to breeding. Heat stress for 10 or 20 minutes resulted in the normal fertilization and cleavage of the resulting embryo but exposure to elevated temperature for 30 or 40 minutes increased fertilization failure and reduced normal fertilization and cleavage (Chang 1952). Similarly, culture of matured bovine oocytes at 42ºC for 0, 1, 2, or 4 h prior to fertilization had no apparent impact on fertilization or cleavage but blastocyst rates, overall cell numbers, and number of trophoblast cells were reduced for embryos from heat-stressed oocytes (Ju *et al.,* 1998). A subsequent report by the same laboratory examined bovine oocytes matured for 20 to 22 h prior to culture at 40.5, 41.5, or 43ºC for 30 min or 1 h (Ju *et al.,* 1999). Culture at 40.5 and 41.5ºC had no effect on cleavage or progression to blastocyst after fertilization but 43º for 1 h reduced blastocyst rates (Ju et al., 1999). Recent data indicates that reduced embryo development of heat-stressed porcine oocytes may be due to heat-induced perturbation in ability to release calcium in response to activating stimuli such as fertilization by sperm (Tseng and Ju 2009). However, these findings were derived from observation of parthenogenetically-activated (via thimerosal) oocytes rather than those fertilized by sperm (Tseng and Ju 2009). Use of

chemical activation rather than sperm could hamper data interpretation as our laboratory has shown higher embryo development of heat stressed oocytes when chemically activated versus fertilized (Edwards et al., unpublished). Others have improved viability of heat-stressed oocytes by microinjection of HSP70 transcripts, which prevented heat-induced reductions in embryo development (Hendrey and Kola 1991).

Specific alterations occurring within matured oocytes in response to heat stress have been coincident with reduced developmental potential after fertilization and may be responsible for some of the observed depressions in fertility of hyperthermic animals. Specifically, exposure of matured porcine oocytes (with first polar body present) to 41.5ºC for 1, 2, or 4 h resulted in duration-dependent increases in chromosomal abnormalities, abnormal meiotic spindles, decreases in pericytoplasmic microtubules and actin-based transzonal processes, and altered actin vitelline rings (Ju and Tseng, 2004). Other alterations including reduced membrane integrity and increased morphological abnormalities were observed after in vitro exposure of MII murine oocytes (16 h post-hCG injection) to 42 to 43ºC for 1 h (Hendrey and Kola 1991). Reduced membrane integrity was also observed after heat stress of ovulated murine oocytes (14 to 16 h post-hCG injection) *in vitro* for 1 h at 42 to 43ºC followed by a 2 h recovery period at 37ºC (Hahnel *et al.*, 1986). Examination of *de novo* protein synthesis in heat-stressed matured oocytes from mice found no impact of heat stress on uptake of L-[^{35}S]-methionine but reduced its incorporation, indicating a heat-induced reduction in protein synthesis (Hahnel *et al.*, 1986).

SUMMARY: A TENTATIVE THESIS REGARDING THE MOLECULAR EFFECTS OF HEAT STRESS DURING OOCYTE MATURATION

For the body of work described hereafter, it was hypothesized that some of the deleterious effects of exposure of maturing oocytes to elevated temperature on subsequent embryo development may be due to alterations in transcripts within the oocyte and/or its surrounding cumulus cells. Evidence supporting this thesis is reviewed in Chapter 2. First, heat stress before, during, and after meiotic maturation reduces protein synthesis by the oocyte. Reduced protein synthesis may be due to alterations in translational machinery or perturbation of maternal RNA, since protein synthesis prior to embryonic genome activation is dependent upon transcripts stored within the ooplasm during oocyte growth. Second, oocytes are largely incapable of responding to elevated temperature exposure during meiotic maturation at the transcriptional level, as RNA synthesis ceases after GVBD. This further indicates the importance of a "healthy" store of RNA within the ooplasm to support subsequent development. Third, heat stress in other cell types has been demonstrated to perturb RNA polyadenylation and integrity (by degradation) which may alter its ability to be translated. Taken together, data suggest the potential for heat-induced disruption of the oocyte RNA. Thus, the overall objective of this dissertation was to determine the effects of heat stress during meiotic maturation on RNA within the maturing oocyte as well as that of the surrounding cumulus cells.

CHAPTER 3: General Features of RNA from Gametes versus Cumulus Cells

ABSTRACT

Despite numerous studies using microcapillary electrophoresis to examine RNA integrity before characterizing transcriptome profiles and specific transcripts of gametes, few reports provided data regarding general features of total RNA (i.e., rRNA ratio or the size distribution of RNA). Results provide additional insight regarding molecular features of gamete RNA and how they compare to cumulus cell RNA. In particular, the RNA integrity number and ratios for rRNA, 18S/fast region and 18S/inter region were significantly lower in total RNA from oocytes versus cumulus cells. Lower values for 18S/fast region ratio suggested that oocyte total RNA had increased abundance of smaller RNA relative to 18S rRNA than RNA from their surrounding cumulus. Extensive efforts demonstrated that oocyte RNA features were repeatable whether maturation occurred *in vitro* or *in vivo*, and were similar between the nuclear stages examined. Features of oocyte RNA were conserved across six mammalian species, as were differences from their respective surrounding cumulus. Profiles of sperm RNA lacked discernible ribosomal RNA peaks, and were conserved across species. Because the oocyte and spermatozoon are highly specialized cells representing different molecular entities required for proper embryo development, features obtained likely represent real differences in gamete versus cumulus RNA.

INTRODUCTION

Upon resumption of meiosis and germinal vesicle breakdown, oocytes become transcriptionally quiescent and must rely upon pools of RNA accumulated during the growth

phase for protein synthesis (Wassarman and Letourneau 1976). Transcriptional inactivity and reliance upon maternal RNA stores persist until after fertilization and, in some species, until the third cleavage division (Telford *et al.*, 1990). Stress-induced perturbations in the total amount of polyadenylated RNA in oocytes during maturation were coincident with reductions in developmental competence (Chapter 4). Before performing microarray analyses to identify the impact on individual transcripts, assessment of RNA integrity was an important first step. To this end, initial efforts using microcapillary electrophoresis suggested differences between oocyte and cumulus RNA (Payton *et al.*, 2006). Specifically, rRNA ratio and RNA integrity number (RIN) values were lower in total RNA from oocytes compared to cumulus cells. This serendipitous finding raised concerns regarding integrity of oocyte RNA.

Since cumulus cells were those that surrounded the oocyte and were processed at the same time, in the same manner, by the same individual, a series of investigations were performed to address the fundamental question, Do features of oocyte RNA differ from those of surrounding cumulus which are more somatic cell-like? In this regard, extensive effort was taken to determine whether RNA features were oocyte specific or an artifact related to procedures or origin (i.e., from oocytes of antral follicles not otherwise destined to ovulate). Specifically, bovine oocyte RNA from different stages of in vitro maturation was evaluated in retrospect using data originating from different studies. When available, cumulus RNA characteristics were also included for comparison to RNA from oocytes. Additional effort was then put forth to examine 1) conservation of oocyte RNA features among six species (bovine, porcine, ovine, murine, canine, and feline), 2) features of total RNA in oocytes undergoing maturation *in vivo*, and 3) resultant profiles after amplifying polyadenylated RNA from bovine

oocytes and surrounding cumulus cells as an indirect means to assess mRNA quality. In addition, total RNA from sperm of four different mammalian species (bovine, porcine, ovine, and human) was assessed.

MATERIALS AND METHODS

General methods for extraction, quantification, and generation of electropherograms of total RNA

Unless indicated otherwise, total RNA was isolated using PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA, USA) as per manufacturer's specifications. Oocytes were denuded of cumulus by vortexing (2.5 to 4.5 min for bovine, ovine, porcine, feline and canine and ~60 sec for murine oocytes) in a HEPES-buffered medium (Parrish *et al.*, 1988) containing 0.3% (w/v) polyvinyl alcohol (PVA) and 0.3 mg/ml hyaluronidase. Cumulus-free oocytes (confirmed by visual examination of each individual oocyte) were transferred in 3.5 µl HEPES-PVA as pools of 34 to 50 oocytes (mode = 50) into nuclease-free 0.6 ml microcentrifuge tubes before addition of extraction buffer. Cumulus cells were washed in HEPES-PVA before pelleting for lysis. After addition of extraction buffer, all samples were incubated at 42ºC for 30 min, and frozen at -80ºC until RNA isolation. TURBO DNase (Ambion, Inc., Austin, TX, USA) was added to the RNA column (2 or 4 units for 30 min at 37ºC) as per suggestion of PicoPure kit. In the majority of instances, the number of RNA pools was included in tables appearing in results section. In cases where data were presented in results section text, number of RNA pools was provided in relevant methods section. An experimental unit was considered to be a group (pool) of oocytes.

Total RNA from oocytes and cumulus cells was quantified using either NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) or Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) as indicated in subsequent sections. Samples quantified using RiboGreen were analyzed in triplicate and the concentration was determined using a standard curve (1 to 400 ng/ml). Electropherograms were generated with the 2100 Bioanalyzer by loading 1 µl of total RNA (0.74 to 47.19 ng for oocytes (mode = 9 ng) and 1.2 to 110 ng for cumulus (mode = 1.2 ng)) onto a RNA 6000 Pico LabChip (Agilent Technologies, Santa Clara, CA, USA).

Electrophoretic separation and detection of RNA via laser-induced fluorescence allowed for assessing RNA integrity by examining the size distribution of RNA molecules (Mueller *et al.,* 2004). An electropherogram is divided into multiple regions (Schroeder *et al.,* 2006) as indicated in Figure 3-1. The pre-region precedes the marker (M) and corresponds to RNA sizes < 25 nt. The 5S-region includes 5S rRNA, tRNA, and other RNAs slightly less than 200 nt, while the fast-region spans RNA sizes approximately 200 to 1500 nt. The 18S-region includes 18S rRNA and other RNA > 1600 to < 1800 nt whereas the 28S-region encompasses 28S rRNA and other RNA sizes ranging from approximately 3200 to < 3600 nt. RNA sizes between the 18S- and 28S-regions comprise the inter-region. A region of RNA inclusive of precursor rRNA was present in some samples at approximately 4000 nt. The post-region includes RNA sizes larger than 4500 nt. The Bioanalyzer software generates 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 ranging from 1 (completely degraded) to 10 (intact) taking into account all the regions of the electropherogram (Schroeder *et al.,* 2006). In addition, relative amounts of RNA

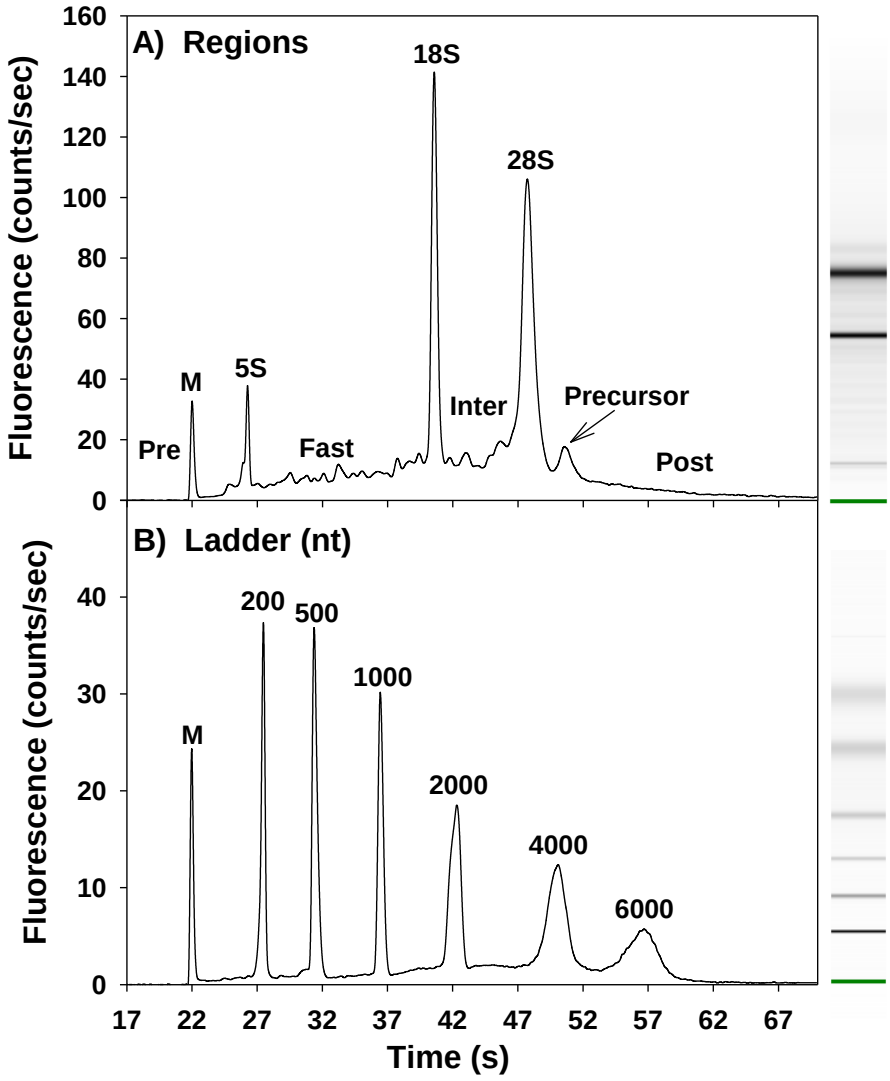


Figure 3-1. Regions of an electropherogram. Segments of an electropherogram (A) and ladder (B; run on every Pico LabChip), and respective gel images are provided for ease of interpretation of sample electropherograms. The ladder is comprised of discrete RNA sizes (nucleotides; nt) that can be used to estimate the size of RNA within each region. M, marker.

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 nt; 18S/fast region ratio). Since 28S rRNA is typically degraded before 18S and is generally coincident with increased abundance of RNA sizes in the inter region (Schroeder *et al.,* 2006), an 18S/inter region ratio was also calculated.

Although samples were within the qualitative range of the Nano (5 to 500 ng/µl) and Pico (50 to 5000 pg/µl) LabChips, visualization of characteristics was concentration dependent. For example, initial efforts to load approximately 10 ng/µl of oocyte RNA onto the Nano LabChip failed to produce an electropherogram or calculate a ratio for rRNA or RIN value. Moreover, subsequent serial dilutions of cumulus total RNA loaded onto Pico LabChips showed use of concentrations at the lower limit of the chip were sufficient only for visualizing regions corresponding to rRNA peaks, while use of concentrations in the middle to upper limits showed RNA sizes in majority of regions (i.e., the 5S, fast, 18S, inter-region, 28S, precursor and post-regions). Thus, visualization of RNA sizes in the 5S and fast regions of oocyte samples depended on loading concentrations falling within the middle to upper limits of the Pico LabChip.

Features of total RNA from bovine oocytes and associated cumulus cells

In vitro maturation (IVM) procedures were performed as previously described (Schrock *et al.,* 2007). Total RNA (concentration, RIN and ratios for rRNA, 18S/fast region, 18S/ inter region and electropherogram) from GV-stage oocytes and after 12 or 24 h of IVM from different experiments were evaluated in retrospect. Although fewer in number, total RNA samples from cumulus cells surrounding oocytes were also examined. Total RNA was quantified using

RiboGreen. To ensure electropherograms were representative of RNA, not DNA, total RNA from a subset of oocyte samples and surrounding cumulus was incubated at 37ºC for 20 min in RNase I_f (100 units; New England BioLabs, Ipswich, MA, USA) or TE buffer (10 mmol l^{-1} Tris-Cl, 1 mmol l^{-1} EDTA). Additionally, electropherograms of total RNA from oocytes with zona pellucida-intact were compared to a subset of samples from zona pellucida-free oocytes (0.5% pronase).

In an effort to address concerns regarding time required for ovary transport to the laboratory (~4.5 h) on RNA integrity, total RNA from oocytes collected immediately after ovary removal from animals at the abattoir was compared to total RNA from oocytes collected after transport of ovaries to the laboratory at ~26ºC (typical) or on ice and quantified using NanoDrop. Furthermore, electropherograms were examined after total RNA was isolated from oocyte using four different commercially available kits designed to produce highly concentrated RNA from limited sample sizes (PicoPure; Mini RNA isolation kit, Zymo Research Corporation, Orange, CA, USA; RNAqueous Micro kit, Ambion; and RNeasy Micro RNA kit, Qiagen Inc, Valencia, CA, USA) and quantified using NanoDrop.

To determine the extent to which RNA concentration influenced certain features, 8 different pools of oocyte RNA (quantified using NanoDrop) were loaded onto Pico LabChips at ~4.5 oocytes/µl (50 oocytes in 11 µl elution) or diluted to 2, 1, or 0.5 oocytes/µl and then analyzed. Because the ability to discern features of total RNA diminished as oocyte equivalents decreased, oocyte and cumulus total RNA was directly compared after loading equivalent amounts onto the Pico LabChip. To do so, total RNA from cumulus samples (n = 18 different pools) was diluted to the same concentration as oocyte samples (n = 17 different pools; 5 to 9

ng/µl as determined by NanoDrop). Finally, as an indirect means to assess mRNA quality, total RNA isolated from bovine oocytes and surrounding cumulus using PicoPure kit was analyzed using the Pico LabChip after NanoDrop quantification. Thereafter, polyadenylated RNA contained within 25 ng of total RNA was amplified using the Ovation RNA Amplification System V2 (NuGEN Technologies, Inc., San Carlos, CA, USA) as per manufacturer's instructions. Size distribution of cDNA product sizes and RIN values in oocyte and cumulus samples were then compared using Nano LabChips.

Features of total RNA from GV-stage oocytes in other mammalian species

Germinal vesicle-stage oocytes were obtained from antral follicles of feline, canine, and ovine ovaries that would have otherwise been discarded after ovariohysterectomies using procedures similar to that previously described (Lawrence *et al.,* 2004). Porcine oocytes were collected from abattoir-derived ovaries of nonpregnant sows and kept separate according to follicle size: 1) large (~ 8 to12 mm; likely preovulatory as ovarian corpora lutea appeared to be undergoing luteolysis (Edwards and Hansen 1996), 2) medium (~ 3 to 8 mm), and 3) small (< 3 mm). After isolation, RNA was quantified using RiboGreen.

Features of total RNA from in vivo *matured murine oocytes of prepubescent/superovulated or sexually mature females*

In vivo-derived MII oocytes were collected from prepubescent-superovulated or sexually mature CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA, USA) by personnel at the Columbia Reproductive Biology Laboratory (University of Missouri, Columbia, MO, USA) in accordance with Animal Care Quality Assurance. Females (3 to 4 weeks of age) were

superovulated as previously described (Nagy *et al.,* 2003). Collection of oocytes from ovarian follicles when hCG would have otherwise been administered allowed for comparison of total RNA from GV-stage versus MII (collected 12 to 13 h after hCG injection) oocytes. Since previous studies have demonstrated that superovulation procedures reduce developmental potential of oocytes (Blondin *et al.,* 1996) and that oocytes from prepubertal animals are not as developmentally-competent as those from adults (Khatir *et al.,* 1996), total RNA from oocytes of nonsuperovulated, sexually mature females (6 to 10 weeks of age; mated with vasectomized males) was also examined (oocytes obtained ~2 h post-ovulation). Oocyte and cumulus lysates were shipped overnight on dry ice and stored at -80ºC until RNA isolation. Isolated RNA was quantified using RiboGreen.

Total RNA from bovine, ovine, porcine, and human sperm

Semen was generously provided by Ultimate Genetics (Wheelock, TX, USA) and ARS-USDA National Animal Germplasm Program (Fort Collins, CO, USA), or was purchased from Fairfax Cryobank (Fairfax, VA). Frozen sperm was thawed at 34ºC for 40 s (bovine), 37ºC for 30 s (ovine), 50ºC for 20 s (porcine), or at room temperature for 20 min (human) before washing through a discontinuous Percoll gradient (bovine, ovine, and porcine: 45/90% (v/v); 2000 *g* 10 min; human: 40/80% (v/v); 1500 *g* for 20 min). Human sperm was washed twice in Dulbecco's phosphate buffered saline (500 *g* for 10 min) and once in 0.1% sodium dodecyl sulfate (v/v)/0.5% Triton-X (v/v). Approximately 100,000 sperm per sample were lysed in 100 µl extraction buffer. Isolation of RNA was as described previously except that 16 units of TURBO DNase were used. Total RNA of sperm from each of six animals for ovine (58 to 82% motile) and porcine (23 to 50% motile), four animals for bovine (78 to 96% motile), and two human

subjects (34 to 63 % motile) were examined. To ensure electropherograms were representative of RNA, not DNA, total RNA from bovine sperm was RNase treated as described previously.

Data Analyses

For each oocyte and cumulus sample, total RNA, rRNA ratio, RIN, 18S/fast region ratio, and 18S/inter region ratio were calculated. Bovine samples collected across maturation were analyzed as a completely randomized design (CRD) using PROC MIXED (SAS 9.1, SAS Institute, Inc., Cary, NC) with main effect of oocyte stage (GV-stage, 12 h or 24 h of IVM) and a covariate for number of oocytes within each sample. Murine oocyte samples were analyzed as a CRD with main effect of treatment (superovulated GV vs. MII; or nonsuperovulated-MII vs. superovulated-MII) included in the statistical model. Analysis of all oocytes compared to all cumulus samples was a randomized block design (RBD) with adjusted degrees of freedom (Kenward-Rogers method) for unbalanced data. Main effect of cell type and random effect of day of sample processing were included in the model. Sperm RNA data were analyzed as a RBD with main effect of species and a random effect of day of examination included in model. Data were presented as least squares means with standard errors of the mean. In cases where sample size was limiting, raw means with standard errors were reported.

RESULTS

Summary of experimental endpoints

Representative images of an electropherogram indicating each region and an RNA ladder (run on each Pico LabChip) were provided in Figure 3-1 for ease of interpreting results. For

each oocyte or cumulus sample, concentration or total amount of RNA, RIN and rRNA ratio was calculated. The proportion of 18S rRNA to smaller RNA sizes (i.e., those between ~200 to 1500 nt) was evaluated by calculating an 18S/fast region ratio to estimate the abundance of smaller RNA sizes in a given sample relative to 18S rRNA. Since 28S rRNA can be degraded before 18S and is typically coincident with increased abundance of RNA sizes in the inter region (Schroeder *et al.*, 2006), an 18S/inter region ratio was also calculated.

Features of total RNA from bovine oocytes and associated cumulus

Concentration or total amount of RNA, RIN, and ratios for rRNA, 18S/fast region and 18S/inter region did not change during *in vitro* maturation (IVM; Table 3-1, Comparison A), and was not influenced by ovary transport conditions (Table 3-1; Comparison C) or the presence of the zona pellucida before placing samples in extraction buffer (Table 3-1; Comparison B). Total RNA from surrounding cumulus had rRNA ratios and RIN values typical of somatic cells which were consistent across IVM (Table 3-1; Comparison A) and did not appear to be influenced by ovary transport conditions (Table 3-1; Comparison C). Interestingly however, when the features of total RNA from oocytes versus surrounding cumulus were visually compared, several differences were noted. Namely, RIN values and ratios for rRNA, 18S/fast region and 18S/inter region were numerically lower in oocytes versus cumulus when RNA was isolated using the PicoPure Isolation Kit (Table 3-1, Comparisons A & C). Differences in rRNA ratio were obvious when comparing oocyte versus cumulus electropherograms (Figure 3-2). RNase treatment resulted in fragments too small to detect in oocytes (i.e., < 25 nt; Figure 3-2, inset a) and shifted RNA sizes towards the marker in cumulus samples (< 200 to < 3000 nt; Figure 3-2, inset b).

Table 3-1. Features of total RNA in bovine oocytes and cumulus cells.

			Oocytes						Cumulus Cells			
	Comparison	**No. of RNA Pools**	**Total RNA[1]**	**rRNA Ratio**	**RIN[2]**	**18S/fast region ratio[3]**	**18S/inter region ratio[4]**	**No. of RNA Pools**	**rRNA Ratio**	**RIN[2]**	**18S/fast region ratio[3]**	**18S/inter region ratio[4]**
A†	GV-stage	10	3.20 ± 0.73	0.71 ± 0.07	4.25 ± 0.69	0.21 ± 0.10	0.63 ± 0.03	3	1.40 ± 0.22	6.73 ± 0.47	0.54 ± 0.18	2.04 ± 0.61
	12 h IVM	8	2.91 ± 0.82	0.75 ± 0.10	3.77 ± 1.03	0.19 ± 0.14	0.67 ± 0.04	5	1.30 ± 0.17	7.73 ± 0.41	0.76 ± 0.14	2.48 ± 0.70
	24 h IVM	28	2.82 ± 0.45	0.66 ± 0.08	4.14 ± 0.80	0.22 ± 0.11	0.62 ± 0.02	8	1.47 ± 0.15	7.31 ± 0.31	0.63 ± 0.12	2.47 ± 0.62
	P-value	---	0.90	0.10	0.71	0.84	0.62		0.75	0.32	0.61	0.21
B†	ZP intact	4	1.77 ± 0.06	0.71 ± 0.04	3.84 ± 0.16	0.19 ± 0.01	0.48 ± 0.01	0	---	---	---	---
	ZP removed	4	1.68 ± 0.06	0.74 ± 0.04	3.58 ± 0.16	0.17 ± 0.01	0.48 ± 0.01	0	---	---	---	---
	P-value	---	0.37	0.66	0.32	0.10	0.98					
C‡	Abattoir	3	1.11 ± 0.74	0.80 ± 0.04	4.47 ± 0.14	0.22 ± 0.02	0.67 ± 0.06	0	---	---	---	---
	4ºC	4	2.34 ± 0.64	0.88 ± 0.04	4.55 ± 0.12	0.23 ± 0.02	0.66 ± 0.05	2	1.55 ± 0.05	8.15 ± 0.05	1.01 ± 0.001	1.25 ± 0.05
	26ºC	2	2.64 ± 0.90	0.95 ± 0.05	4.65 ± 0.17	0.23 ± 0.03	0.65 ± 0.07	1	1.2	8.1	1.10	1.54
	P-value	---	0.39	0.16	0.73	0.96	0.99		---	---	---	---
D‡	PicoPure	2	3.32 ± 0.26	0.85 ± 0.05	5.0 ± 0.2	0.24 ± 0.01	0.56 ± 0.01	1	1.5	8.5	1.20	1.64
	Ambion	1	1.26	0.7	6.2	0.39	0.47	1	1.5	6.7	0.35	0.79
	Qiagen	1	10.10	0.4	5.2	0.33	0.51	1	1.5	7.1	0.41	1.26
	Zymo	1	1.55	0.9	6.2	0.42	0.69	0	---	---	---	---
E‡	GV-stage	8	3.49 ± 0.41	0.73 ± 0.10[b]	3.88 ± 0.08[b]	0.17 ± 0.02[b]	0.53 ± 0.02[b]	8	1.76 ± 0.10[a]	8.38 ± 0.08[a]	0.99 ± 0.02[a]	1.36 ± 0.02[a]
	P-value	---`	---	---	---	---	---		< 0.0001	< 0.0001	< 0.0001	< 0.0001

P-values refer to values within a comparison and column except for comparison E. [ab] Values significantly different ($P < 0.05$) within a row

A: Features of total RNA from GV-stage oocytes and those undergoing IVM for 12 or 24 h (least squares means ± SEM)

B: Total RNA was isolated from GV-stage oocytes collected at abattoir or after ovary transport to the laboratory at 4º or 26ºC (least squares means ± SEM; cumulus- raw means ± SEM)

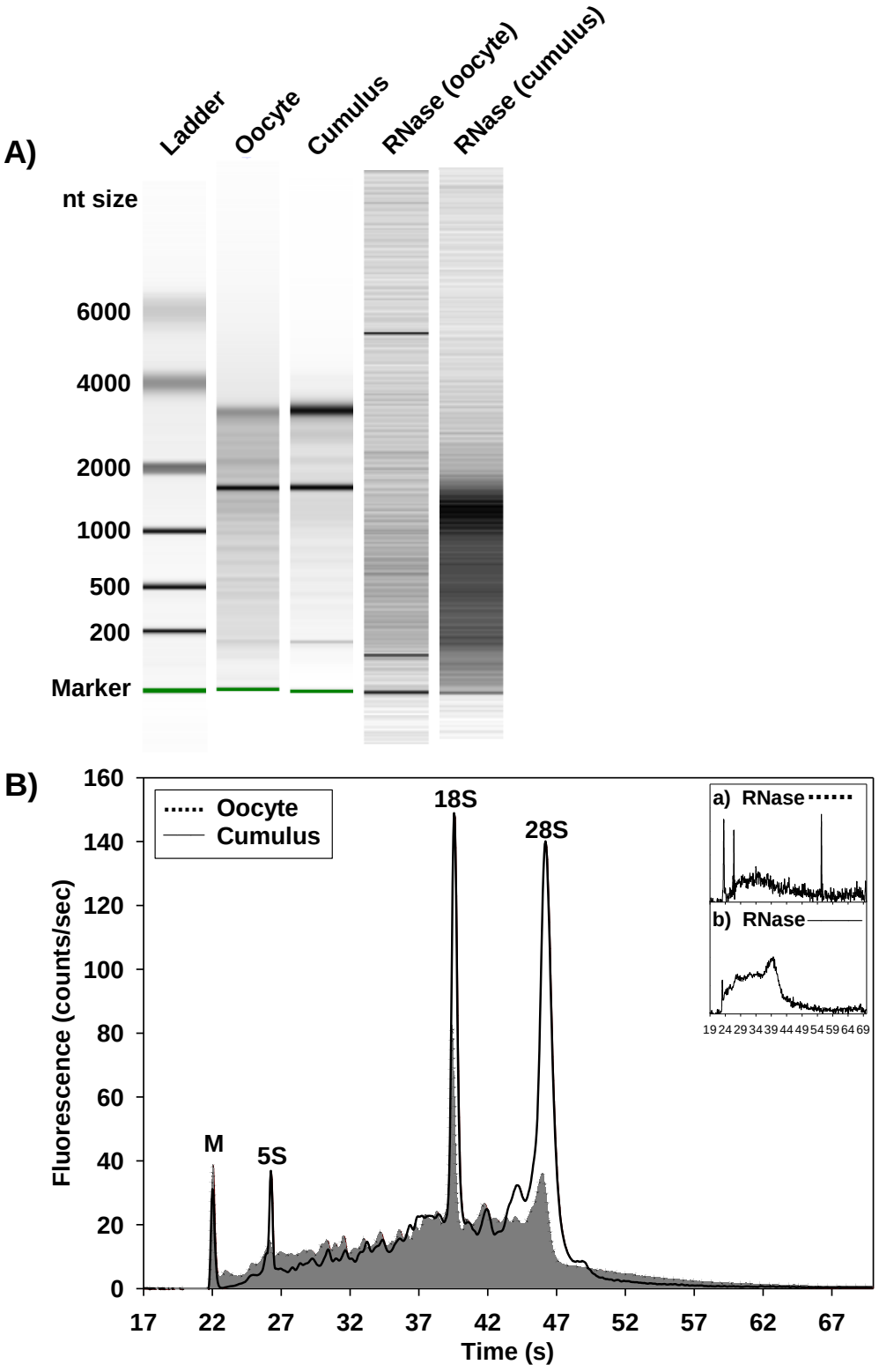
C: Least squares means ± SEM for total RNA from oocytes with or without zona pellucida (ZP)

D: Raw means ± SEM for total RNA of oocytes and cumulus isolated with different RNA isolation kits

E: Least squares means ± SEM for total RNA from GV-stage oocytes and surrounding cumulus that was subsequently amplified to determine mRNA integrity

Total RNA was quantified using Ribogreen (†) or NanoDrop (‡). [1]Total RNA = ng/oocyte; [2]RIN = RNA integrity number; [3]Area of 18S rRNA region divided by area of fast region; [4]Area of 18S rRNA region divided by area of inter region

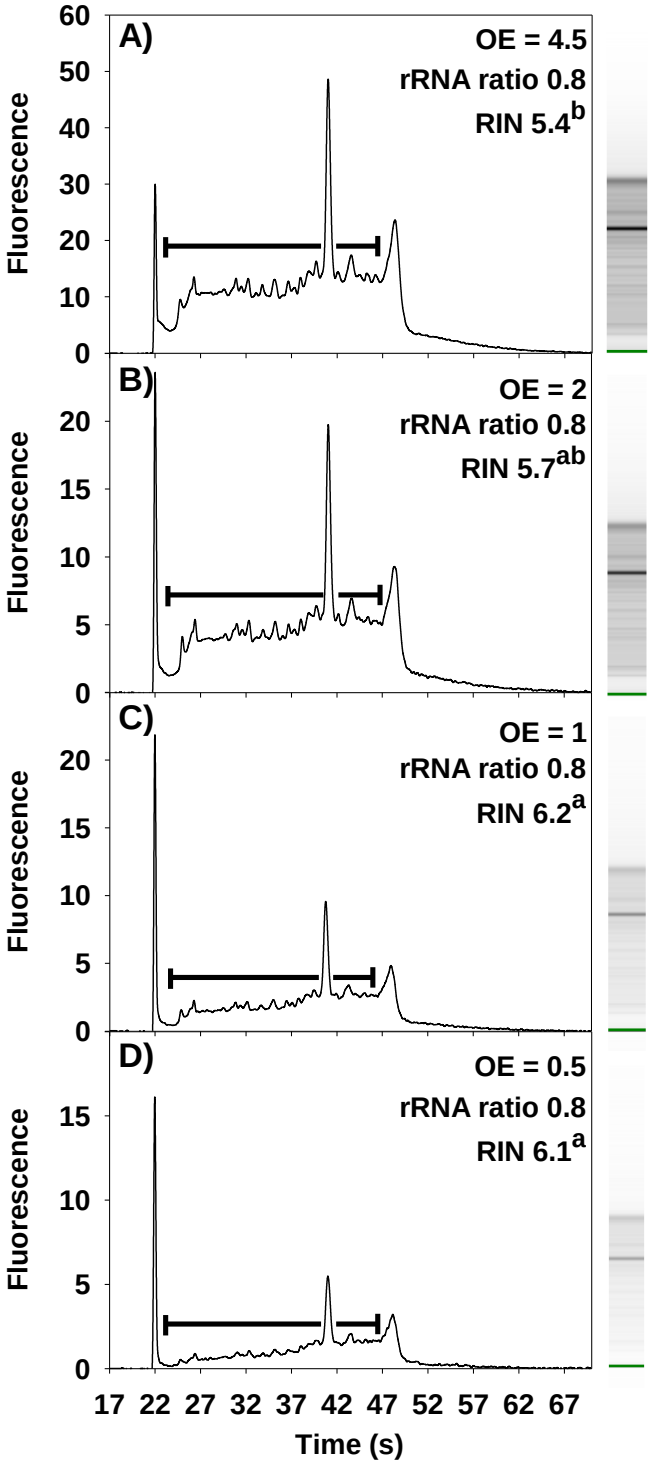
Figure 3-2. Representative gel images and electropherograms of total RNA from 24 hIVM bovine oocytes and their surrounding cumulus loaded at equivalent amounts (~5 ng). (A) Gel images for oocytes and cumulus cells before and after RNase treatment. (B) Electropherograms of oocyte (dotted line with gray shading) and cumulus (solid line) total RNA. Insets are electropherograms from RNase-digested total RNA from 24 hIVM oocytes (a) and cumulus (b). 5S represents 5S rRNA and other RNA species of similar size. M, marker.


A)
Ladder
Oocyte
Cumulus
RNase (oocyte)
RNase (cumulus)
nt size
6000
4000
2000
1000
500
200
Marker
B)
Oocyte
Cumulus
18S
28S
M
5S
a) RNase
b) RNase
19 24 29 34 39 44 49 54 59 64 69
Fluorescence (counts/sec)
Time (s)
0
20
40
60
80
100
120
140
160
17
22
27
32
37
42
47
52
57
62
67

Use of other commercially available kits reported to produce highly concentrated total RNA from limited sample sizes (i.e., Mini RNA isolation kit, Zymo Research Corporation; RNAqueous Micro kit, Ambion; or RNeasy Micro RNA kit, Qiagen Inc) produced features similar to those obtained after PicoPure isolation of oocyte total RNA (Table 3-1; Comparison D). When compared to cumulus samples, total RNA from oocytes had numerically lower RIN values and ratios for rRNA and 18S/inter region. Ability to distinguish a peak in the 5S region differed among kits (images not shown). Specifically, addition of 1.25 volumes of ethanol to oocyte lysates was coincident with a discernible peak in the 5S region (~200 nt) with fewer RNA sizes appearing in the fast region (> 200 nt to 1500 nt) when using Ambion's RNA isolation kit. Repeatability of features was also examined after total RNA was isolated with PicoPure but diluted to ~4.5, 2, 1, or 0.5 oocyte equivalents before loading onto the PicoLab Chip (Figure 3-3). Ratios for rRNA ratio (0.78, 0.76, 0.76, and 0.81 for 4.5, 2, 1, and 0.5 oocytes, respectively; SEM = 0.04; P = 0.70) and 18S/inter region (0.70, 0.70, 0.70, and 0.66 for 4.5, 2, 1, and 0.5 oocytes, respectively; SEM = 0.04; P = 0.52) were similar regardless of oocyte equivalent and consistent with other efforts reported in Table 3-1. In contrast, RIN (5.35, 5.71, 6.19, and 6.14 for 4.5, 2, 1, and 0.5 oocytes, respectively; SEM = 0.31) increased as number of oocyte equivalents decreased (1 and 0.5 oocyte equivalents versus 4.5; P = 0.01) as did the 18S/fast region ratio (0.27, 0.29, 0.42, and 0.54 for 4.5, 2, 1, and 0.5 oocytes, respectively; SEM = 0.08; P = 0.02). In other words, ability to discern abundance of smaller RNA sizes was somewhat dependent on number of oocyte equivalents (i.e., concentration) in a given sample (see Figure 3-3).

Because ability to discern features of total RNA diminished as oocyte equivalents decreased, oocyte and cumulus total RNA was directly compared after loading equivalent

Figure 3-3. Representative electropherograms of total RNA (fluorescent counts/sec) from ~4.5 (A), 2 (B), 1 (C), and 0.5 (D) bovine oocyte equivalents (OE) originating from one oocyte pool. Note the diminished ability to distinguish signal in fast and inter regions as RNA concentration decreased. Least squares means for rRNA ratio and RIN values are provided. Values with different superscripts ($^{a, b}$) were significantly different ($P < 0.05$). Gel-like images corresponding to each electropherogram are provided to the right of each panel.


A)
OE = 4.5
rRNA ratio 0.8
RIN 5.4b
B)
OE = 2
rRNA ratio 0.8
RIN 5.7ab
C)
OE = 1
rRNA ratio 0.8
RIN 6.2a
D)
OE = 0.5
rRNA ratio 0.8
RIN 6.1a
Fluorescence
Time (s)
17 22 27 32 37 42 47 52 57 62 67

amounts onto the Pico LabChip. Ratios for rRNA (0.67 vs. 1.63; SEM = 0.05), 18S/fast region (0.33 vs. 0.75; SEM = 0.08), and 18S/inter region (0.63 vs. 1.27; SEM = 0.10) as well as RIN values (5.27 vs. 7.79; SEM = 0.28) were lower for oocyte total RNA than for cumulus RNA ($P < 0.0001$).

To assess the extent to which polyadenylated RNA within oocyte versus cumulus total RNA was suitable for downstream analyses (i.e., qPCR or microarray), cDNA product sizes after amplification with the Ovation RNA Amplification System were compared. General features of total RNA from oocyte and cumulus samples are reported in Table 3-1 and differed ($P < 0.0001$; Comparison E) as per other efforts. Notably, however, distribution of amplified cDNA products was similar (Figure 3-4) with sizes centered around 1000 nt (range of 200 to ~4000 nt with majority between 200 to 2000 nt).

Features of total RNA from germinal vesicle (GV)-stage oocytes in other mammalian species

Concentration of total RNA ranged from 1.2 to 4.6 ng/oocyte (Table 3-2). The rRNA ratio for bovine oocytes was somewhat intermediate to other species with porcine and murine exhibiting the lowest ratios (Table 3-2). In general, oocyte electropherograms were similar across species (data not shown), except that those generated for canine and feline oocytes had a higher 28S rRNA peak which is consistent with higher values reported in Table 3-2. Ratios for 18S/fast region and 18S/inter region and RIN values were consistent across species when using the PicoPure RNA isolation kit (Table 3-2). However, within a species, RIN values and ratios for rRNA, 18S/fast region, and 18S/inter region were numerically lower in oocytes than in surrounding cumulus (Table 3-2).

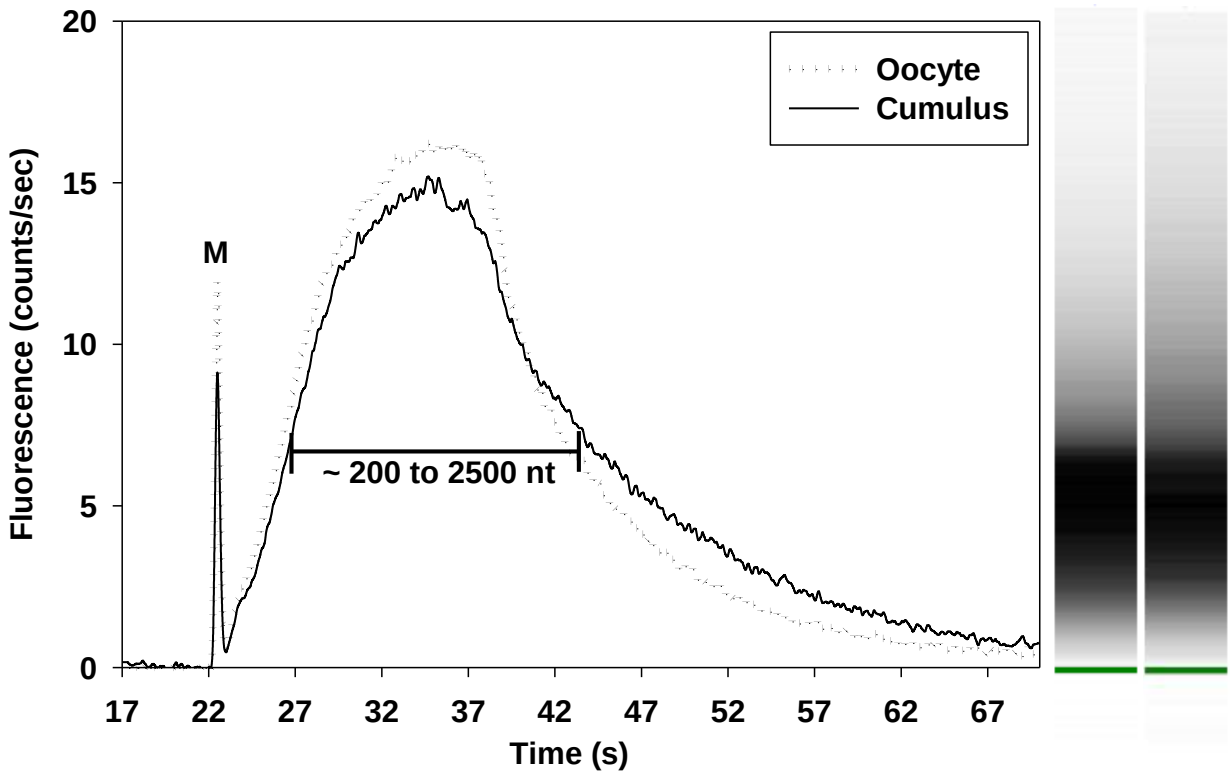


Figure 3-4. Representative electropherograms and gel images of cDNA from amplified polyadenylated RNA (Nano LabChip) originating from bovine oocytes (dotted line) and surrounding cumulus (solid line). Gel-like images (from left to right) are of oocyte and cumulus amplified cDNA.

Table 3-2. Features of total RNA* in GV-stage oocytes and surrounding cumulus.

Species	Oocytes						Cumulus			
	No. of RNA Pools	Total RNA[1] (ng/oocyte)	rRNA Ratio	RIN[2]	18S/Fast Region Ratio[3]	18S/Inter Region Ratio[4]	No. of RNA Pools	rRNA Ratio	RIN[2]	18S/Fast Region Ratio[3]	18S/Inter Region Ratio[4]
Bovine	**10**	2.56 ± 0.52	0.79 ± 0.03	4.92 ± 0.30	0.26 ± 0.04	0.63 ± 0.08	3	1.67 ± 0.07	6.73 ± 0.77	0.54 ± 0.18	1.16 ± 0.19
Ovine	**2**	4.26 ± 1.40	0.90 ± 0.20	4.90 ± 1.10	0.24 ± 0.08	0.74 ± 0.15	1	1.2	8.3	1.30	1.54
Porcine (Overall)	**16**	3.76 ± 0.43	0.51 ± 0.05	4.18 ± 0.20	0.23 ± 0.01	0.64 ± 0.05	4	1.25 ± 0.17	7.18 ± 0.45	0.39 ± 0.11	0.87 ± 0.14
Large follicles	2	3.03 ± 0.97	0.35 ± 0.15	4.10 ± 0.50	0.17 ± 0.03	0.70 ± 0.02	2	1.20 ± 0.00	7.05 ± 0.45	0.46 ± 0.16	0.98 ± 0.01
Medium follicles	9	3.76 ± 0.52	0.48 ± 0.06	3.97 ± 0.30	0.23 ± 0.02	0.55 ± 0.06	1	1.7	8.3	0.53	1.07
Small follicles	5	4.05 ± 1.05	0.64 ± 0.09	4.53 ± 0.36	0.25 ± 0.01	0.79 ± 0.04	1	0.9	6.3	0.12	0.44
Feline	**9**	2.41 ± 0.36	0.99 ± 0.04	5.26 ± 0.21	0.24 ±0.03	0.83 ± 0.13	1	1.5	7.1	0.54	1.37
Canine	**2**	3.10 ± 0.14	1.40 ± 0.10	5.80 ± 0.10	0.21	0.76	1	1.8	7.0	0.42	1.04
Murine	**3**	1.18 ± 0.29	0.63 ± 0.06	4.30 ± 0.23	0.14 ± 0.01	0.60 ± 0.08	4	1.08 ± 0.31	7.58 ± 0.57	0.83 ± 0.15	2.05 ± 0.23

*Raw means ± SEM

[1]Total RNA was quantified using NanoDrop

[2]RIN = RNA integrity number

[3]Area of 18S rRNA region divided by area of fast region

[4]Area of 18S rRNA region divided by area of inter region

Features of total RNA from in vivo *matured murine oocytes of prepubescent/superovulated or sexually mature females*

To examine features of total RNA from *in vivo* matured oocytes, a murine model was chosen. Since previous studies have demonstrated that superovulation procedures reduce developmental potential of oocytes (Blondin *et al.,* 1996) and that oocytes from prepubertal animals are not as developmentally-competent as those from adults (Khatir *et al.*, 1996), total RNA from oocytes of nonsuperovulated, sexually mature females was also examined. Total amount of RNA, RIN and ratios for rRNA, 18S/fast region and 18S/inter region did not differ between 1) GV-stage versus metaphase II (MII) oocytes from prepubescent-superovulated females ($P > 0.20$) and 2) MII-oocytes from prepubescent-superovulated females versus those from sexually mature nonsuperovulated females ($P > 0.30$; Table 3-3). Features of cumulus RNA were similar regardless of origin ($P > 0.30$), but differed from those reported for oocyte total RNA ($P < 0.05$; Table 3-3).

Overall comparison of total RNA from oocytes versus cumulus

To provide a more definitive comparison of the features of oocyte versus cumulus total RNA, data from different comparisons were combined into one large data set and statistically analyzed (n = 128 oocyte and n= 46 cumulus samples). When this was done, the rRNA ratio (0.71 vs. 1.48; SEM = 0.04), RIN value (4.75 vs. 7.68; SEM = 0.15), 18S/fast region ratio (0.24 vs. 0.75; SEM = 0.03), and 18S/inter region ratio (0.62 vs. 1.39; SEM = 0.03) were significantly lower in total RNA from oocytes versus cumulus cells ($P < 0.0001$). Lower values for the 18S/fast region ratio suggest that oocyte total RNA has an increased abundance of smaller RNA sizes relative to 18S rRNA compared to surrounding cumulus.

Table 3-3. Characteristics of total RNA from murine oocytes and surrounding cumulus.*

			Oocytes						Cumulus			
Animal	**Oocyte Stage**	**No. of RNA Pools**	**Total RNA (ng/oocyte)**	**rRNA Ratio**	**RIN[1]**	**18S/Fast Region Ratio[2]**	**18S/Inter Region Ratio[3]**	**No. of RNA Pools**	**rRNA Ratio**	**RIN[1]**	**18S/Fast Region Ratio[2]**	**18S/Inter Region Ratio[3]**
Prepubescent/ Superovulated	**GV-stage**	3	1.78 ± 0.59	0.67 ± 0.22	4.30 ± 0.43[b]	0.14 ± 0.12[b]	0.60 ± 0.16[b]	4	1.08 ± 0.19	7.58 ± 0.37[a]	0.83 ± 0.11[a]	2.05 ± 0.14[a]
Prepubescent/ Superovulated	**MII**	3	1.35 ± 0.53	0.40 ± 0.22[b]	3.53 ± 0.43[b]	0.08 ± 0.12[b]	0.42 ± 0.16[b]	3	1.37 ± 0.22[a]	7.97 ± 0.43[a]	0.90 ± 0.12[a]	1.71 ± 0.16[a]
Sexually Mature/ Nonsuperovulated	**MII**	3	2.22 ± 0.61	0.33 ± 0.22[b]	3.30 ± 0.43[b]	0.10 ± 0.12[b]	0.41 ± 0.16[b]	2	1.35 ± 0.27[a]	7.20 ± 0.53[a]	0.52 ± 0.15[a]	1.35 ± 0.20[a]

*Statistical comparisons of prepubescent/superovulated GV-stage vs. MII oocytes (row 1 vs. row 2) and prepubescent/superovulated vs. sexually mature/nonsuperovulated MII oocytes (row 2 vs. row 3) were made. In addition, oocytes and cumulus within a stage (within a row) were compared. Data are reported as least squares means ± SEM. [ab]Values significantly different ($P < 0.05$) within a row

[1]RIN = RNA integrity number

[2]Area of 18S rRNA region divided by area of fast region

[3]Area of 18S rRNA region divided by area of inter region

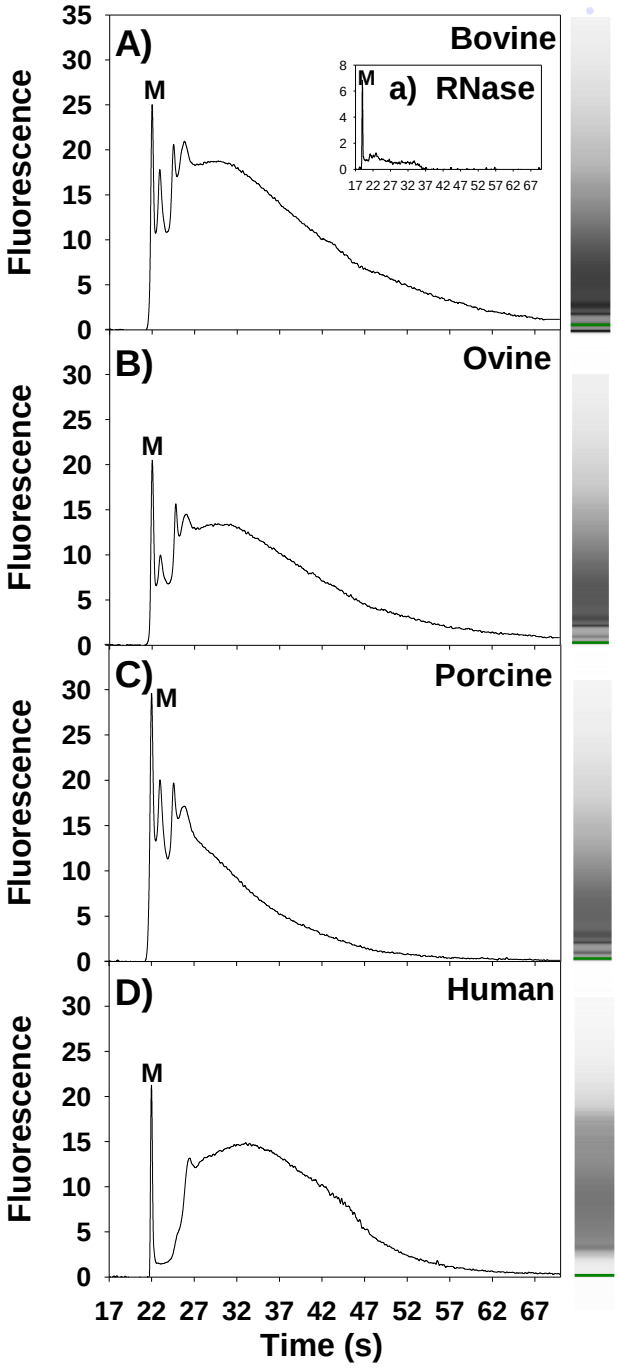
Features of total RNA from bovine, ovine, porcine, and human sperm

Notably, total RNA from sperm lacked rRNA peaks precluding calculation of rRNA ratio. RIN values did not different across species (2.28 ± 0.05 for bovine, ovine, and porcine, 2.15 ± 0.06 for human; P = 0.29). Distribution of RNA sizes in total RNA from sperm was similar between bovine and ovine with 3 distinct peaks located from the marker to immediately past the 5S-region and a broad peak extending from approximately 200 nt to > 4000 nt (Figure 3-5, panels A, B). The distribution of RNA sizes in total RNA from porcine sperm was more variable (3 peaks near the marker and generally a broad peak extending to approximately 2000 nt were noted; Figure 3-5, panel C). Size distribution in human sperm RNA was similar to that obtained from livestock species with the exception that only one distinct peak was observed near the marker followed by a broad peak extending to approximately 4000 nt (Figure 3-5, panel D). RNase treatment digested most RNA into fragments too small to be detected (Figure 3-5, panel A, inset a).

DISCUSSION

Despite numerous studies reporting use of microcapillary electrophoresis to examine RNA integrity before characterizing transcriptome profiles and specific transcripts, few reports, if any (depending on species) provided data regarding general features of total RNA (i.e., rRNA ratio, RIN, or the size distribution of RNA). Results described herein provide additional insight regarding molecular features of gamete RNA and how they compare to cumulus cell total RNA. To this end, it was shown that RIN values and ratios for rRNA, 18S/fast region, and18S/inter region were significantly lower in oocytes versus surrounding cumulus when total RNA was isolated using a column-based approach. Lower ratios for 18S/fast region and18S/inter region

Figure 3-5. Representative electropherograms of total RNA from sperm (fluorescent counts/sec). Note the lack of rRNA peaks. Inset in panel A is RNase-treated total RNA from bovine. Gel-like images corresponding to each electropherogram are provided to the right of each panel. M, marker.


A)
Bovine
M
a) RNase
B)
Ovine
C)
Porcine
D)
Human
Fluorescence
Time (s)

support the view that oocyte total RNA has an increased abundance of RNA sizes ranging from ~200 to 1500 nt relative to 18S rRNA. While actual values varied, oocyte RNA features were repeatable whether maturation occurred *in vitro* or *in vivo*, and were similar between the nuclear stages examined (i.e., GV-stage vs. MII). Furthermore, features of oocyte RNA were conserved across the six mammalian species examined (bovine, ovine, porcine, canine, feline and murine) yet differed from surrounding cumulus.

Certain features of oocyte RNA may be conserved in other species, as Kocabas et al. (2006) presented gel-like images of total RNA from *in vivo* matured human oocytes displaying a lower rRNA ratio than the "expected" (i.e., at least 1.5 for intact RNA, Schroeder *et al.*, 2006). Also, Elis et al. (2008) showed electropherograms of total RNA from the germinal disc region of chicken oocytes displaying an "atypical state" (i.e., lower rRNA ratio) between ovulation and oviposition. In that study, somatic-like features of total RNA were not noted until after embryonic genome activation (Elis *et al.*, 2008). Mindful of this, it is plausible for observed RNA features to be unique to an oocyte and of potential importance for development. Elis et al. (2008) proposed a similar hypothesis after replicating the "atypical" state of total RNA from the germinal disc region (devoid of granulosa cells) using different extraction methods.

In retrospect, differences in features of total RNA from oocytes versus cumulus may be expected given the unique role and transcriptional state of the fully-grown oocytes. For example, these oocytes are contained within growing antral follicles and are, in large part, transcriptionally inactive while cumulus cells are transcriptionally active (Tirone et al., 1997). Furthermore, oocytes contain maternal pools of RNA, protein, and energy stores that accumulated during the growth phase (Bachvarova 1974; Engel and Franke 1976; Sternlicht and Schultz 1981), a period during which features of oocyte total RNA are more like somatic cells (Bachvarova 1974; Jahn

et al., 1976; Bachvarova and De Leon 1977, 1980; Bachvarova 1981; Brower *et al.,* 1981; Brower and Schultz 1982). Thereafter, maternal stores are critical for providing sustenance not only while the "quiescent" oocyte is resident within antral follicle (GV-stage), but during maturation, after ovulation, fertilization, and up through embryonic genome activation. Despite reductions in maternal stores of polyadenylated mRNA by > 50% after resumption of meiosis (Chapter 4, De Leon *et al.,* 1983; Lequarre *et al.,* 2004), total amount of RNA does not change during maturation (this study, Lequarre *et al.,* 2004). This is likely because mRNA comprises a small portion of the total (this study, Lequarre *et al.,* 2004) thereby explaining why general features were similar regardless of nuclear stages examined in the present study.

The functional significance of a lower than "expected" rRNA ratio in oocytes is unclear but is not likely due to an active process to degrade 28S rRNA, particularly since rRNA ratio does not change during oocyte maturation and persists up through early cleavage stages after fertilization (Chapter 4, Elis *et al.,* 2008). Because the rRNA ratio is lower during a time when the oocyte or early embryo is reliant upon maternal stores (i.e., transcriptionally quiescent), it is tempting to speculate that this feature offers some level of translational control. To this end, rRNA has been hypothesized in other cell types to preferentially select certain mRNAs for translation (Mauro and Edelman 2007). The extent to which this may occur in oocytes is uncertain as de novo protein synthesis has been demonstrated in several species including murine (Hahnel *et al.,* 1986) and bovine (Edwards and Hansen 1996). Moreover, lower than expected rRNA ratios in other cell types do not necessarily impact protein synthetic capabilities. For example, ribosomes from mouse brain subjected to nucleases maintained greater than 80% function as determined by in vitro translation assays, even when all but 2% of rRNAs were at least partially degraded (Grove and Johnson 1973, 1974). In addition, rat liver ribosomes were

found to retain 30% of their original activity while having negligible amounts of intact 28S rRNA (Huvos *et al.,* 1970).

Even if lower than expected rRNA ratios in oocytes reflect some level of degradation, physiologically-relevant or not, results showing virtually identical size distribution of cDNA products after amplification of polyadenylated RNA from oocytes versus surrounding cumulus verify that the mRNA contained within is of sufficient "integrity" for downstream analyses. Elis et al. (2008) came to a similar conclusion (i.e., mRNA within total RNA having low rRNA ratios is largely intact) using gel electrophoresis to visualize products after radiolabel of reverse transcription products. Apparent lack of mRNA degradation despite reduced rRNA ratio may explain why perturbations in relative abundance of maternal transcripts as detected by microarray or qPCR (Patel et al., 2007) can correlate with functional differences in the oocyte (Lee *et al.,* 2009).

While presence is now widely accepted (Miller and Ostermeier 2006), total RNA in mature spermatozoa, unlike that from oocytes, lacked discernible 18S and 28S rRNA peaks with majority of sizes falling between 200 to 2000 nt which agrees with other reports (Ostermeier *et al.,* 2002; Grunewald *et al.,* 2005; Gilbert *et al.,* 2007; Yang *et al.,* 2009). Because mature spermatozoa are transcriptionally-inactive (Grunewald *et al.,* 2005) like fully grown oocytes, RNA may represent remnants of transcripts from spermiogenesis (Tanaka and Baba 2005) or that which may have been stored for eventual use. In this regard, sperm total RNA contains full-length polyadenylated transcripts (Gilbert *et al.,* 2007), antisense RNA (Ostermeier *et al.,* 2005), and microRNA (Ostermeier *et al.,* 2005; Amanai *et al.,* 2006). Most recent reports would support a role in sperm function and embryo development (Ostermeier *et al.,* 2004; Amanai *et al.,* 2006; Gur and Breitbart 2006, 2007, 2008).

Differences in RNA isolation procedures exist with most commercially-available kits claiming proprietary chemistries expected to result in "superior RNA recovery" (personal communication with each manufacturer's technical support). Mindful of this, certain features of total RNA from gametes or cumulus may differ or be excluded depending on isolation procedure utilized. For example, a defined 5S peak was noted after oocyte RNA was isolated with Ambion's kit but not after PicoPure use. A prominent 5S peak was noted for cumulus cell RNA regardless of isolation kit. Also, while the 18S/fast region ratio was lower in total RNA from oocytes versus surrounding cumulus after use of the PicoPure, this difference was not noted after use of the Ambion or Qiagen kit. Since data described herein were predominantly derived using a column-based procedure to isolate total RNA (i.e., reliant on RNA binding to a membrane or matrix), RNA from murine oocytes and surrounding cumulus isolated with TRIzol® reagent was also examined. Although numbers were limited and values were higher than reported herein, rRNA ratio was lower in total RNA from oocytes versus surrounding cumulus (unpublished). However, unlike data presented in this study, RIN values of total RNA from oocytes ranged from 7 to 8.3 which were more consistent with cumulus cell total RNA (7.1 to 9.0). While disparity is difficult to explain, retrospective comparison of features of total RNA from mature spermatozoa also suggest procedural-dependent differences (i.e., this study and Grunewald et al. (2005) compared with Gilbert et al. (2007, bovine) and Yang et al. (2009, porcine)). Because procedures utilized herein were not optimized for isolating sizes < 200 nt, smaller RNA sizes in oocyte versus cumulus total RNA (e.g., microRNA, piwiRNA and snoRNA) were not compared.

This study showed that certain features of total RNA from oocytes differed from those of cumulus cells. Although lower than expected rRNA ratios appear to be a ubiquitous feature of oocyte RNA, relevance of this feature to developmental competence remains unclear and

warrants further investigation. Given that an oocyte and sperm must ultimately come together to create a new individual, it is intuitive for each to have very different molecular entities from each other and from cumulus cells. Despite differences in features, RNA from both sperm (Ostermeier *et al.*, 2002; Ostermeier *et al.*, 2005; Gilbert *et al.*, 2007) and oocytes (Misirlioglu *et al.*, 2006; Mourot *et al.*, 2006) is useful for deriving meaningful data (e.g., qPCR and microarrays) that might correlate with functional differences at the protein level (Gur and Breitbart 2006; Patel *et al.*, 2007; Lee *et al.*, 2009).

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Bioanalyzer. The authors are also grateful to Ultimate Genetics, ARS-USDA National Animal Germplasm Program, and Mike Buuck at Fairfax Cryobank for provision of sperm samples.

CHAPTER 4: Effects of Direct Exposure to a Physiologically-Relevant Elevated Temperature During Meiotic Maturation on Polyadenylated RNA, RNA Sizes, and Specific Transcripts in Bovine Oocytes and Surrounding Cumulus Cells

ABSTRACT

Heat-induced reductions in developmental competence after direct exposure of oocytes to a physiologically-relevant elevated temperature (41ºC) have been coincident with reduced protein synthesis. Since heat stress perturbs RNA integrity and adenylation status in other cell types, it was hypothesized that heat stress during meiotic maturation may alter maternal stores of RNA within the oocyte and/or its surrounding cumulus cells. The objective was to examine the effect of heat stress during bovine oocyte maturation on total RNA, polyadenylated RNA, RNA size distribution, and abundance of eight specific transcripts important for meiotic maturation and heat shock response in oocytes and surrounding cumulus cells during *in vitro* maturation and in resultant 4 to 8-cell and blastocyst stage embryos after fertilization. Exposure of cumulus-oocyte complexes to 41ºC during the first 12 h of maturation did not alter amount of total RNA, polyadenylated RNA, RNA sizes, or individual transcripts per oocyte. Transcripts for HSP70 were perturbed by heat stress in cumulus. Differences in RNA from embryos were more related to embryo stage than exposure to heat stress during maturation. A subset of replicates, in which oocytes had reduced developmental potential independent of heat stress (i.e., developmentally-challenged), provided an additional model of oocyte competence to examine general characteristics inherent to competent (> 30% blastocyst after fertilization) and challenged (< 20% blastocyst) oocytes. Developmentally-challenged oocytes had reduced abundance of individual transcripts compared to competent oocytes while cumulus transcript abundance was

similar except for abundance of cyclin B1. Overall, exposure to 41ºC during meiotic maturation did not perturb oocyte and cumulus RNA characteristics examined.

INTRODUCTION

Females exposed to environmental heat stress experience infertility (Dutt 1963; Tompkins *et al.,* 1967; Dunlap and Vincent 1971; Badinga *et al.,* 1985). In cattle, this is largely due to heat-induced increases in rectal temperatures, which may meet or exceed 41ºC (Monty and Wolff 1974; Ealy *et al.,* 1993). Elevated rectal temperatures are not without consequence as they have been negatively correlated with pregnancy rates (Ulberg and Burfening 1967; Monty and Wolff 1974; Biggers *et al.,* 1987).

Effects of environmental heat stress to reduce fertility are pronounced when occurring during or near estrus, 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 period has been associated with reductions in early embryonic development (i.e., morula and blastocyst-stage embryos, Putney *et al.,* 1989a) and increases in embryonic loss occurring near the time of placental attachment (Cartmill *et al.,* 2001) after fertilization. The negative impact of hyperthermia during estrus may be attributable to indirect effects on the maternal environment (e.g., alterations in follicular development, hormonal profiles, and other factors as reviewed by Rensis and Scaramuzzi 2003) as well as to direct effects on the oocyte.

In this regard, direct exposure of oocytes to a physiologically-relevant elevated temperature of 41ºC during meiotic maturation may reduce early embryo development by 30 to 65% (Edwards and Hansen 1996, 1997; Lawrence *et al.,* 2004; Roth and Hansen 2004a, b;

Edwards *et al.,* 2005; Schrock *et al.,* 2007) in a manner similar to that observed after exposure of heifers to heat stress sufficient to elevate rectal temperatures to 41ºC (Putney *et al.,* 1989a). Furthermore, data have also demonstrated that the few embryos developing from heat-stressed oocytes are not as developmentally-competent as those from non-heat stressed oocytes (Cartmill *et al.,* 2001; Edwards *et al.,* 2009).

Examination of nuclear stage in more than 2,200 bovine oocytes after direct exposure to elevated temperature determined that reduced development of heat-stressed oocytes after fertilization is not due to an inability to undergo meiotic maturation (i.e., progress to MII, Edwards *et al.,* 2005). Rather, heat stress of oocytes accelerates nuclear and cytoplasmic maturation (Edwards *et al.,* 2005). Schrock et al. (2007) determined that heat-stressed oocytes were most competent to develop to the blastocyst-stage if fertilized 7.3 h earlier than non-stressed oocytes. Thus, addition of sperm to heat-stressed oocytes at the same time as non-stressed oocytes results in the fertilization of oocytes that have been arrested at MII longer (i.e., aged). Given that the fertile lifespan of the oocyte is finite, heat-induced acceleration of developmentally-important processes is of concern and could be responsible for some of the observed heat-induced reductions in embryo development. In support of this, aging of MII oocytes reduces developmental potential (Ward *et al.,* 2002; Agung *et al.,* 2006) similar to that observed after application of heat stress during the first 12 h of meiotic maturation.

While the mechanisms underlying heat-induced reductions in developmental competence of oocytes after fertilization remain unclear, poor embryo development has been coincident with a 30 to 50% reduction in *de novo* synthesis of intracellular proteins in bovine oocytes (Edwards and Hansen 1996). Similar consequences have been reported for other species. Specifically, exposure of murine oocytes to 43ºC immediately after removal from ovarian follicles [i.e., at

germinal vesicle (GV)-stage] reduced *de novo* protein synthesis by 30 to 80%, depending upon duration of heat stress (Curci *et al.,* 1987). Heat stress of matured [i.e., metaphase II(MII)] murine oocytes at 43ºC for 1 h resulted in an approximately 85% reduction in *de novo* protein synthesis (Hahnel *et al.,* 1986). Any perturbation in protein synthesis during meiotic maturation would be problematic as translation is required for GV breakdown (GVBD), chromatin condensation, and progression to MII (Sirard *et al.,* 1989).

While the mechanisms responsible for heat-induced perturbations in protein synthesis within oocytes are not currently known, data from other cell types suggest alterations in translational machinery (reviewed by Clemens 2001; Spriggs *et al.,* 2008) or RNA (reviewed by Voellmy 1994; Bond 2006). For example, heat stress of somatic cells perturbed the phosphorylation status of translation initiation factors (Duncan and Hershey 1989), altered functionality of internal ribosomal entry sites located on transcripts (Kim and Jang 2002), and reduced the proportion of ribosomes actively engaged in protein synthesis (Duncan and Hershey 1989). Furthermore, heat stress of Drosophila cells altered RNA stability (Yost *et al.,* 1990) and adenylation status (Dellavalle *et al.,* 1994). Heat-induced perturbation of transcript adenylation has also been reported for avian and mammalian somatic cells (Mezquita *et al.,* 1998). Transcript alterations after heat stress are concerning as oocytes are transcriptionally-quiescent after GVBD (Rodman and Bachvarova 1976; Fair *et al.,* 1997; Lodde *et al.,* 2008) and, thus, must rely upon pools of maternal RNA previously accumulated during oocyte growth for protein synthesis (Wassarman and Letourneau 1976) during oocyte maturation, fertilization, and up to embryonic genome activation (8 to 16-cell stage in bovine, Barnes and First 1991).

Given the above, the overall objective of this series of experiments was to examine RNA populations in heat-stressed and non-stressed oocytes. In the first study, total RNA and poly(A)

RNA were examined at 12 and 24 h of in vitro maturation (hIVM). A second study examined poly(A) RNA in oocytes at 12, 16, 20, and 24 hIVM. The third study evaluated the impact of heat stress on maturing oocytes to alter the size distribution of RNA molecules contained within total RNA, individual transcripts in oocytes and surrounding cumulus, and the potential for consequences, if any, to carry over to embryos derived from heat-stressed oocytes. Specific transcripts were chosen based upon their importance in oocyte maturation [cyclin B1 (Draetta *et al.*, 1989; Labbe *et al.*, 1989); growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, Eppig 2001; McNatty *et al.*, 2005); poly(A) polymerase (PAP, Raabe *et al.*, 1991)] and/or response to heat stress [heat shock protein 70 (HSP70, reviewed by Hartl 1996)].

MATERIALS AND METHODS

Oocyte collection and selection criteria

Bovine ovaries were purchased from a commercial abattoir (Browns Packing Company; Gaffney, SC, USA). Oocytes were collected as previously described (Lawrence *et al.*, 2004). Only cumulus-oocyte complexes (COC) 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 COC cultured together within one well of a 4-well Nunclon culture dish (Fisher Scientific; Pittsburgh, PA, USA).

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

After removal from antral follicles, COC were randomly allotted to one of six treatments as indicated in Figure 4-1. A subset of GV-stage oocytes (Figure 4-1, treatment 1) was immediately denuded of associated cumulus by vortexing [4.5 min in HEPES-PVA (HEPES-TL (Parrish et al., 1988) supplemented with 0.3% polyvinyl alcohol (Sigma-Aldrich; St. Louis, MO, USA), 0.20 mM sodium pyruvate (Sigma), and 0.5X penicillin/streptomycin (Millipore; Billerica, MA, USA)]. The number of oocytes recovered and were visibly lysed was recorded. Each oocyte was manipulated individually under a stereomicroscope to ensure complete removal of cumulus before lysing as pools of 50 in 100 µl lysis buffer (Stratagene; La Jolla, CA USA) and storing at -80ºC. Two additional groups were matured for 12 h at either 38.5º or 41.0ºC (Figure 4-1, treatments 2 and 3, respectively) before denuding [4.5 min vortex in HEPES-PVA with 0.3% hyaluronidase (Sigma)] and lysis as for GV-stage oocytes. Remaining COC were matured at 38.5ºC for 24 hIVM (Figure 4-1, treatment 4), 41.0ºC for 12 hIVM followed by 38.5ºC for remaining 12 hIVM (treatment 5), or 41.0ºC for 24 hIVM (treatment 6; positive control to assess effects of extreme heat stress). At 24 hIVM, COC were denuded and processed as at 12 hIVM. This design allowed for examination of effects of heat stress as well as changes occurring during meiotic maturation.

RNA was isolated from frozen lysates using the Absolutely RNA Microprep kit (Stratagene) as per manufacturer's instructions. Elution volume was 30 µl. Total RNA was assessed using the RiboGreen RNA-Specific Quantitation kit with DNase I (Molecular Probes; Eugene, OR, USA) with a Synergy HT microplate reader (BioTek Instruments, Inc.; Winooski, VT, USA) using an eight-point low-range assay (0-100 ng/ml; interassay CV of 8.7%). Amount

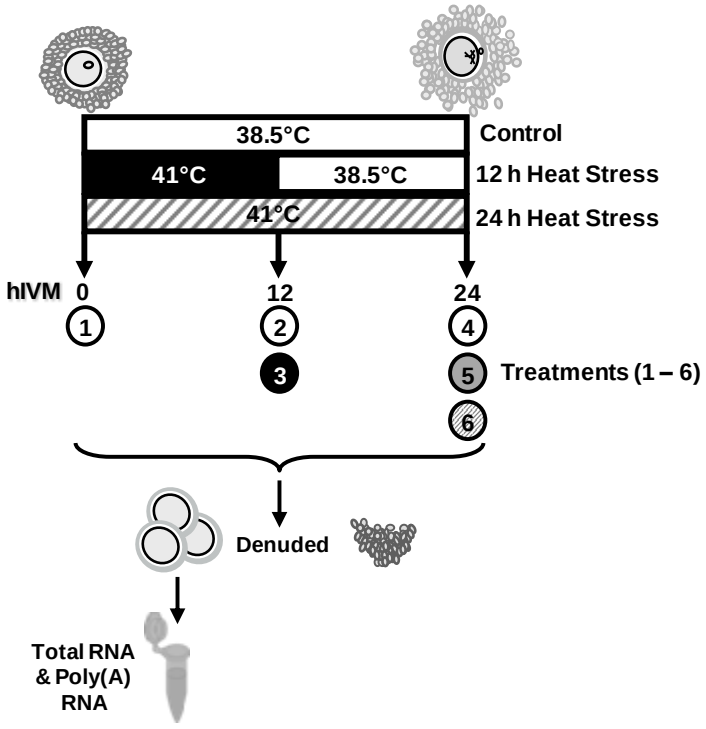


Figure 4-1. Schematic of design for experiment 1. Cumulus-oocyte complexes were randomly allotted to culture at 38.5ºC for 0 (GV-stage), 12 or 24 hIVM (treatments 1, 2, and 4; indicated by white circles). Additional subsets of oocytes were matured at 41.0ºC for 12 h (treatment 3; indicated by black circle) or cultured for another 12 h at 38.5ºC (treatment 5; indicated by gray circle). An additional subset of oocytes was cultured at 41.0ºC for the entire 24 hIVM (treatment 6; indicated by hatched circle). All oocytes were denuded of cumulus at indicated time and stored in lysis buffer at -80ºC until RNA extraction and analysis of total RNA and poly(A) RNA.

of poly(A) RNA was quantified using the Poly(A) mRNA Detection System (Promega Corporation; Madison, WI, USA) as per manufacturer's instructions using a six-point standard curve ranging from 0 to 160 pg/µl (interassay CV of 6.9%). Depending on experimental replicate, each sample was assayed in either duplicate or triplicate using a total of 10 µl of RNA. Results for total RNA and poly(A) RNA were expressed on a per oocyte basis. In addition, the proportion of total RNA comprised of poly(A) RNA was calculated. Experiment was replicated on 4 different occasions between December and January and included a total of 12 pools (n = 50 oocytes/pool) per each treatment (n = 600 oocytes/treatment) for a total of 3,600 oocytes.

Experiment 2: Abundance of poly(A) RNA in control and heat-stressed oocytes at GV-stage, 12, 16, 20, and 24 hIVM

Cumulus-oocyte complexes were randomly assigned to a total of 9 treatments involving culture at 38.5 or 41.0ºC as indicated in Figure 4-2. A group of GV-stage COC (Figure 4-2, treatment 1; immediately after removal from ovarian follicles) was denuded of surrounding cumulus cells (4.5 min vortex in HEPES-PVA) and examined individually to ensure complete cumulus removal. Cumulus-free GV-stage oocytes were frozen at -80ºC as pools of 60 in lysis buffer (Stratagene). Control groups were placed in culture at 38.5ºC for 12 (Figure 4-2, treatment 2), 16 (treatment 4), 20 (treatment 6), or 24 hIVM (treatment 8). Remaining COC were heat-stressed at 41ºC for the first 12 hIVM and denuded (4.5 min vortex in HEPES-PVA with 0.3% hyaluronidase; Figure 4-2, treatment 3) or placed at 38.5ºC until 16 (treatment 5), 20 (treatment 7), or 24 hIVM (treatment 9). Pools of cumulus-free oocytes (n = 60/pool) were frozen at -80ºC in lysis buffer.

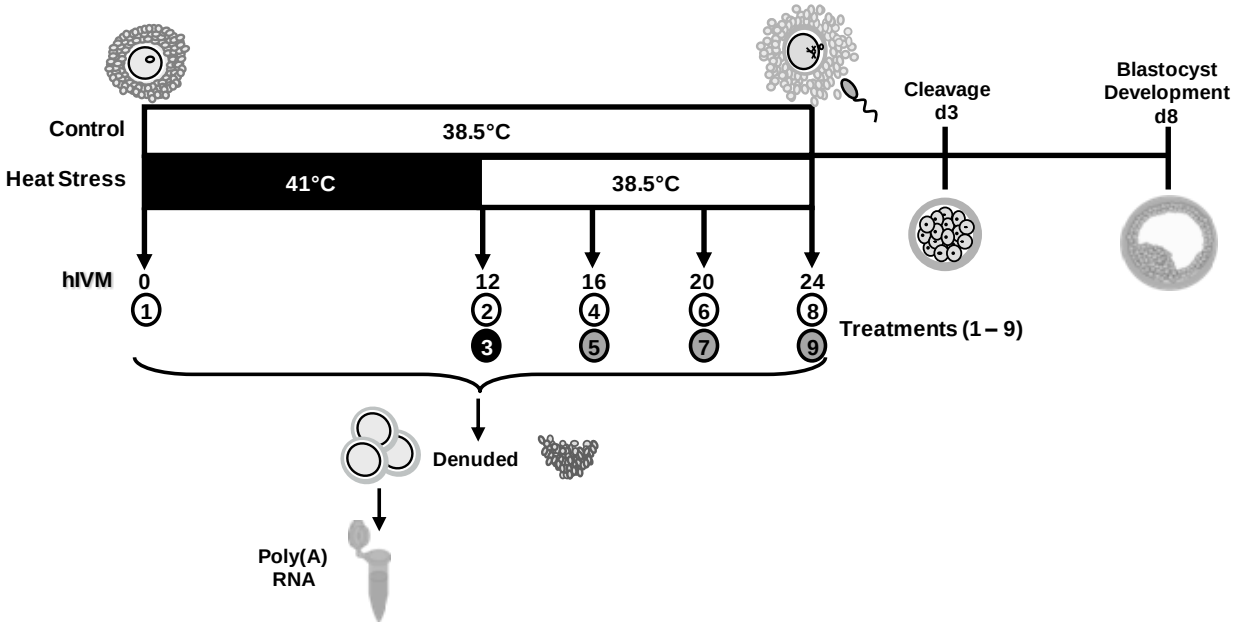


Figure 4-2. Schematic of design for experiment 2. Cumulus-oocyte complexes (COC) were collected and randomly allotted to one of 9 treatments. Soon after removal from ovarian follicles, GV-stage oocytes (treatment 1) were denuded of surrounding cumulus and frozen in lysis buffer at -80ºC. Additional COC were culture at 38.5ºC (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 41.0ºC for the first 12 hIVM and denuded immediately (treatment 3; indicated by black circle) or cultured at 38.5ºC 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 -80ºC until RNA extraction and analysis of poly(A) RNA. For 5 of the 13 replicates, a subset underwent fertilization at 24 hIVM; cleavage and blastocyst development were assessed on days 3 and 8, respectively.

Total RNA, isolated using Absolutely RNA Microprep kit (Stratagene) as per manufacturer's instructions, was eluted in 30 µl. Poly(A) RNA quantified using the Poly(A) mRNA Detection System (Promega) as per manufacturer's instructions using a six-point standard curve ranging from 0 to 160 pg/µl (interassay CV of 7.8%). In 6 replicates, RNA from GV-stage oocytes was above the highest standard (160 pg/µl) despite subsequent inclusion of a higher standard (200 pg/µl). Therefore, the highest point on the standard curve (160 or 200 pg/µl) within a given assay was used as the value for those 6 samples.

On five different occasions, subsets of control and heat-stressed oocytes underwent IVF and were allowed to develop to the blastocyst stage as previously described (Edwards et al., 2005, 2009; Schrock et al., 2007) to document the extent to which heat stress reduced developmental potential. Frozen semen was generously provided by Ultimate Genetics (Wheelock, TX, USA) and Harrogate Genetics International (Harrogate, TN, USA). Experiment was replicated on 13 different occasions (n = 60 oocytes/pool; 780 total per treatment) between January and June for a total of 7,020 oocytes.

Experiment 3: Size distribution of RNA molecules and relative abundance of developmentally-important transcripts in control and heat-stressed oocytes and their surrounding cumulus cells

Immediately after removal from ovarian follicles (GV-stage) or after in vitro maturation at 38.5 or 41.0ºC for 12 or 24 h, subsets of oocytes were denuded of cumulus by vortexing (4.5 min in HEPES-PVA with 0.3% hyaluronidase), lysed in groups of 50 in 25 µl RNA extraction buffer (MDS Analytical Technologies/Molecular Devices; Sunnyvale, CA, USA), and incubated at 42ºC for 30 min as per manufacturer's instructions before storage at -80ºC until further analysis (Figure 4-3, treatments 1 through 5). Cumulus cells from each group of 50 oocytes were

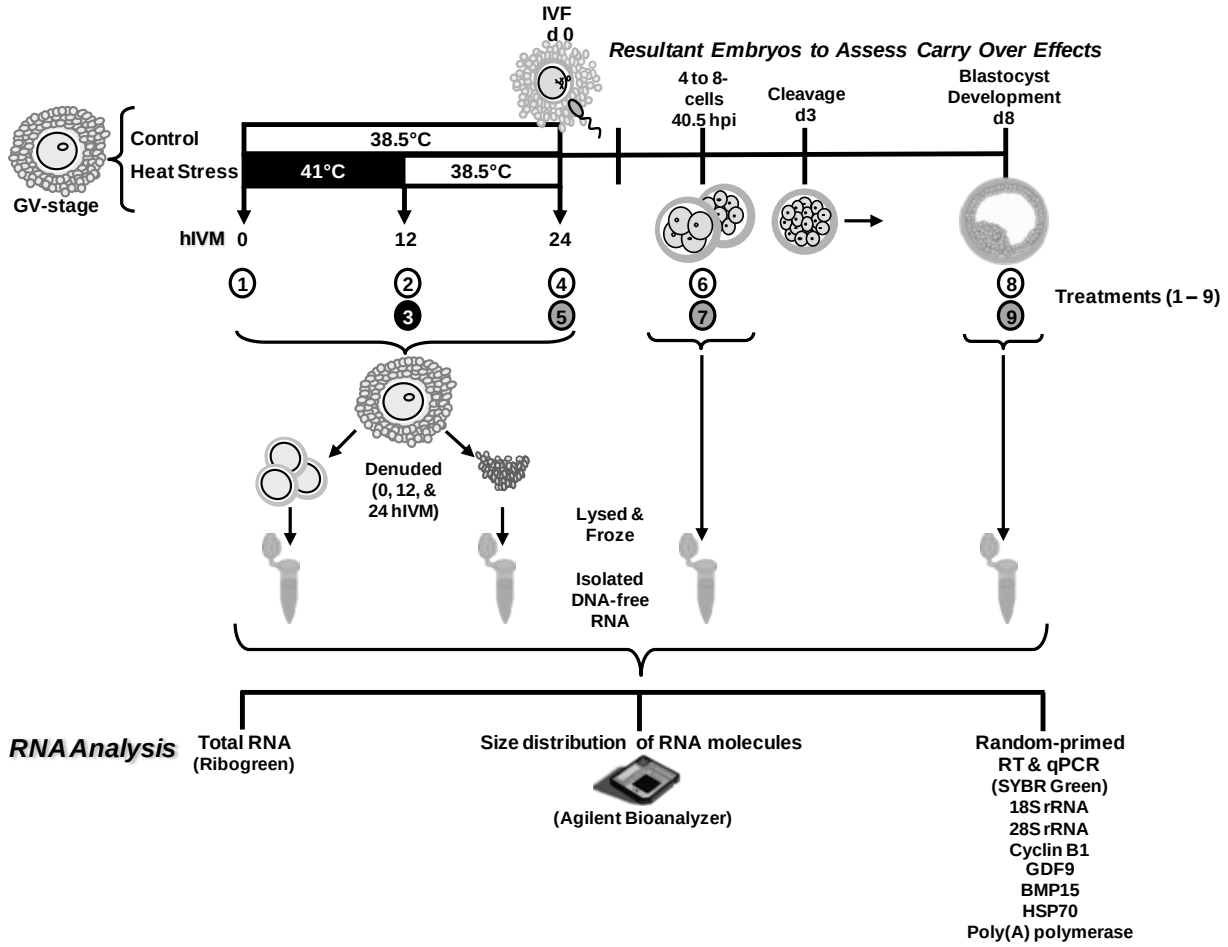


Figure 4-3. Schematic of design for experiment 3. Cumulus-oocyte complexes were randomly allotted to culture at 38.5 for 0 (GV-stage), 12 or 24 hIVM (indicated by white circles). Others were matured at 41.0ºC for 12 h and removed for analysis (indicated by black circle) or cultured for another 12 h at 38.5ºC (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 -80ºC until RNA extraction and analysis of total RNA, RNA integrity, and individual transcripts. Remaining were subjected to IVF and denuded 18-20 hours 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.

kept separate according to treatment origin, washed in HEPES-PVA, and pelleted for lysis in 200 µl extraction buffer as per Figure 4-3. Additional subsets of COC underwent *in vitro* fertilization at 24 hIVM, were denuded of associated cumulus and spermatozoa at 18-20 h post-insemination (hpi), and then placed in KSOM as previously described (Edwards et al., 2005, 2009; Schrock et al., 2007). At approximately 40.5 hpi, a subset of 4 and 8-cell embryos from control and heat-stressed oocytes (n = 34 to 117/pool/replicate, mean = 58.1) were removed from culture and lysed in 25 µl extraction buffer (Figure 4-3, treatments 6 and 7). Cleavage and blastocyst development were assessed in other subsets at 44 to 77 hpi and 166.5 to 198.25 hpi, respectively, to determine developmental potential and to assess extent to which the impact of heat stress on the oocyte during meiotic maturation may carry over to later stage embryos. Blastocysts were assigned stage and quality scores as per Schrock et al. (2007) according to guidelines set by the International Embryo Transfer Society (Robertson and Nelson 1998) before lysis in 25 µl extraction buffer (n = 3 to 35/pool/replicate, mean = 12.6).

RNA was extracted using PicoPure RNA isolation kit (MDS Analytical Technologies/Molecular Devices) as per manufacturer instructions and was DNase-treated on the column using TURBO DNase [4 (oocytes and embryos) to 16 units (cumulus), Applied Biosystems/Ambion, Austin, TX, USA] before elution. DNA-free RNA was eluted in an 11 or 20 µl volume, for oocytes/embryos and cumulus, respectively. Total RNA was quantified using Quant-iT RiboGreen RNA Reagent and kit (Invitrogen Corporation, Carlsbad, CA, USA). Samples (1 µl total RNA) were diluted 200X and assayed in triplicate in 50 µl volume on a TBS-380 fluorometer (Turner BioSystems, Inc., Sunnyvale, CA, USA) and the average used to compute total RNA per oocyte from simple linear regression of a standard curve (1 to 400 ng/ml; interassay CV= 9.1% and 2.5% for 2000- and 200-fold dilution assays, respectively).

Size distribution of RNA molecules within isolates was assessed by microcapillary electrophoretic separation and laser-induced fluorescence using RNA 6000 Pico Lab Chips analyzed on a 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA) as per Chapter 3. For each sample (1 µl of eluted RNA), rRNA ratio and RNA integrity number [RIN; algorithm-based value ranging from 1 (completely degraded) to 10 (intact) taking into account entire electropherogram] were calculated. In addition, the proportion of 18S rRNA to smaller RNA sizes (i.e., those in the fast region between ~200 to 1500 nt) was evaluated by calculating an 18S/fast region ratio after smear analysis within Bioanalyzer software. This was done to estimate the abundance of smaller RNA sizes in a given sample relative to 18S rRNA. An 18S/inter region ratio was also calculated as 28S rRNA may be degraded before 18S and is typically coincident with increased abundance of RNA sizes in the inter region (i.e, RNA between the 18S and 28S peaks, Schroeder et al., 2006).

Quantitative PCR analysis (qPCR) was utilized to assess relative abundance of individual transcripts important for meiotic maturation and heat stress response (Table 4-1). Primers were designed using Primer Express (Applied Biosystems, Inc.; Foster City, CA, USA) or FastPCR (28S and HSP70 only; version 4.0.27; Institute of Biotechnology; University of Helsinki, Finland) from bovine sequences in GenBank. Prior to reverse transcription, oocyte and embryo RNA samples were spiked with 12 pg green fluorescent protein (GFP) RNA per oocyte or embryo as an exogenous control for normalization of transcript abundance. For cumulus, GFP RNA was added at 500 pg per 100 ng total RNA. Green fluorescent protein construct with T7 promoter was designed by Laura Good-Abercrombie and generously provided by Dr. Neal Stewart. Plasmid DNA was linearized with EcoR1 (Fisher) and in vitro transcribed (4 h at 37ºC) using the MEGAscript kit (Ambion). Template DNA was removed (2 units TURBO DNase; 15

Table 4-1. 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)
18S rRNA	AY779625	AAGACGGACCAGAGCGAAAG	GGTCGGAACTACGACGGTATCT	100
28S rRNA	AY639443.1	TCTGGTGGAGGTCCGTAGCGGT	TGCGTTTGCGAGAGCGCCAGCT	139
BMP15	NM001031752	TGGAACATGTTGGGCAAAAG	AACTCACGAACCTCACTACCTCTTG	100
Cyclin B1	L26548	TCAGATTACTGCAGGAGACCATGT	ACTCCAACCAGCTGCAGCAT	100
GDF9	NM_174681	TTGCCTGGCTCTGTTTTCCT	CAAGTCTCAGCCTCAGATTCCA	100
HSP70[a]	NM203322	ACGTCGTTGATCCTGTGGGCCGT	ACCTTGCCGTGCTGGAACACC	147
PAP	X63436	AGCAGCCTCGACTTGTCTATGG	GAGAGCTGCCAGAACTGTTCAA	100

[a]Primers specific for bovine HSP70-1 (HSPA1A) and HSP70-2 (HSPA1B) located in tandem on chromosome 23 (Gallagher *et al.*, 1993)

min at 37ºC) and RNA purified and precipitated using MEGAclear kit (Ambion). Pelleted GFP RNA was resuspended in Tris-EDTA (pH 7.4), quantified with Ribogreen, and stored at -80ºC. RNA integrity was checked using a Nano LabChip analysis (Agilent) and product produced a single peak of expected size (~872 nt product). For qPCR, a 102 bp product was amplified using the following primers: 5'-CAACTTCAAGACCCGCCACA-3' (forward) and 5'-TCTGGTAAAAGGACAGGGCCA-3'(reverse). Reverse transcription was performed with 500 ng random hexamers (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 (37ºC for 1 h, 94ºC for 10 min) were cooled to 4ºC before storage at -20ºC. 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, products were sequenced using an Applied Biosystems 3730 DNA Analyzer at the University of Tennessee Molecular Biology Resource Facility. 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 Lonergan *et al.,* 2003; Donnison and Pfeffer 2004; Bettegowda *et al.,* 2006; Vigneault *et al.,* 2007; Racedo *et al.,* 2008) 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 60ºC or 63ºC (28S and HSP70) for 30 sec and 72ºC for 30 sec followed by a dissociation curve. Samples were calibrated to GV-stage oocytes or cumulus from GV-stage oocytes and analyzed using the ΔΔCt method.

Experiment 3 was replicated on 23 different occasions between January and May and October to December [1,150 oocytes per each of 5 treatments (total of 5,750 oocytes), 1,177 to 1,233 4 to 8-cells per treatment (total of 1,401 4 to 8-cells), and 290 to 303 blastocysts per treatment (total of 593 blastocysts)].

Statistical Analyses

For each study, all results are presented as least squares means (LSM) ± standard error of the mean (SEM) unless otherwise indicated. Data obtained in the first study were analyzed as a randomized complete block with main effect of treatment and random effects of date of oocyte collection and replicate within day of collection using PROC GLIMMIX of SAS (SAS 9.1, SAS Institute, Inc., Cary, NC, USA) with a normal distribution. Total RNA, poly(A) RNA, and the proportion of total RNA comprised of poly(A) RNA were calculated. To assess the effect of maturation time on RNA pools, contrasts were run comparing GV-stage to 12 and 24 hIVM at 38.5ºC. The effect of culturing oocytes at an elevated temperature of 41.0ºC for the first 12 hIVM on RNA pools was determined with *a priori* contrasts to assess effects of culture time (12 or 24 hIVM), culture temperature (38.5º or 41ºC), and the interaction. Mean separation for all analyses was by Fisher's protected (F-protected) least significant difference test and was in agreement with separation by contrasts. Data are reported as LSM ± standard error of the difference.

Data from the second study were analyzed as a randomized complete block using PROC GLIMMIX (SAS) 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, the effect of HS on poly(A) at each time point, and the interaction of maturation time

and temperature. Replicates with corresponding development data were also analyzed separately to assess effects of poly(A) RNA levels during oocyte maturation on subsequent ability to develop into blastocyst stage embryos. Mean separation for all analyses was by F-protected least significant difference test and was in agreement with separation by contrasts.

For the third study, embryo developmental data were analyzed as a randomized complete block with main effect of temperature and random effect of replicate using PROC GLIMMIX (SAS) 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 an incomplete block (4 to 8-cell embryos not present in every replicate) with main effect of treatment, random effect of replicate, and covariate for amount of RNA per qPCR reaction as an additional normalize (since samples were loaded on a per oocyte or embryo basis). Cumulus RNA data were analyzed as a randomized block design with main effect of treatment and random effect of replicate using PROC GLIMMIX with a normal distribution (SAS). Experimental endpoints included total RNA (amount per oocyte or embryo or ng/µl for cumulus), rRNA ratio, RIN, 18S/fast region ratio, 18S/inter region ratio, and relative abundance of individual transcripts. Although mean separation for all analyses was by F-protected least significant difference test, a factorial treatment design with main effects of hIVM (12 or 24 hIVM) and temperature (38.5 or 41.0ºC), and the effect of maturation time (GV-stage vs. 12 and 24 hIVM) were of interest. These were assessed by contrasts and were in agreement with mean separation. To further examine potential changes in RNA sizes during meiotic maturation, electropherograms of GV-stage and 24 hIVM oocytes (matured at 38.5ºC) from developmentally-competent pools were divided into 3 second segments (i.e., 17 to 20 sec, 20.05

to 23 sec, etc. up to 69.95 sec). Peaks for 18S and 28S rRNA were each counted as one segment. The average fluorescent intensity within each block was compared for GV-stage versus matured oocytes using PROC GLIMMIX with a normal distribution (SAS) with main effects of oocyte stage and time segment, covariate for RNA concentration, and random effect of replicate.

To determine if heat stress during maturation altered the expression pattern of a transcript across development (i.e., GV-stage up to blastocyst-stage embryo), qPCR data for replicates with heat-induced reductions in blastocyst development of ≥ 20% were analyzed using multisource nonlinear mixed model regression (PROC NLMIXED; SAS). Quadratic (18S and 28S only; y = (int_C + dummy * int_HS) + (b_C + dummy * b_HS) * hour + (b_Cq + dummy * b_HSq) * $hour^2$ + block) and exponential decay (all other transcripts; y = (int_C + dummy * int_HS) + alpha * exp ((b_C + dummy * b_HS) * hour) + block) equations were used to fit transcript abundance curves for control and heat stress treatments (y = relative transcript abundance, int_C = intercept of control/38.5ºC, dummy = heat stress/41ºC treatment, int_HS = intercept of heat stress, alpha = overall intercept, exp = exponential, b_C = linear slope of control, b_HS = linear slope of heat stress, b_Cq = quadratic slope of control, b_HSq = quadratic slope of heat stress, hour = oocyte or embryo stage in hours post collection, and block = replicate).

Differences in ability of oocytes to become blastocyst-stage embryos for unknown reasons independent of heat stress allowed for comparison of amount of total RNA, RNA sizes, rRNA ratios, RIN values, and relative abundance of individual transcripts for developmentally-competent (blastocyst development > 30%) and –challenged (blastocyst development < 20%) GV-stage oocytes as an additional model of competence. After ranking each of the 23 replicates by blastocyst development in those oocytes matured at 38.5ºC, the 6 replicates with highest

blastocyst rates were compared to the 6 replicates with the lowest blastocyst development. Data were analyzed as a completely randomized design (CRD) with main effect of competence (developmentally-competent or –challenged). For qPCR data, developmentally-competent and -challenged replicates were paired with the developmentally–competent replicate used as the calibrator for the developmentally-challenged replicate, analyzed as a CRD using PROC GLIMMIX with a normal distribution, and log-transformed as necessary for normality. Nonlinear regression was performed as indicated above without blocking.

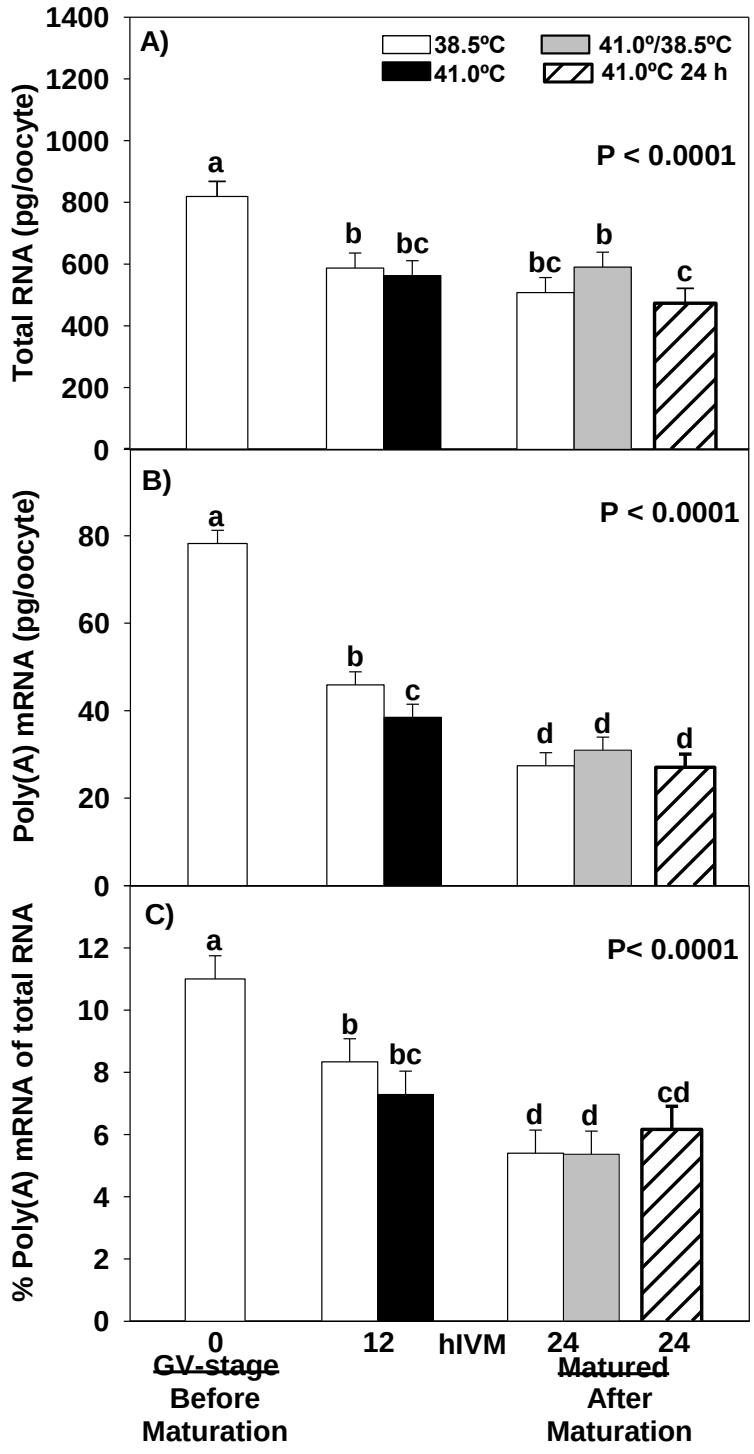
RESULTS

Abundance of total RNA and poly(A) RNA in GV-stage or control and heat-stressed oocytes at 12 and 24 hIVM

Recovery (96.4 to 99.1%; SEM = 0.7%; P = 0.19) and lysis (1.3 to 2.0 ; SEM = 0.6%; P = 0.88) of oocytes after cumulus denudement were similar regardless of hIVM (GV-stage or after 12 or 24 hIVM) or IVM temperature (38.5ºC or 41.0ºC). Total RNA per oocyte differed depending on duration of maturation (hIVM; P < 0.0001). Specifically, total RNA per oocyte was higher at GV-stage than at 12 hIVM (P < 0.0001) but did not change during the remainder of maturation (P = 0.11 for 12 hIVM versus 24 hIVM; Figure 4-4, panel A). Amount of total RNA per oocyte was not altered by maturing oocytes at 41ºC for 12 or 24 hIVM (Figure 4-4, panel A).

Abundance of poly(A) RNA also differed depending on when oocytes were examined during meiotic maturation (P < 0.0001; Figure 4-4, panel B). Levels were highest in GV-stage oocytes, lower by 12 hIVM (P < 0.0001), and lowest at 24 hIVM (P < 0.0001; Figure 4-4, panel B). Exposure of oocytes to 41.0ºC for the first 12 hIVM reduced poly(A) RNA per oocyte at 12

Figure 4-4. Total and poly(A) RNA in oocytes matured at 38.5º or 41.0ºC for 0 (GV-stage oocytes), 12 or 24 hIVM. A) Total RNA in pg per oocyte. B) Poly(A) RNA in pg per oocyte. C) Proportion of total RNA comprised of poly(A) RNA. Least squares means are reported ± the standard error of the difference between the means. Treatment differences indicated by letter designation at significance level indicated for each panel. White bars indicate culture at 38.5ºC for 0, 12, or 24 hIVM. Black bar indicates heat stress for 12 h. Gray bar indicates heat-stress for 12 h and culture at 38.5ºC thereafter. Hatched bar indicates heat stress for the entire 24 hIVM.


A)
38.5ºC
41.0ºC
41.0º/38.5ºC
41.0ºC 24 h
P < 0.0001
Total RNA (pg/oocyte)
1400
1200
1000
800
600
400
200
0
a
b
bc
bc
b
c
B)
P < 0.0001
Poly(A) mRNA (pg/oocyte)
80
60
40
20
0
a
b
c
d
d
d
C)
P< 0.0001
% Poly(A) mRNA of total RNA
12
10
8
6
4
2
0
a
b
bc
d
d
cd
0
12
hIVM
24
24
GV-stage
Before
Maturation
Matured
After
Maturation

hIVM compared to respective control (Figure 4-4, panel B). However, by 24 hIVM, total amount of poly(A) RNA was similar regardless of maturation temperature (P = 0.24; Figure 4-4, panel B).

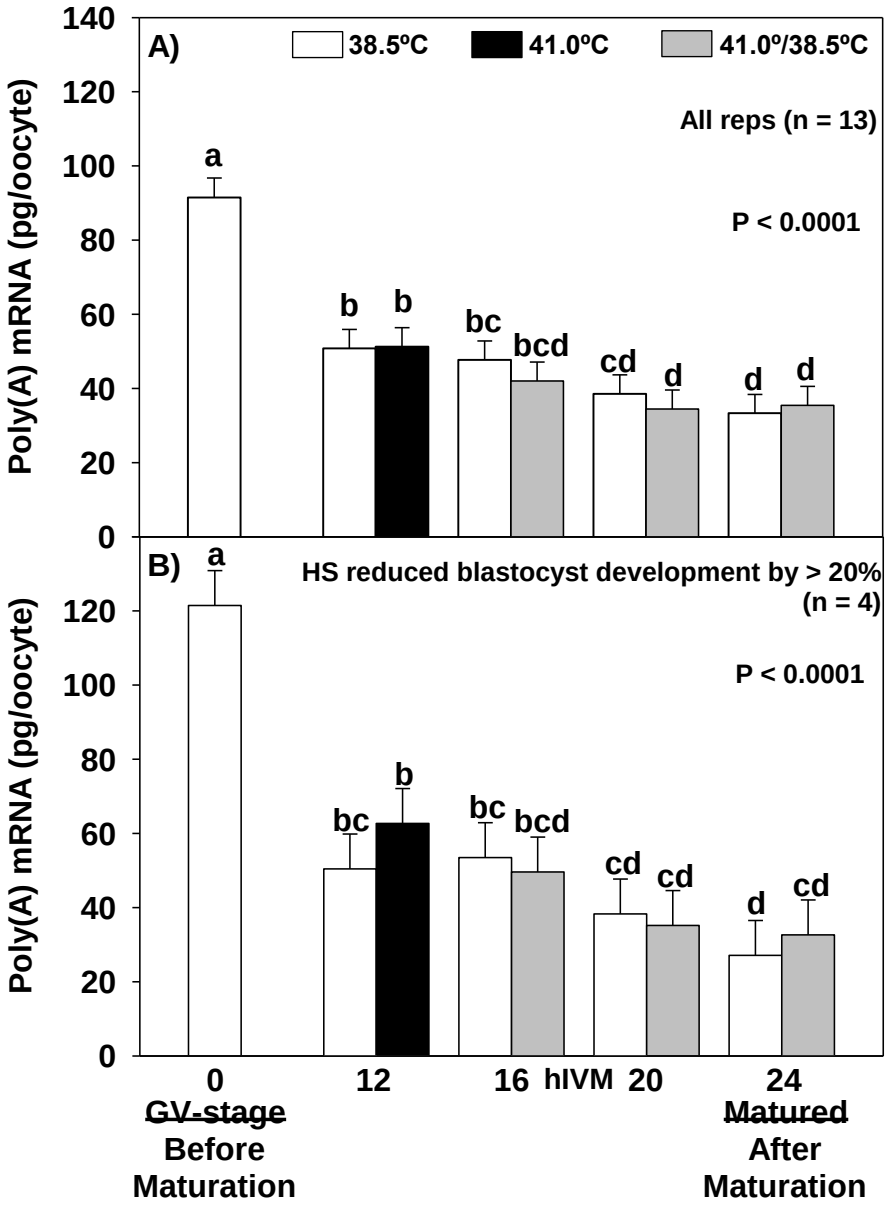
The proportion of total RNA that was polyadenylated (% poly(A) RNA) was also examined. In GV-stage oocytes, 11% of total RNA was poly(A) RNA (Figure 4-4, panel C). This proportion decreased to 8.3% and 5.4% by 12 and 24 hIVM, respectively (Figure 4-4, panel C). There was no impact of heat stress exposure for the first 12 hIVM or for the entire 24 hIVM to alter the proportion poly(A) RNA of total RNA compared to respective controls (Figure 4-4, panel C).

Abundance 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 were similar regardless of hIVM or IVM temperature examined. 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; Figure 4-5, panel A). Poly(A) RNA levels per oocyte continued to decline up to 24 hIVM (Figure 4-5, panel A). There was no impact of heat stress exposure during the first 12 hIVM to affect poly(A) RNA levels at any of the time point examined (P > 0.25; Figure 4-5, panel A).

To provide a more precise test of the hypothesis that heat-induced alterations in poly(A) RNA may explain a portion of heat-induced reductions in embryo development after fertilization, only those experimental replicates whereby heat stress reduced blastocyst rates by ≥

Figure 4-5. Poly(A) RNA within oocytes matured at 38.5º or 41.0ºC for up to 24 hIVM. White bars indicate culture at 38.5ºC 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 38.5ºC thereafter. Data from all 13 replicates are provided in panel A. . Data from the four replicates having heat-induced reductions in blastocyst development are provided in panel B. Least squares means ± standard error of the mean are shown with treatment differences indicated by letter designation at significance level indicated for each panel.


A)
38.5ºC
41.0ºC
41.0º/38.5ºC
All reps (n = 13)
P < 0.0001
Poly(A) mRNA (pg/oocyte)
140
120
100
80
60
40
20
0
a
b
b
bc
bcd
cd
d
d
d
B)
HS reduced blastocyst development by > 20%
(n = 4)
P < 0.0001
a
bc
b
bc
bcd
cd
cd
d
cd
0
12
16
hIVM
20
24
GV-stage
Before
Maturation
Matured
After
Maturation

20% were utilized (n = 4 replicates; 30.6 vs. 20.4% blastocyst development; SEM = 4.2%; P = 0.01). Similar to previous results, poly(A) RNA was highest in GV-stage oocytes and decreased as maturation progressed (Figure 4-5, panel B). Even when the effect of heat stress was to reduce development by ≥ 20%, there was no impact of 41ºC exposure during the first 12 hIVM to alter poly(A) RNA per oocyte (Figure 4-5, panel B).

Size distribution of RNA molecules and relative abundance of developmentally-important transcripts in control and heat-stressed oocytes and their surrounding cumulus cells

Of the 23 replicates performed, 17 had blastocyst development ≥ 20% after fertilization. Within these, the effect of heat stress to reduce embryo development ranged from 0 to 55.3%. To provide a more precise test of the hypothesis that heat-induced alterations in RNA may explain a portion of heat-induced reductions in embryo development after fertilization, only those experimental replicates whereby heat stress reduced blastocyst rates by ≥ 20% were utilized (n = 7).

Neither recovery and lysis of oocytes after denuding nor ability to undergo cleavage and development to 8 to 16-cell embryos after fertilization were altered by heat stress application during the first 12 hIVM (Table 4-2). However, exposure of maturing oocytes to 41ºC during the first 12 hIVM reduced the proportion developing to the blastocyst stage 8 days after fertilization (Table 4-2). Blastocysts from heat-stressed oocytes had similar stage and quality scores as those from non-stressed oocytes (Table 4-2).

Control and heat-stressed oocytes utilized for RNA isolation had similar recovery (95.3 to 97.8 %; SEM = 1.3%; P = 0.62) and lysis (0.6 to 2.2%; SEM = 0.6%; P = 0.52) after denuding regardless of hIVM examined. However, the proportion having residual cumulus cells after

Table 4-2. Embryo development for developmentally-competent oocytes with heat-induced blastocyst reductions of ≥ 20% (n = 7 reps).

		18 to 20 hpi		66 to 74 hpi		167 to 193 hpi			
IVM temperature	Total COC	Recovered (%)	Lysed (%)	Cleaved (%)	8 to 16-cell* (%)	Blast/PZ (%)	Blast/Cleaved (%)	Stage	Quality
38.5ºC	446	97.25 ± 0.97	1.61 ± 0.60	80.46 ± 2.25	81.99 ± 2.59	29.76 ± 2.26[a]	36.92 ± 2.90[a]	6.20 ± 1.32	1.13 ± 0.09
41.0ºC	469	96.87 ± 1.05	0.44 ± 0.31	80.00 ± 2.28	79.01 ± 2.82	19.13 ± 1.94[b]	23.79 ± 2.52[b]	6.22 ± 1.32	1.30 ± 0.09
	P-value	0.74	0.13	0.87	0.38	0.004	0.01	0.92	0.20

* Proportion of cleaved embryos

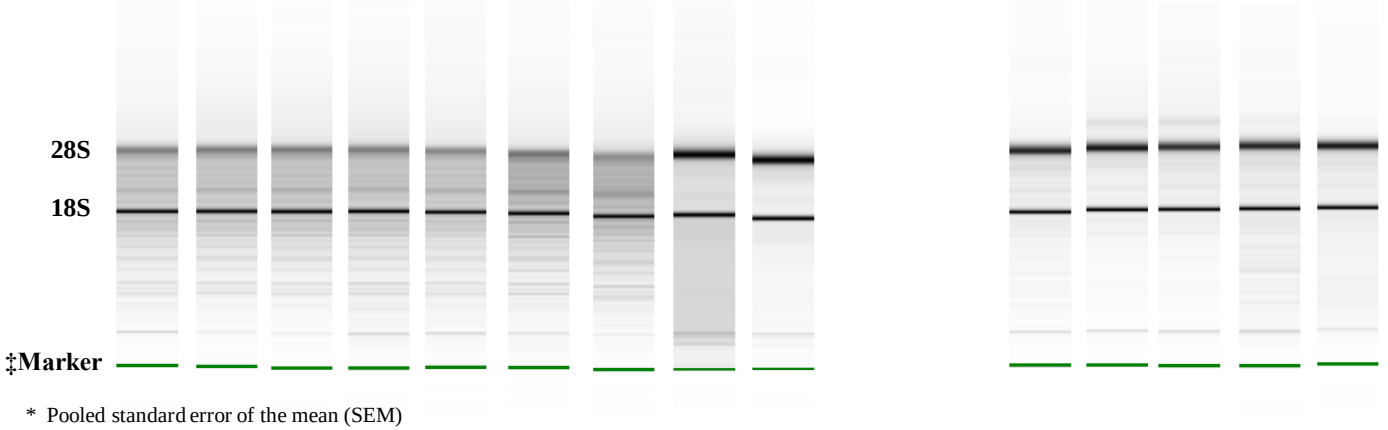
[ab] Denotes differences at $P < 0.05$

denuding decreased as hIVM increased (12.4 % at GV-stage, 5.4 and 4.7 % for 38.5 and 41.0ºC at 12 hIVM, and 2.0 and 1.1 % for 38.5 and 41.0ºC at 24 hIVM, respectively; SEM = 1.9 %; P = 0.002). Total RNA per oocyte did not change during meiotic maturation and was not altered by exposure to 41ºC during the first 12 hIVM (Table 4-3). Four to 8-cell embryos had similar amounts of total RNA as oocytes but this value increased significantly in blastocyst-stage embryos derived from heat-stressed oocytes (Table 4-3). Values for RIN and ratios for rRNA, 18S/fast region, and 18S/inter region were comparable in oocytes and 4 to 8-cell embryos regardless of maturation temperature (Table 4-3). In contrast, blastocysts had higher rRNA and 18S/inter region ratios than oocytes or 4 to 8-cells but RIN values were not different (Table 4-3). Ratio of 18S/fast region was lower in blastocysts from heat-stressed oocytes compared to all other treatment groups (Table 4-3). Total RNA, ratios for rRNA, 18S/fast region, and 18S/inter region as well as RIN values were similar in surrounding cumulus regardless of timing of oocyte maturation or application of heat stress (Table 4-3). No heat-induced alterations in electrophoretic profiles were observed at any stage of development as indicated by gel-like images below Table 4-3. However, total RNA from blastocysts was more like that of cumulus cells than oocytes or 4 to 8-cell embryos.

As a next step, abundance of individual transcripts was examined. There was an overall trend for the relative abundance of 18S rRNA, 28S rRNA, HSP70, cyclin B1, BMP15, PAP, and GDF9 transcripts to be similar in GV-stage and matured oocytes but lower in 4 to 8-cell and blastocyst-stage embryos (Figure 4-6, panels A to G). Exposure to 41ºC for the first 12 hIVM did not alter the abundance of any transcripts examined in oocytes or resultant embryos after fertilization (Figure 4-6). For cyclin B1, BMP15, PAP, and GDF9, relative abundance was

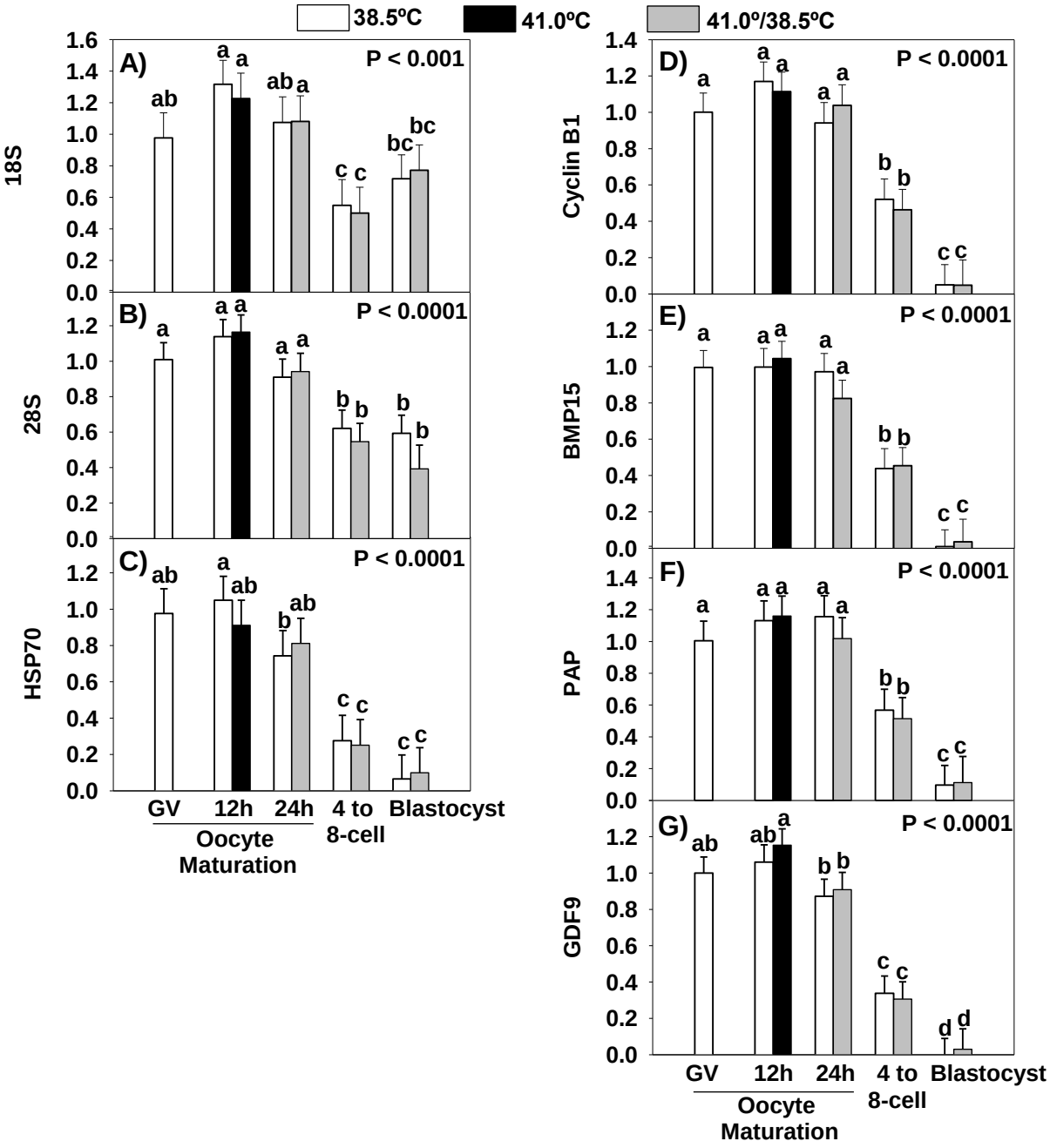
Table 4-3. Characteristics of total RNA from developmentally-competent oocytes having heat-induced reductions in blastocyst development of ≥ 20% (n = 7 reps).

	Oocyte					Embryo						Cumulus						
Variables	**GV-stage**	**12 hIVM**	**12 hIVM**	**24 hIVM**	**24 hIVM**	**4 to 8-cell**	**4 to 8-cell**	**Blast**	**Blast**	**SEM***	***P***	**GV-stage**	**12 hIVM**	**12 hIVM**	**24 hIVM**	**24 hIVM**	**SEM***	***P***
IVM temp (ºC)	---	**38.5**	**41.0**	**38.5**	**41.0**	**38.5**	**41.0**	**38.5**	**41.0**	---	---	---	**38.5**	**41.0**	**38.5**	**41.0**	---	---
Total RNA**	1.82^{c}	2.18^{bc}	2.10^{bc}	2.41^{bc}	2.28^{bc}	2.64^{bc}	3.26^{bc}	3.80^{b}	6.43^{a}	0.81	0.0001	54.97	52.27	73.34	62.53	86.04	18.55	0.41
rRNA ratio	0.86^{b}	0.76^{b}	0.78^{b}	0.74^{b}	0.82^{b}	0.80^{b}	0.70^{b}	1.86^{a}	1.76^{a}	0.12	<0.0001	1.62	1.74	1.79	1.74	1.60	0.11	0.60
RIN	5.33	4.99	5.04	5.10	5.12	4.47	3.94	7.19	6.30	0.50	0.10	8.13	8.54	8.11	8.31	8.51	0.36	0.35
18S/fast region ratio	0.31^{a}	0.28^{a}	0.28^{a}	0.29^{a}	0.23^{a}	0.28^{a}	0.22^{ab}	0.22^{a}	0.10^{b}	0.04	0.02	0.91	0.98	0.95	1.00	1.29	0.18	0.20
18S/inter region ratio	0.69^{b}	0.66^{b}	0.64^{b}	0.65^{b}	0.63^{b}	0.52^{bc}	0.51^{bc}	0.85^{a}	0.42^{c}	0.06	0.002	1.19	1.75	1.47	1.74	1.68	0.24	0.24



* Pooled standard error of the mean (SEM)
** ng per oocyte/embryo or ng/µl (cumulus)
[abc] Different superscripts within a row indicate significant differences (P < 0.05) for oocytes & embryos
‡Images are representative

Figure 4-6. Relative abundance of transcripts per oocyte or embryo for replicates (n = 7) where culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development (≥ 20%) after fertilization. White bars indicate culture at 38.5ºC for 0, 12, or 24 hIVM or embryos derived from oocytes cultured at 38.5ºC. Black bar indicates heat stress for 12 h. Gray bar indicates heat-stress for first 12 hIVM and culture at 38.5ºC thereafter. A) 18S rRNA, B) 28S rRNA, C) HSP70, D) Cyclin B1, E) BMP15, F) PAP, and G) GDF9. Least squares means ± standard error of the mean are shown with treatment differences indicated by letter designation at significance level indicated for each panel.


38.5ºC
41.0ºC
41.0º/38.5ºC
A)
P < 0.001
18S
B)
P < 0.0001
28S
C)
P < 0.0001
HSP70
D)
P < 0.0001
Cyclin B1
E)
P < 0.0001
BMP15
F)
P < 0.0001
PAP
G)
P < 0.0001
GDF9
GV
12h
24h
4 to 8-cell
Blastocyst
Oocyte Maturation

lower in blastocysts than 4 to8-cell embryos (Figure 4-6, panels D to G). Regression analysis was also performed to better clarify changes in transcript abundance from onset of meiotic maturation to blastocyst-stage embryos and showed quadratic behavior of 18S and 28S rRNA (Figure 4-7, panels A and B) and exponential decay of HSP70, cyclin B1, BMP15, PAP, and GDF9 (Figure 4-7, panels C to G). There was no impact of exposure to 41 ºC during the first 12 hIVM on the pattern of transcript abundance for the stages of development examined (Figure 4-7).

In surrounding cumulus cells, relative quantity of 18S rRNA did not change as oocyte maturation progressed (Figure 4-8, panel A). However, 28S rRNA was lower in cumulus from GV-stage oocytes compared to those matured for 12 hIVM (Figure 4-8, panel B). There was no effect of heat stress during the first 12 h of maturation on 18S or 28S rRNA transcript abundance. For HSP70, there was a significant interaction between maturation time and temperature ($P = 0.005$). Specifically, relative abundance of HSP70 was higher in cumulus from heat-stressed oocytes at 12 hIVM compared to controls but differences was no longer detected at 24 hIVM (Figure 4-8, panel C). Quantity of cyclin B1 transcripts decreased in step-wise fashion as maturation progressed with no impact of heat stress (Figure 4-8, panel D). Transcripts for PAP in cumulus cells were significantly lower at the onset of maturation than at 12 or 24 hIVM but were not affected by 41ºC (Figure 4-8, panel E). No transcripts for BMP15 or GDF9 were detected in cumulus cell RNA.

Differences in ability of oocytes to become blastocyst-stage embryos for unknown reasons independent of heat stress allowed for comparison of developmentally-competent (blastocyst development > 30%) and –challenged (blastocyst development < 20%) GV- stage

Figure 4-7. Patterns of relative transcript abundance over development as assessed by regression analysis for replicates (n = 7) whereby culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development (≥ 20%) after fertilization. 18S (A) and 28S (B) displayed polynomial behavior while HSP70 (C), Cyclin B1 (D), BMP15 (E), PAP (F), and GDF9 (G) exhibited exponential decay of transcript abundance over oocyte maturation and early embryonic development. Solid line reflects culture throughout at 38.5ºC while dashed line was cultured at 41.0ºC for the first 12 hIVM but 38.5ºC thereafter. Arrows indicate data points from which regression analysis derived the equations (0, 12, and 24 hIVM as well as 40.5 h post-IVF (64.5 h after collection) for 4 to 8-cells and 192 h post-IVF (216 h after collection) for blastocysts).

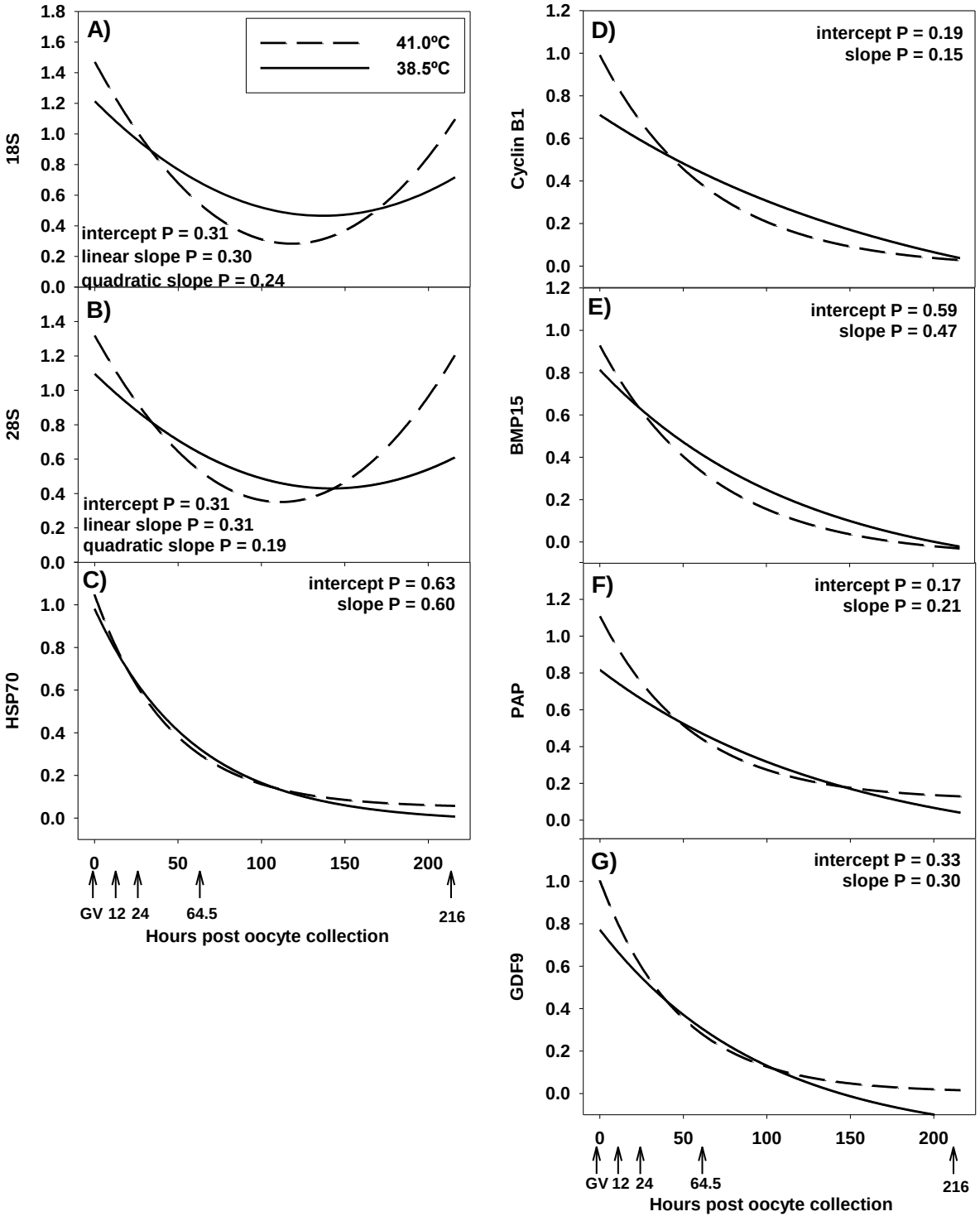
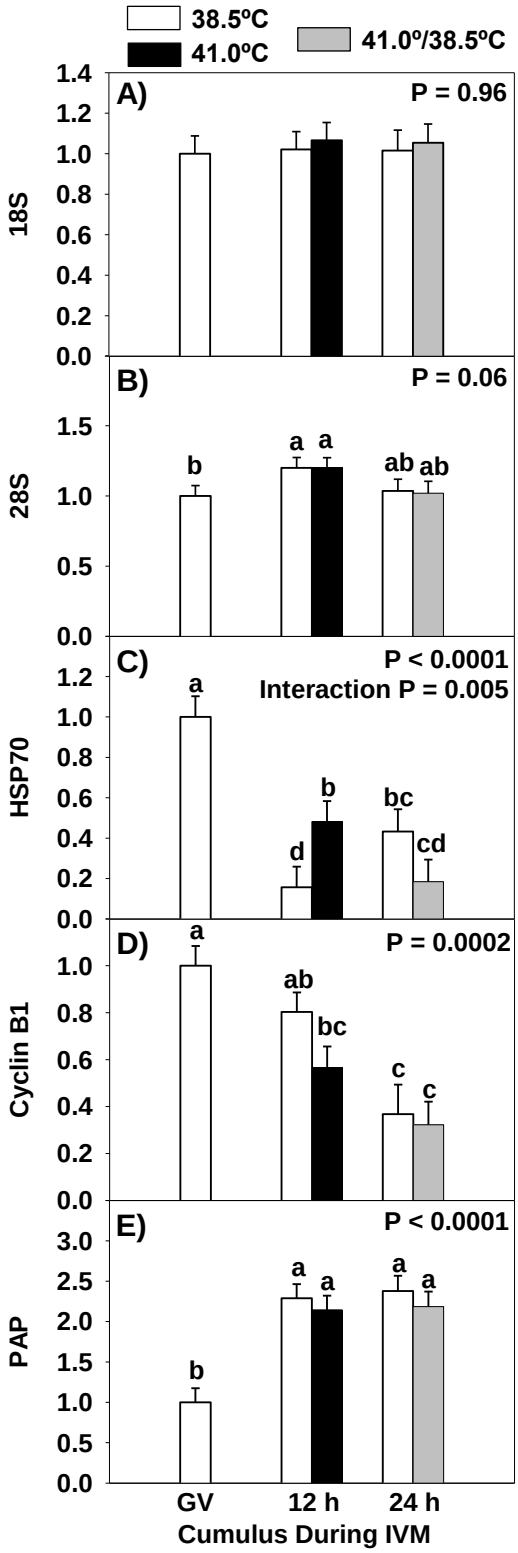

A)
41.0ºC
38.5ºC
18S
intercept P = 0.31
linear slope P = 0.30
quadratic slope P = 0.24
B)
28S
intercept P = 0.31
linear slope P = 0.31
quadratic slope P = 0.19
C)
HSP70
intercept P = 0.63
slope P = 0.60
D)
Cyclin B1
intercept P = 0.19
slope P = 0.15
E)
BMP15
intercept P = 0.59
slope P = 0.47
F)
PAP
intercept P = 0.17
slope P = 0.21
G)
GDF9
intercept P = 0.33
slope P = 0.30
0 50 100 150 200
GV 12 24 64.5 216
Hours post oocyte collection

Figure 4-8. Relative abundance of transcripts for surrounding cumulus cells of oocytes whereby culture at 41.0ºC during the first 12 h of maturation reduced blastocyst development (≥ 20%) after fertilization. A) 18S rRNA, B) 28S rRNA, C) HSP70 (significant interaction between culture temperature and time of maturation), D), Cyclin B1, and E) PAP. White bars indicate culture at 38.5ºC 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 38.5ºC for the remaining 12 hIVM. Least squares means ± standard error of the mean are shown with treatment differences indicated by letter designation at significance level indicated for each panel.


38.5ºC
41.0ºC
41.0º/38.5ºC
A)
P = 0.96
18S
B)
P = 0.06
28S
C)
P < 0.0001
Interaction P = 0.005
HSP70
D)
P = 0.0002
Cyclin B1
E)
P < 0.0001
PAP
GV
12 h
24 h
Cumulus During IVM

oocytes as an additional model of competence. After ranking each of the 23 replicates by blastocyst development in those oocytes matured at 38.5ºC, the 6 replicates with highest

Figure 4-7. Blastocyst rates were compared to the 6 replicates with the lowest blastocyst development. No differences were observed for recovery or lysis of PZ at denuding, nor for ability of PZ to cleave or progress to the 8 to 16-cell stage (Table 4-4). However, the proportion of PZ becoming blastocyst-stage embryos was significantly lower for challenged than competent oocytes (Table 4-4). Stage and quality of blastocysts were not affected by developmental potential of GV-stage oocytes (Table 4-4).

Total RNA, rRNA ratio, and RIN values were not different for RNA from developmentally-competent and -challenged GV-stage oocytes (Table 4-5). Similarly, cumulus cells from developmentally-competent and –challenged oocytes had similar total RNA concentration, rRNA ratio, and RIN values (Table 4-5). Electrophoretic profiles of GV-stage oocytes were not influenced by subsequent ability to become blastocysts as evidenced by gel-like images below Table 4-5.

Marked differences were observed in relative abundance of some of the individual transcripts examined in developmentally-competent versus -challenged GV-stage oocytes and their surrounding cumulus (Figure 4-9). Specifically, 18S was more abundant in developmentally-challenged oocytes while HSP70, cyclin B1, BMP15, PAP, and GDF9 were reduced (Figure 4-9, panel A). Surrounding cumulus from developmentally-challenged oocytes had reduced relative levels of Cyclin B1 (Figure 4-9, panel B). Regression analysis indicated that the responsiveness of some transcripts differed between developmentally-competent and -challenged oocytes (Figure 4-10). Developmentally-challenged oocytes had increased intercepts

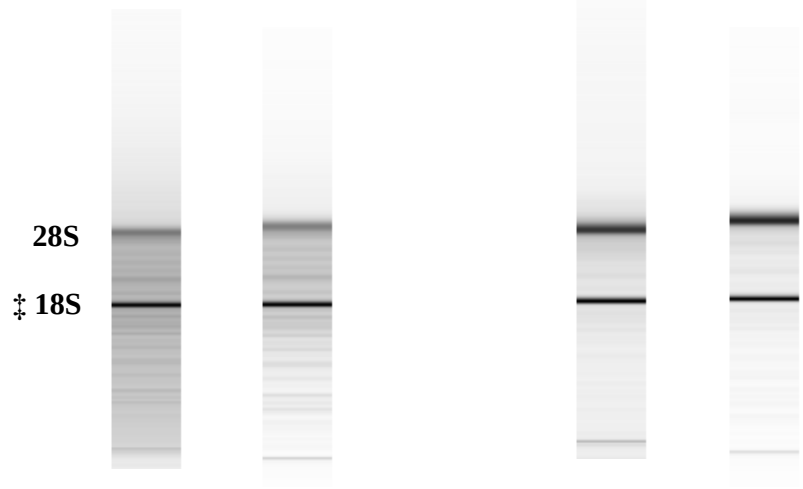
Table 4-4. Embryo development for developmentally-competent vs. challenged oocytes.

		18 to 20 hpi		50 to 76 hpi		167 to 193 hpi			
Developmental Potential	Total COC	Recovered (%)	Lysed (%)	Cleaved (%)	8 to 16-cell* (%)	Blast/PZ (%)	Blast/Cleaved (%)	Stage	Quality
Competent	376	96.01 ± 1.33	1.60 ± 0.65	80.00 ± 2.38	83.71 ± 2.09	32.73 ± 2.58[a]	40.91 ± 1.84[a]	6.31 ± 0.24	1.06 ± 0.14
Challenged	384	97.54 ± 0.80	1.04 ± 0.52	77.74 ± 3.98	83.59 ± 4.70	13.65 ± 1.87[b]	17.56 ± 1.92[b]	5.98 ± 0.24	1.36 ± 0.13
	P-value	0.33	0.52	0.63	0.98	0.0002	< 0.0001	0.35	0.15

* Proportion of cleaved embryos

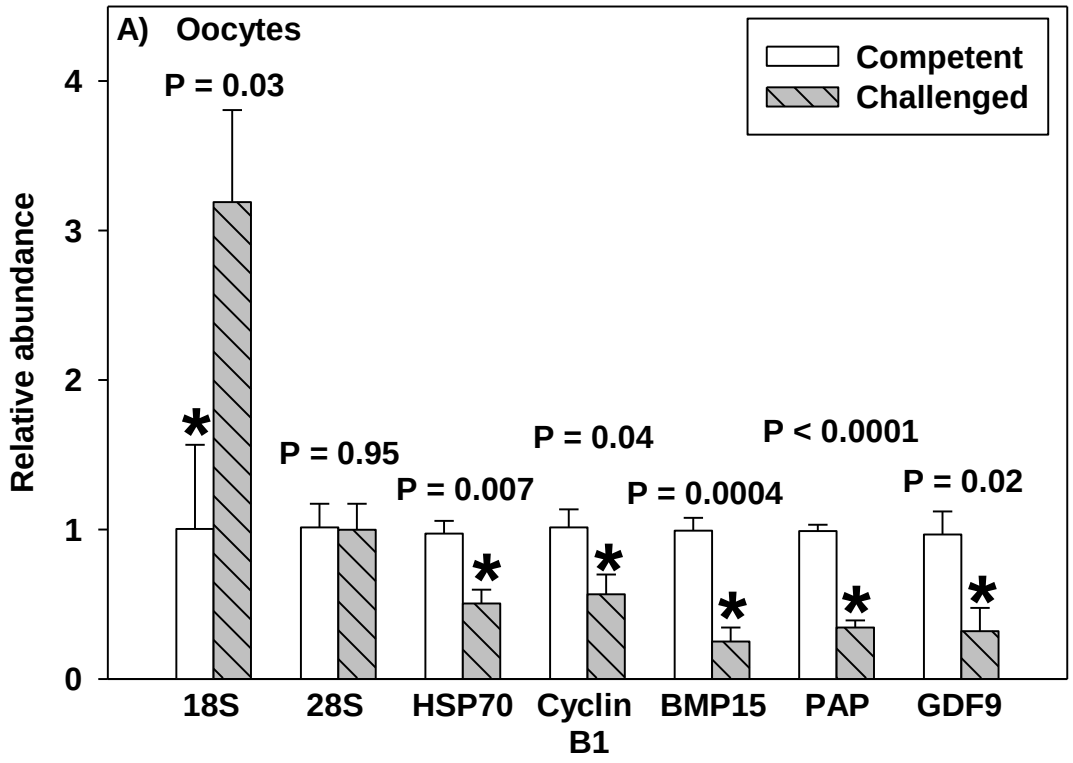
Table 4-5. Characteristics of total RNA from developmentally-competent versus challenged GV-stage oocytes (n = 6 reps).

	GV-stage oocytes				Cumulus			
Variables	**Competent**	**Challenged**	**SEM***	***P***	**Competent**	**Challenged**	**SEM***	***P***
Total RNA**	2.36	2.12	0.48	0.73	45.49	27.05	15.22	0.18
rRNA ratio	0.81	0.82	0.05	0.89	1.85	1.85	0.18	0.85
RIN	4.67	5.41	0.48	0.30	8.60	8.42	0.35	0.72
18S/fast region ratio	0.28	0.32	0.05	0.58	1.11	0.90	0.20	0.49
18S/inter region ratio	0.75[a]	0.66[b]	0.02	0.03	1.55	1.33	0.17	0.41



* Pooled standard error of the mean (SEM)
** ng per oocyte/embryo or ng/µl (cumulus)
[abc] Different superscripts within a row indicate significant differences (P < 0.05) for oocytes
‡Images are representative

Figure 4-9. Relative abundance of transcripts in developmentally-competent (n = 6 replicates) or -challenged (n = 6 replicates) GV-stage oocytes (A) and surrounding cumulus (B). P-value and asterisk above each set of columns indicate significance of difference.


A) Oocytes
Competent
Challenged
Relative abundance
0
1
2
3
4
P = 0.03
P = 0.95
P = 0.007
P = 0.04
P = 0.0004
P < 0.0001
P = 0.02
*
18S
28S
HSP70
Cyclin B1
BMP15
PAP
GDF9

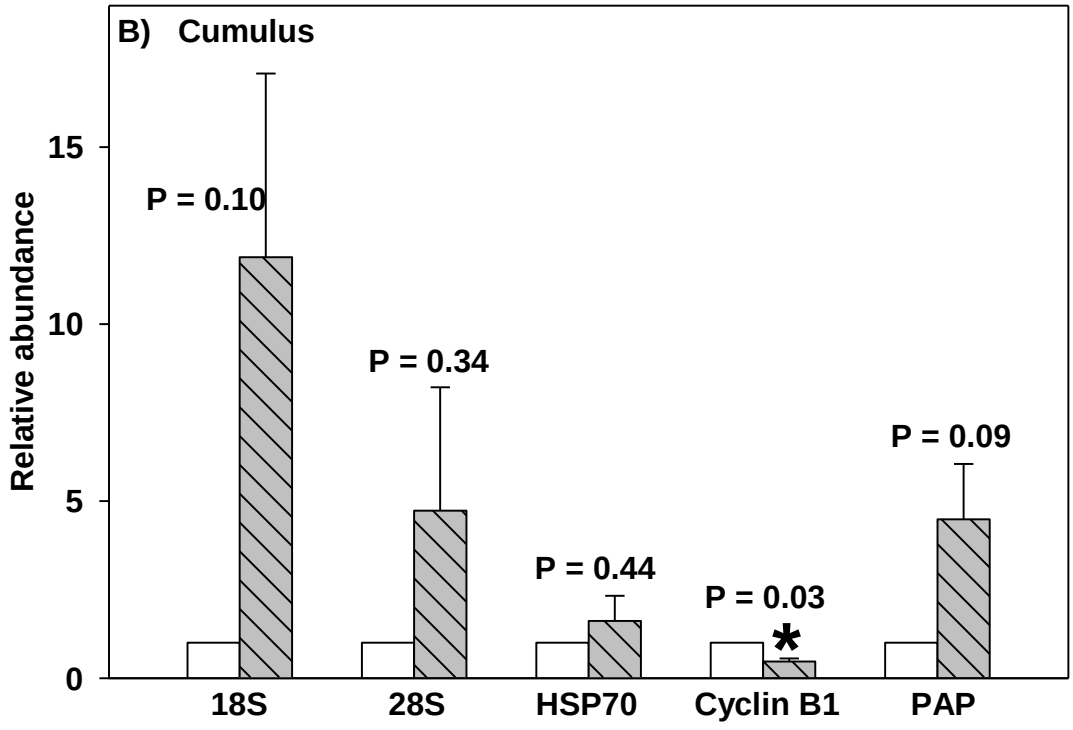
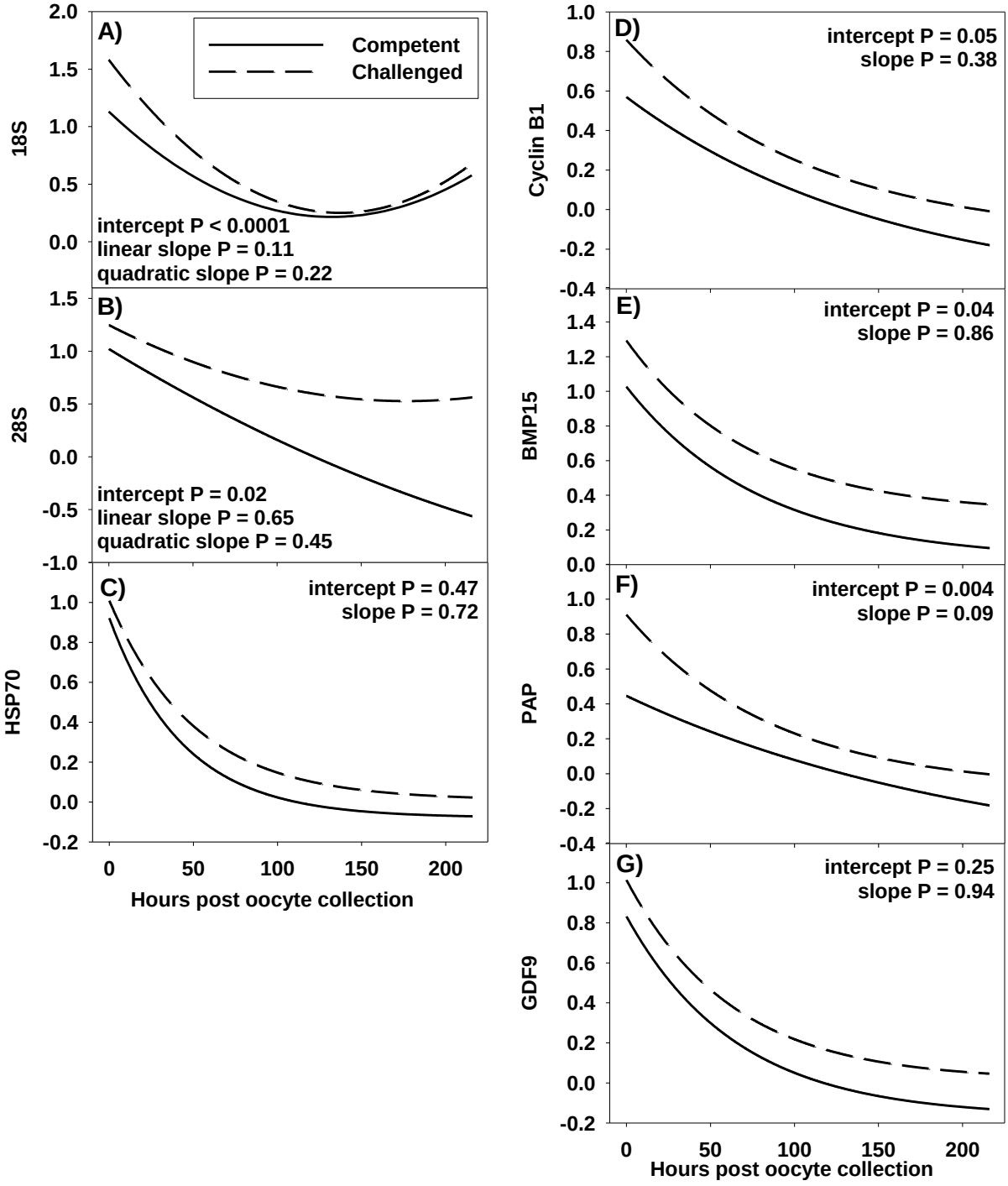

B) Cumulus
Relative abundance
0
5
10
15
P = 0.10
P = 0.34
P = 0.44
P = 0.03
P = 0.09
*
18S
28S
HSP70
Cyclin B1
PAP

Figure 4-10. Patterns of relative transcript abundance over development of developmentally-competent versus developmentally-challenged oocytes and resultant embryos as determined by regression analysis. 18S (A) and 28S (B) displayed polynomial behavior while cyclin B1 (C), BMP15 (D), GDF9 (E), HSP70 (F), and PAP (G) exhibited exponential decay of transcript abundance over oocyte maturation and early embryonic development. Data points from which regression analysis derived the equations presented were 0, 12, and 24 hIVM as well as 40.5 h post-IVF (64.5 h after collection) for 4 to 8-cells and 192 h post-IVF (216 h after collection) for blastocysts.


A)
Competent
Challenged
18S
intercept P < 0.0001
linear slope P = 0.11
quadratic slope P = 0.22
B)
28S
intercept P = 0.02
linear slope P = 0.65
quadratic slope P = 0.45
C)
HSP70
intercept P = 0.47
slope P = 0.72
D)
Cyclin B1
intercept P = 0.05
slope P = 0.38
E)
BMP15
intercept P = 0.04
slope P = 0.86
F)
PAP
intercept P = 0.004
slope P = 0.09
G)
GDF9
intercept P = 0.25
slope P = 0.94
Hours post oocyte collection
Hours post oocyte collection

for 18S ($P < 0.0001$; Figure 4-10, panel A), 28S ($P = 0.02$; Figure 4-10, panel B), cyclin B1 ($P = 0.05$; Figure 4-10, panel D), BMP15 ($P = 0.04$; Figure 4-10, panel E), and PAP ($P = 0.004$; Figure 4-10, panel G).

DISCUSSION

Results in this study describe specific fractions of bovine oocyte and cumulus RNA (> 200 nt) during oocyte maturation and embryo development, and the immediate effects of 41ºC during the first 12 hIVM on those RNA populations. Additional efforts to examine RNA in embryos from control and heat-stressed oocytes allowed for assessing the potential for perturbations, if any, to carryover to early embryos. To our knowledge, this is the first study to examine the effects of a physiologically-relevant elevated temperature on global populations of oocyte and cumulus RNA in this manner. Apart from heat stress findings, differences in developmental potential of morphologically-equivalent oocyte pools (developmentally-competent vs. –challenged) provided a model of oocyte competence that allowed for the unique opportunity to examine general characteristics of RNA inherent to oocytes with high and low ability to progress to the blastocyst-stage of embryo development.

The overall hypothesis of these experimental efforts was that a portion of the reductions in embryo development observed after heat stress application during meiotic maturation may be due to alterations in maternal stores of RNA within the ooplasm and/or in RNA of surrounding cumulus cells. As a more direct test of this hypothesis, only those experimental replicates having heat-induced reductions in blastocyst development (when possible) were evaluated for RNA characteristics. However, even when blastocyst development was reduced by ≥ 20%, no heat-induced alterations in poly(A) levels, RNA size distribution, or relative abundance of individual

transcripts were observed. Heat stress altered HSP70 expression in cumulus cells but this finding was consistent regardless of the developmental potential of heat-stressed oocytes after fertilization. Together, data suggests that heat-induced reductions in *de novo* protein synthesis by oocytes are not likely due to a global impact of elevated temperature to alter RNA integrity (i.e., size distribution of RNA molecules) or levels of specific transcripts but may be due to altered abundance of transcripts having a poly(A) tail, as seen in Experiment 1 but not Experiment 2. Reduced protein synthesis may also be due to other factors such as initiation of translation (Kim and Jang 2002) or active involvement of ribosomes in protein synthesis (Duncan and Hershey 1989).

In retrospect, apparent lack of heat-induced consequences on maternal RNA was not surprising given that most of the detrimental effects from exposure of maturing oocytes to elevated temperature are not observed until after several embryonic cleavage divisions. Specifically, overall cleavage rates ~72 h after fertilization were not altered (Edwards and Hansen 1996, 1997; Lawrence *et al.,* 2004; Edwards *et al.,* 2005; Schrock *et al.,* 2007; Edwards *et al.,* 2009) but compaction (Edwards *et al.,* 2009) and blastocyst-formation were reduced for embryos derived from heat-stressed oocytes (Edwards and Hansen 1996, 1997; Lawrence et al., 2004; Roth and Hansen 2004a, b; Edwards et al., 2005; Schrock et al., 2007). Protein synthetic profiles of heat-stressed oocytes demonstrating few and slight differences in specific proteins compared to controls (Edwards and Hansen 1996, 1997; West-Rispoli *et al.,* 2006) support the idea that elevated temperature does not have "global" effects on oocyte RNA. This is in agreement with other studies examining transcript abundance using other models of developmental competence. Specifically, Thelie et al. (2007) found that differences in

polyadenylation status accounted for previously described differences in relative abundance of specific transcripts between *in vivo* and *in vitro* matured bovine oocytes as overall levels of transcripts examined did not change in oocytes. An alternative competence model utilizing cow versus calf oocytes found higher levels of follistatin mRNA (Patel *et al.*, 2007) and protein (Lee *et al.*, 2009) were associated with increased developmental potential. Additional research is necessary to further elucidate what, if any, changes occur at the level of individual transcripts in response to heat stress that could account for reduced embryo development after fertilization.

Although the effects of heat stress on oocyte and cumulus RNA in the studies reported herein were minimal for the endpoints examined, the experimental design permitted discovery of changes in RNA during oocyte and early embryo development. Perhaps one of the more interesting observations relates to the size distribution of RNA molecules contained within oocytes and 4 to 8-cell embryos versus blastocysts and cumulus cells. Previous work found that oocytes and cumulus have very different RNA profiles during meiotic maturation (see Chapter 3) in agreement with that reported herein. However, this work extends those results by characterizing RNA in early embryos near the time of major embryonic genome activation and after compaction and blastocoele formation. Similar ratios for rRNA, 18S/fast region, and 18S/inter region as well as RIN values for RNA from 4 to 8-cell embryos compared to that of oocytes, but different from that of blastocysts, likely indicates the less differentiated state of oocytes and early cleavage-stage embryos and ability to undergo transcription. Oocytes after GVBD and early embryos are transcriptionally-quiescent, relying upon maternal pools of RNA stored within the ooplasm (Wassarman and Letourneau 1976) for protein synthesis until embryonic genome activation (occurring at the 8 to 16-cell stage in bovine embryos, Memili *et*

al., 1998). However, blastocysts are capable of RNA synthesis, like cumulus cells (Tirone *et al.,* 1997) which is likely reflected in similar electrophoretic profiles. A recent report found that morula also have RNA appearing more like somatic cells than that observed for oocytes or embryos up to the 8-cell stage (Gilbert *et al.,* 2009).

Finding such different RNA profiles during early embryo development is intriguing and is probably reflective of developmentally-important events occurring during this time. For instance, rRNA synthesis in bovine embryos is not reactivated until the third embryonic cell cycle (i.e., during progression from 4 to 8-cells, Hyttel *et al.,* 2000; Jakobsen *et al.,* 2006) which may explain lower rRNA ratios in RNA from oocytes and 4 to 8-cell embryos than in blastocysts. It is unclear what impact this may have on protein synthesis, however, as 75 to 80% of murine oocyte ribosomes are non-polysomal (i.e., not actively involved in translation, De Leon *et al.,* 1983) and intact 28S rRNA may not be necessary for translation to occur (Huvos *et al.,* 1970; Grove and Johnson 1973, 1974). The lack of heat-induced alterations in RNA integrity at any stage examined indicates that a physiological heat stress of 41.0ºC does not elicit the more detrimental effects, like RNA degradation, noted in somatic cells exposed to supraphysiological temperatures (Yost *et al.,* 1990).

When using the PicoPure kit for RNA isolation, total RNA did not change during meiotic maturation, consistent with previous findings for bovine oocytes (Chapter 3, Lequarre *et al.,* 2004). This was not the case if RNA was isolated using the Stratagene kit as total RNA was decreased at 12 hIVM but remained similar thereafter. The disparity is difficult to explain but may be related to differences in RNA isolation kits utilized and efficiency of each to isolate RNA of different sizes. Even so, the amount of RNA obtained for GV-stage and matured oocytes was

in agreement with others (~2 ng/oocyte, Bilodeau-Goeseels and Schultz 1997; Lequarre *et al.*, 2004). Amount of total RNA was similar in oocytes and 4 to 8-cell embryos but increased in blastocyst stage embryos, consistent with previous reports (Gilbert *et al.*, 2009).

Global populations of poly(A) RNA were reduced by half during oocyte maturation, also consistent with others (Lequarre *et al.*, 2004) using bovine oocytes and the same methodology. While these reductions were slightly more than that reported by Biase et al. (2008) using qPCR for relative quantification of poly(A) RNA in bovine oocytes (~30% reduction from GV-stage to 18 hIVM), declines in poly(A) RNA of ~50% during oocyte maturation have been reported for murine oocytes (De Leon *et al.*, 1983). Additionally, the reductions in poly(A) RNA observed during maturation herein were similar to those reported for murine (19% at GV-stage and 10% in matured oocytes, De Leon *et al.*, 1983) and bovine oocytes (Lequarre *et al.*, 2004).

In the first experiment, exposure of oocytes to 41ºC during the first 12 hIVM reduced the abundance of poly(A) RNA at 12 hIVM but not at 24 hIVM. To ascertain the time period at which differences no longer become apparent, a second study was performed examining 12, 16, 20, and 24 hIVM. However, no effect of heat stress to reduce poly(A) RNA was observed. This disparity is difficult to explain but may be related to collection of oocytes in different years, increased days of oocyte collection in the second study, or other unknown reasons. Regardless, relevance of potential heat-induced reductions in poly(A) RNA at 12 hIVM in relation to developmental potential are questionable as a numerical increase in poly(A) RNA was observed for heat-stressed oocytes despite heat-induced blastocyst reductions of > 30% (Figure 4-5, panel B). In retrospect, this is not surprising as Lequarre et al. (2004) demonstrated that GV-stage and

matured cow and calf oocytes had similar levels of poly(A) RNA. Yet, calf oocytes are known to have low developmental competence (Khatir *et al.,* 1998).

When examining the abundance of specific transcripts important for meiotic maturation and response to heat stress exposure, no impact of heat stress was noted but there were changes as early embryo development proceeded. In general, relative abundance of 18S and 28S rRNA transcripts assessed by qPCR was higher in oocytes than in 4 to 8-cell and blastocyst-stage embryos. This is perplexing in light of microcapillary electrophoretic profiles indicating higher rRNA ratios in blastocysts than in oocytes and 4 to 8-cell embryos. However, it should be noted that RNA profiles were generated after analyzing 1 µl total RNA while qPCR data were generated after analyzing 0.1 oocyte or embryo equivalents, correcting for RNA concentration, and normalizing to an exogenous control. Ratios of rRNA are also qualitative while qPCR is quantitative in relative terms.

In the present study, several transcripts were examined by qPCR. Cyclin B1 was selected as a gene of interest based upon its importance in meiotic maturation as the regulatory subunit of maturation promoting factor necessary for meiotic progression (reviewed by Dekel 2005). The relative abundance of cyclin B1 transcripts did not change during IVM and was not impacted by exposure to 41ºC for the first 12 hIVM. Lack of change in this transcript is in agreement with others examining bovine oocytes during meiotic maturation (Lonergan *et al.,* 2003).

Transcripts for two oocyte-specific factors, bone morphogenic protein 15 (BMP15 or GDF9b) and growth and differentiation factor 9 (GDF9), were also examined based upon their ability to improve developmental potential when added to culture media (Hussein *et al.,* 2006) and their importance in cumulus expansion (reviewed by Gilchrist *et al.,* 2008). Specifically,

studies in murine oocytes found that a factor secreted by the oocyte was responsible for FSH-induced production of hyaluronan by cumulus cells (Salustri *et al.,* 1990). Subsequent effort demonstrated that members of the transforming growth factor superfamily, namely BMP15 (Gueripel *et al.,* 2006) and GDF9 (Dragovic *et al.,* 2005), facilitate FSH-mediated gene expression necessary for cumulus expansion during oocyte maturation (Dragovic *et al.,* 2005). It was hypothesized that these factors may be altered by elevated temperature as hyaluronic acid production by cumulus cells during expansion is reduced in response to heat stress (Lenz *et al.,* 1983). However, oocytes demonstrating heat-induced reductions in subsequent blastocyst development had similar levels of both BMP15 and GDF9 throughout maturation. Lack of change in BMP15 and GDF9 during maturation is in agreement with others (Lonergan *et al.,* 2003). Blastocyst-stage embryos had low to undetectable levels of both transcripts, similar to previous reports (Pennetier *et al.,* 2004). The lack of GDF9 and BMP15 transcripts in cumulus cells in the present study is in agreement with previous findings by others (Sendai *et al.,* 2001).

Poly(A) polymerase is responsible for polyadenylation of RNA by addition of AMP molecules to the 3' end of transcripts (McDevitt *et al.,* 1988), a critical function during meiotic maturation (reviewed by Kashiwabara *et al.,* 2008). Transcripts were examined in an effort to provide insight into changes in levels of poly(A) RNA over meiotic maturation. There was no impact of heat stress on relative abundance of PAP mRNA, nor did levels change during meiotic maturation. This is in agreement with previous studies demonstrating no change in PAP levels in GV-stage and matured oocytes (Racedo *et al.,* 2008). In cumulus cells, PAP transcripts increased significantly from the beginning of IVM to 12 hIVM and remained high at 24 hIVM. This pattern was noted regardless of developmental potential of the oocytes from which the

cumulus cells were derived and suggests an increased need for transcript polyadenylation, likely for heightened translation of transcripts necessary for cumulus expansion (reviewed by Richards 2007), as maturation progresses.

Heat shock protein 70 (HSP70) is a molecular chaperone important in the stress response (Hartl 1996), such as that elicited by exposure to elevated temperature, and is decreased in oocytes exposed to a physiological heat stress (Edwards and Hansen 1997). When using developmentally-competent oocytes, no heat-induced alterations in mRNA abundance were observed in oocytes, as reported previously (Kawarsky and King 2001), even when the effect of heat stress during the first 12 hIVM was to reduce subsequent blastocyst development. Cumulus cells exposed to 41.0ºC displayed higher HSP70 mRNA at 12 hIVM but lower at 24 hIVM compared to controls, indicative of a transcriptionally-mediated heat shock response. This response to heat stress is likely independent of the developmental potential of the oocytes (i.e., ability to progress to blastocyst stage embryos after fertilization) from which the cumulus cells were derived (data not shown). Specifically, the pattern of transcript abundance (higher at 12 but lower at 24 hIVM) was observed consistently in replicates yielding high and low blastocyst development.

For each RNA endpoint examined (RNA size distribution and abundance of specific transcripts), marked differences were noted between oocytes and blastocyst-stage embryos. Finding such changes in RNA from oocytes compared to blastocysts may be reflective of a "weeding out" effect. Oocyte pools are comprised of only a small fraction of oocytes that ultimately become blastocysts. Later stage embryo pools are derived from only those oocytes with higher developmental competence. Thus, it may be that individual oocytes with the ability

to become blastocysts after fertilization have different RNA characteristics than those arresting at a prior stage. This theory is difficult to test, however, given the sensitivity of current technology and lack of knowledge regarding what factors are responsible for conferring developmental competence but will be interesting to pursue as methodological sensitivity improves.

Much research is focused on identifying "markers" of oocyte developmental potential. While certain transcripts and proteins are known to be necessary for early development (such as Oct-4, Zuccotti *et al.,* 2008), or are correlative with oocyte competence (such as follistatin, Lee *et al.,* 2009), there is currently no consensus regarding what factors may be reliably utilized as indicators of the ability of oocytes to successfully undergo embryo and fetal development after fertilization. However, recent findings from cumulus cell analysis of developmentally-competent and –challenged oocytes are promising for development of "oocyte competence bioassays" via non-invasive cumulus biopsy (Assidi *et al.,* 2008; Hamel *et al.,* 2008; van Montfoort *et al.,* 2008; Tesfaye *et al.,* 2009).

Regarding developmental potential, the oocytes utilized herein were obtained from antral follicles (3 to 8 mm) of abattoir-derived ovaries from cattle of unknown reproductive status. While this system consistently yields blastocyst rates upwards of 30% (Lawrence *et al.,* 2004; Edwards *et al.,* 2005; Schrock *et al.,* 2007), the vast majority of oocytes do not develop after IVF. On certain occasions, very few proceed to become blastocysts despite morphologically "good" appearance of GV-stage oocytes. Direct comparison of those yielding high rates of blastocysts (> 30%; developmentally-competent) to those with low blastocyst yields (< 20%; developmentally-challenged) is informative of a different aspect of development, independent of heat stress. Results were interesting in that each mRNA examined had a lower relative

abundance in developmentally-challenged oocytes. For cyclin B1, findings from this model of developmental potential are consistent with those from others utilizing a different model of competence, in vivo versus in vitro maturation (whereby in vitro maturation is inferior to in vivo in blastocyst development after fertilization). Specifically, *in vitro* matured bovine oocytes had significantly lower levels of cyclin B1 RNA than *in vivo* matured oocytes (Lonergan *et al.*, 2003).

The pattern of change during development of embryos from developmentally-competent and –challenged oocytes was also different for some of the transcripts, as assessed by regression analysis. In particular, developmentally-challenged oocytes had numerically higher transcript abundance at 12 hIVM than at GV-stage for each transcript examined (data not shown), resulting in a higher intercept for some of the transcripts (Figure 4-10). While transcriptional activity was not monitored in the present study, transcriptionally-mediated increases in mRNA are the likely source of more transcript. Oocytes with reduced developmental potential may initiate IVM with lower relative abundance of mRNA as evidenced for the genes of interest examined. This is suggestive of an inherent threshold of maternal mRNA abundance necessary for successful development. While speculative, there may also be a mechanism in place to allow oocytes to perceive sub-optimal mRNA abundance and initiate a transcriptional burst in an effort to improve developmental potential. However, energetically expensive processes like transcription may serve to reduce ability of oocytes and early embryos to survive until embryonic genome activation. Specifically, the "quiet embryo hypothesis" suggests that embryos able to minimize metabolic activity maximize their ability to undergo further development (reviewed by Baumann *et al.*, 2007).

In summary, exposure of COC to elevated temperature during the first 12hIVM had no consequence on amount of total RNA, poly(A) RNA, size distribution of RNA, or individual transcripts examined in oocytes. Although there were heat-induced alterations in cumulus expression of HSP70 transcripts, this was observed regardless of subsequent blastocyst development. However, data are informative in that exposure to a physiologically-relevant elevated temperature does not induce widespread perturbations in global populations of oocyte and cumulus RNA. This suggests that differences, if any, are at the level of individual transcripts. Thus, microarray analysis is a logical next step in efforts to elucidate potential markers of developmental competence, allowing identification of heat-induced alterations of specific transcript abundance within oocytes and surrounding cumulus.

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CHAPTER 5: Transcriptome Profiles of Oocytes and Cumulus Cells After Direct Exposure to a Physiologically-Relevant Elevated Temperature During Meiotic Maturation

ABSTRACT

Direct exposure of maturing oocytes to a physiologically-relevant elevated temperature reduces embryo development after fertilization coincident with reduced de novo protein synthesis, which may explain a portion of embryo losses. Mechanisms responsible for heat-induced reductions in oocyte protein synthesis are unknown but may be related to alterations in maternal transcripts, which are critical for protein synthesis during meiotic maturation. The impact of heat stress on oocyte transcripts was assessed using microarray analysis. Oocytes were collected before (germinal vesicle (GV)-stage) and after culture at 38.5ºC for 24 h (control) or 41ºC for the first 12 h of meiotic maturation (38.5ºC thereafter). Cumulus cells were also examined due to their intimate connection to oocytes during meiotic maturation. Because oocyte transcripts may or may not have a polyadenylated tail, RNA was amplified by 3'-poly(A) priming or combination of 3'-poly(A) and internal priming before analysis using bovine Affymetrix GeneChip. Numerous transcripts were changed after meiotic maturation of oocytes, consistent with events known to occur in preparation for fertilization, including several that were present at the onset of maturation but not after, and vice versa. The abundance of a subset of transcripts was also dependent upon amplification method used. Heat stress of maturing oocytes altered several transcripts important for mitochondrial function which may impact ability of heat-stressed oocytes to produce energy (i.e., ATP). Cumulus transcript changes mirrored phenotypic changes as expansion occurs during oocyte maturation. Direct application of heat stress to cumulus altered transcripts involved in cell signaling. Assessment of whether transcript changes

translate into functional changes in the oocyte or cumulus will provide additional insight into the consequences of heat stress during meiotic maturation.

INTRODUCTION

Females of a variety of species (sheep, Dutt 1963; pigs, Tompkins et al., 1967; cattle, Cavestany et al., 1985) exposed to elevated ambient temperatures, such as those occurring during the summer months, experience reduced fertility in large part because of heat-induced increases in hyperthermia (reviewed by Hansen and Arechiga 1999). This is particularly problematic in dairy cattle where body temperatures may reach or surpass 41ºC (Gaalaas 1945; Seath and Miller 1946; Monty and Wolff 1974; Roman-Ponce *et al.,* 1981; Turner 1982; Elvinger *et al.,* 1991; Elvinger *et al.,* 1992; Ealy *et al.,* 1993; Wolfenson *et al.,* 1993). Heat-induced reductions in embryo development are prominent when hyperthermia occurs during or near estrus as the oocyte undergoes meiotic maturation in preparation for fertilization within the maternal environment (Stott and Williams 1962; Gwazdauskas *et al.,* 1973; Cavestany *et al.,* 1985; Putney *et al.,* 1989b).

Meiotic maturation, also known as oocyte maturation, is a complex process involving coordinated events occurring at the nuclear, cytoplasmic, and molecular levels in the oocyte (reviewed by Sirard *et al.,* 2006) and is necessary to reduce the copies of maternal chromatin prior to fertilization. Direct exposure of bovine oocytes undergoing meiotic maturation to a physiologically-relevant elevated temperature reduced embryo development after fertilization (Edwards and Hansen 1996; Lawrence *et al.,* 2004; Schrock *et al.,* 2007) to a similar degree as that observed for animals experiencing hyperthermia (Stott and Williams 1962; Cavestany *et al.,* 1985; Putney *et al.,* 1989b). While the underlying mechanisms responsible for reduced

development remain unclear, some of the effects of heat stress may be mediated through cumulus cells, which are intimately associated with the oocyte during the first 9 h of IVM (bovine, Thomas *et al.,* 2004) via gap junctions and focal adhesions. Even after gap junctional breakdown (beginning within the first 3 h of IVM, Thomas *et al.,* 2004) as cumulus begin to expand via secretion of an extracellular matrix (reviewed by Russell and Salustri 2006), cumulus-secreted factors may still influence the oocyte microenvironment (reviewed by Gilchrist *et al.,* 2004) and any perturbation of the oocyte-cumulus relationship impairs subsequent embryo development (Geshi *et al.,* 2000; Wongsrikeao *et al.,* 2005; Yeo *et al.,* 2009). While the effects of heat stress on cumulus cells are largely unknown, exposure to an elevated temperature of 41ºC for 22 h reduced cumulus expansion as assessed by production of hyaluronan (Lenz *et al.,* 1983), a predominant extracellular matrix protein. Cumulus are also transcriptionally-active (Tirone *et al.,* 1997) and capable of responding to heat stress at the transcriptional level with increased HSP70 mRNA immediately following exposure to 41ºC for 12 h (Chapter 4). Furthermore, cumulus may be thermoprotective by enhancing the ability of bovine oocytes to undergo *de novo* protein synthesis (Edwards and Hansen 1996).

In this regard, direct application of heat stress at 41ºC for the first 12 h of meiotic maturation reduced *de novo* protein synthesis by approximately 30%, coincident with 65% heat-induced reductions in blastocyst development (Edwards and Hansen 1996). Similar findings have been observed for mice; exposure of murine oocytes to 43ºC immediately after removal from ovarian follicles (i.e., at germinal vesicle (GV)-stage) reduced *de novo* protein synthesis by 30 to 80% in a duration-dependent manner (Curci *et al.,* 1987). Moreover, heat stress of matured [i.e., metaphase II(MII)] murine oocytes at 43ºC for 1 h resulted in an approximately 85% reduction in *de novo* protein synthesis (Hahnel *et al.,* 1986). Perturbations of this magnitude are

concerning as protein synthesis is critical for meiotic maturation (i.e., progression from GV-stage to MII) and subsequent development after fertilization (Sirard *et al.,* 1989).

It is currently unknown what components within oocytes are perturbed after direct exposure to elevated temperature that may account for the large reductions in protein synthesis. However, studies with other cell types indicate possible alterations in translational machinery and RNA. First, exposure of HeLa cells to 41-42ºC up to 2 h reduced the proportion of ribosomes actively involved in translation by > 50% with concomitant alterations in the phosphorylation status of translation initiation factors (Duncan and Hershey 1989). Second, heat-stressed cells may substitute the default protein synthetic mechanism in favor of internal ribosomal entry site-mediated translation, involving specialized areas located within certain transcripts for ribosomes to bind (reviewed by Jackson 2005). This method of protein synthesis was utilized in HeLa cells cultured at 42ºC for 5 to 15 h (Kim and Jang 2002). Alternatively, heat stress may induce post-transcriptional alterations in RNA, which would be problematic for subsequent translation. In this regard, exposure to supraphysiological elevated temperatures are capable of inducing RNA degradation as reported for α- and β- tubulin after heat stress of the protozoan, *Tetrahymena pyriformis* (Coias *et al.,* 1988). Heat stress has also been demonstrated to alter the adenylation status of transcripts. For example, heat shock protein 70 (HSP70) transcripts are preferentially deadenylated in heat-stressed *Drosophila* cell cultures (Dellavalle *et al.,* 1994) while avian testicular cells exposed to elevated temperature have increased polyadenylation of HSP70 and ubiquitin RNA (Mezquita *et al.,* 1998). Furthermore, exposure of maturing bovine oocytes to 41ºC for the first 12 h of meiotic maturation may reduce the overall abundance of polyadenylated transcripts (Chapter 4).

Any disruption of oocyte RNA could have a severe impact on subsequent development. Specifically, oocytes become transcriptionally-quiescent upon resumption of meiosis and GV breakdown (Rodman and Bachvarova 1976; Fair *et al.,* 1995; Lodde *et al.,* 2008). Thus, they must rely upon pools of RNA accumulated during the growth phase for protein synthesis (Wassarman and Letourneau 1976) during meiotic maturation, fertilization, and early embryonic cleavage divisions (reviewed by Bettegowda and Smith 2007), until embryonic genome activation. This occurs in bovine embryos at the 8 to 16-cell stage, 4 days after resumption of meiosis (Memili *et al.,* 1998). Any impact of heat stress during meiotic maturation to perturb oocyte RNA would be particularly problematic as polyadenylated transcripts are reduced by > 50% as the oocyte matures (Chapter 4, De Leon *et al.,* 1983; Lequarre *et al.,* 2004) via deadenylation and/or degradation. However, total amount of RNA remains relatively constant (Chapters 3 and 4, Lequarre *et al.,* 2004). Further investigation is necessary to clarify what role maternal transcripts may play in heat-induced reductions in oocyte protein synthesis and subsequent development.

Thus, the objective of this study was to characterize the transcriptome profiles of oocytes and their surrounding cumulus cells after meiotic maturation and in relation to exposure to a physiologically-relevant elevated temperature of 41ºC using microarray analysis. Because oocyte RNA may or may not be polyadenylated during meiotic maturation (Brevini-Gandolfi *et al.,* 1999), two different methods of amplification were employed (3'-poly(A) primed or internal and poly(A) primed) in an effort to clarify changes in polyadenylation during meiotic maturation and in relation to heat stress.

MATERIALS AND METHODS

Collection and treatment of oocyte and cumulus samples

A schematic outlining experimental design is provided in Figure 5-1. Cumulus-oocyte complexes (COC) were collected from 3 to 8 mm follicles between October and May as previously described (Lawrence *et al.,* 2004). Only those having multiple layers of tightly compacted cumulus cells and a homogenous cytoplasm were selected for subsequent use. An experimental unit was a group (pool) of COC cultured together within one well of a 4-well Nunclon culture dish (Fisher Scientific; Pittsburgh, PA, USA). A subset of COC was denuded immediately by vortexing [4.5 min in HEPES-PVA (HEPES-TL (Parrish *et al.,* 1988) supplemented with 0.3% polyvinyl alcohol)] and each oocyte was manipulated individually under a stereomicroscope to ensure complete removal of cumulus. These cumulus-free germinal vesicle (GV)-stage oocytes (n = 50 per pool) and their surrounding cumulus were lysed separately in extraction buffer (from PicoPure RNA isolation kit, Molecular Devices, Sunnyvale, CA, USA; 25 or 200 µl for oocytes and cumulus, respectively) and stored at -80ºC until RNA isolation. Remaining COC were cultured in oocyte maturation medium (Schrock *et al.,* 2007) at 38.5ºC for 24 h (control) or 41ºC for 12 h followed by 38.5ºC for 12 h (heat stress; HS) as indicated in Figure 5-1. After 24 h of in vitro maturation (hIVM), a subset of COC from control and HS treatments was denuded (4.5 min vortex in HEPES-PVA with 0.3% hyaluronidase) and processed as described for GV-stage oocytes, yielding separate oocyte (n = 50) and cumulus pools from each treatment. Remaining COC were fertilized with motile sperm (500,000/ml) from a combination of two *Bos taurus* bulls (Angus, Senepol, or Simmental breeds), denuded at 18 to 20 h post-IVF, and placed into KSOM for subsequent culture as previously described

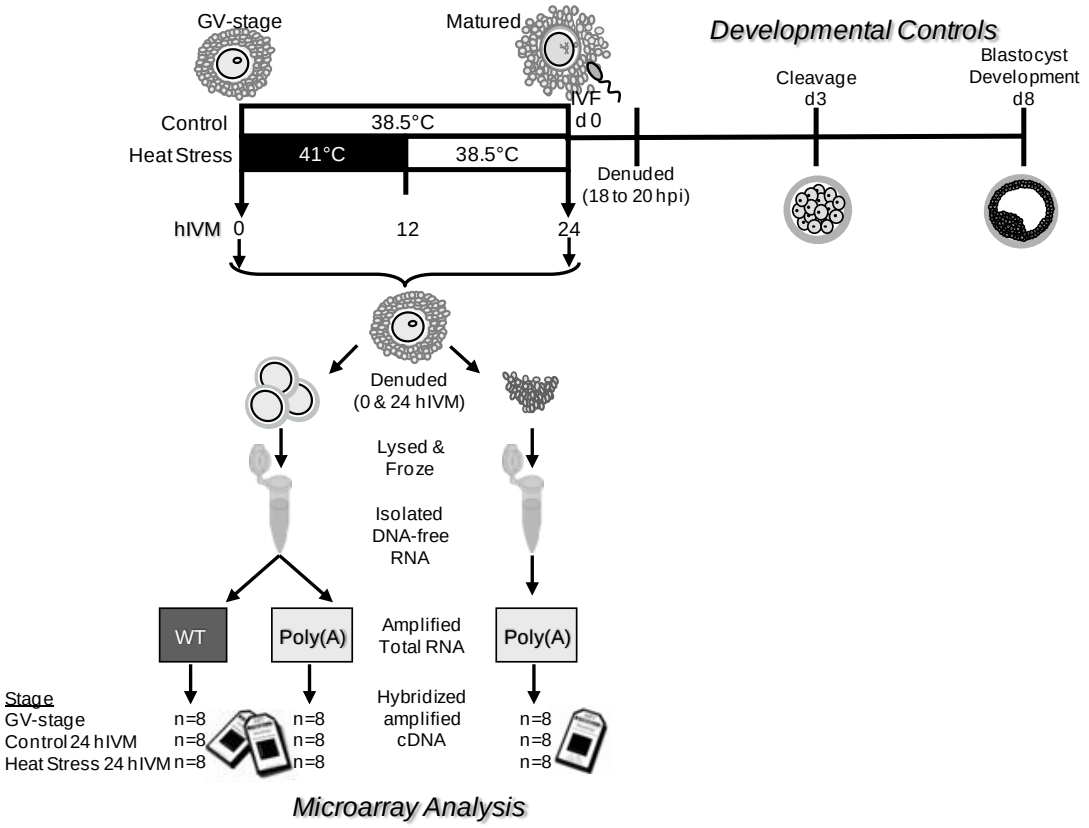


Figure 5-1. Experimental schematic for analysis of oocyte and cumulus transcriptomes. Cumulus-oocyte complexes were randomly allocated to control (38.5ºC for 24 h) or heat stress (41ºC for first 12 hIVM, 38.5ºC for remaining 12 hIVM) treatments. A subset was denuded at GV-stage. Remaining were matured for 24 h before denuding. Oocytes and cumulus were kept separate by stage and treatment, lysed, and stored at -80ºC until RNA isolation. Total RNA was amplified by 3'-poly(A) or internal (whole transcriptome; WT) priming methods prior to biotin-labeling and hybridization to Affymetrix Bovine arrays. Subsets of control and heat-stressed oocytes underwent IVF and subsequent culture to assess developmental potential. Experiment was replicated on 8 different occasions.

(Schrock *et al.,* 2007). Cleavage and blastocyst development were assessed on days 3 (average of 72.25 h post-IVF) and 8 (average of 201 h post-IVF) after fertilization, respectively. Blastocysts were assigned stage and quality scores in accordance with International Embryo Transfer Society standards (Robertson and Nelson 1998).

Of 12 replicates having blastocyst development ≥ 20%, heat-induced reductions in blastocyst development ranged from 0 to 75%. To provide a more precise test of the hypothesis that reductions in embryo development after heat stress exposure during oocyte maturation may be due to heat-induced alterations in oocyte and/or cumulus RNA, only experimental replicates (n = 8 different days of oocyte collection) for which the effect of heat stress was to reduce blastocyst development by > 25% (range 27.1 to 74.9%) were included for microarray analysis.

RNA isolation, amplification, and microarray analysis

Total RNA was isolated from oocytes and cumulus using the PicoPure RNA Isolation kit (Molecular Devices) with on-column DNA digestion (32 units of TURBO DNase, Ambion/Applied Biosystems, Austin, TX, USA). DNase-treated total RNA was eluted in 15 (oocyte) or 25 µl (cumulus) and quantified (NanoDrop ND-1000; Nanodrop Technologies, Wilmington, DE, USA). Integrity of RNA was assessed by microcapillary electrophoresis (RNA 6000 Pico LabChip on 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA) as per manufacturer's instructions with endpoints including RIN value and ratios of rRNA, 18S rRNA to fast region, and 18S rRNA to inter region as previously described (see Chapter 4 for details).

Total RNA yield from oocytes and cumulus was insufficient for microarray analysis without amplification. Because the polyadenylation status of oocyte transcripts may change during meiotic maturation (Chapter 4, Bachvarova and De Leon 1980; Bachvarova *et al.,* 1985;

Lequarre *et al.*, 2004) while the overall abundance of transcripts may remain stable (Su *et al.*, 2007), oocyte RNA was amplified based upon presence of 3' polyadenylation [poly(A)] or was both 3'-poly(A) and internally primed (whole transcriptome; WT). Specifically, total RNA was amplified using either Ovation RNA Amplification System V2 (3'-poly(A) primed; 25 ng oocyte or cumulus total RNA as starting material; NuGEN Technologies, Inc., San Carlos, CA) or WT-Ovation Pico RNA Amplification System (amplified by 3'-poly(A) and internal priming; 10 ng oocyte total RNA as starting material; NuGEN). For cumulus cell samples, 25 ng total RNA was amplified with Ovation RNA Amplification System V2 only. Amplified cDNA was analyzed on RNA 6000 Nano LabChips (Agilent) as per manufacturer's instructions prior to overnight shipment on dry ice to Vanderbilt Microarray Shared Resource (Vanderbilt University, Nashville, TN, USA) for all subsequent processing. Fragmentation and biotinylation were performed using FL-Ovation cDNA Biotin Module V2 (NuGEN; cDNA input of 5 µg for WT amplified and 3.75 µg for poly(A) amplified as per manufacturer's specifications). Samples were then hybridized to GeneChip Bovine Genome Arrays (Affymetrix, Inc., Santa Clara, CA, USA) containing 24,128 probe sets representing ~23,000 transcripts annotated to 11,255 unique Entrez gene identifiers from bovine build 4.0 and 19,000 UniGene clusters (10,775 annotated to a gene) plus 133 control probes. For data acquisition, arrays were scanned using the GeneChip Scanner 3000 7G with AutoLoader and the Affymetrix GeneChip Command Console (Affymetrix). Experiment was designed to be compliant with Minimum Information About a Microarray Experiment (MIAME) standards (Brazma *et al.*, 2001) and replicated 8 different times for a total of 72 chips (Figure 5-1).

Statistical analyses

Embryo development data

Developmental data were analyzed as a randomized complete block with main effect of temperature and random effect of replicate (i.e., day of oocyte collection) using PROC GLIMMIX (SAS 9.1, SAS Institute, Inc., Cary, NC, USA) with a binomial distribution. Stage and quality scores for blastocysts were analyzed similarly but with a normal distribution. Mean separation for all analyses was by F-protected least significant difference test.

Total RNA and amplified cDNA data

Data from microcapillary electrophoresis endpoints were analyzed as a randomized complete block using PROC GLIMMIX with a normal distribution. After amplification, electropherograms were averaged across samples within a treatment and amplification method and overlaid to assess similarities.

Microarray data pre-processing

All quality control (QC) measures, pre-processing and analyses were performed using the statistical computing language R (R Development Core Team, 2008, version 2.8.1) and Bioconductor (Gentleman *et al.*, 2004). Arrays were assessed through standard QC measures for Affymetrix arrays: pseudo-images of the arrays (to detect spatial effects), MA scatter plots of the arrays versus a pseudo-median reference chip, and other summary statistics including histogram and boxplots of raw log intensities, boxplots of relative log expressions and normalized unscaled standard errors, RNA degradation plots, and principal component analysis (Bolstad *et al.*, 2005). Three arrays (i.e., chips) were identified as outliers and not included in the analysis (a 24 hIVM control WT-amplified oocyte, a 24 hIVM HS WT-amplified oocyte, and a GV-stage cumulus; see appendix Figures A1 and A2).

A detection call, which flags each transcript as present, marginal, or absent (Liu *et al.*, 2002; Pepper *et al.*, 2007), was also computed to identify non-expressed probes on all arrays. Detection calls for the probes were calculated with default parameters ($\tau = 0.015$, $\alpha_1 = 0.04$, $\alpha_2 = 0.06$). For each comparison of interest (see details below), all probes called absent were removed from the analysis. Remaining probes were utilized for subsequent differential abundance analysis if called present for at least 6 arrays within each treatment (Table 5- 1). Present calls were similar to those reported by others using the GeneChip Bovine Genome array for oocyte samples (Misirlioglu *et al.*, 2006; Fair *et al.*, 2007). Stage-specific transcripts (i.e., those present at onset of maturation but absent after 24 hIVM and vice versa) were also determined for oocyte and cumulus samples.

A recent study by Harrison et al. (2007) comparing 45 different combinations for background correction, normalization, and summarization of Affymetrix microarray data found the major source of variability in the analysis is the method of summarization used to transform the multiple probe intensities into one measure of expression. To increase specificity, three different summarization methods were used. Transcript intensities were estimated from the probe level data in $\log_2$ scale using the summarization methods VSN (Huber *et al.*, 2002), RMA (Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b), and GCRMA (Wu *et al.*, 2004). Briefly, for VSN summarization, data were adjusted by an affine transformation and $\log_2$ transformed to stabilize the variance. For RMA, following background correction by convolution, data were quantile normalized and summarized by median polish. GCRMA background correction used an affinity measure model based on probe sequences and mismatch intensities. Data were then quantile normalized and summarized by median polish.

Table 5-1. Transcripts called present for at least 6 arrays in different cell types.

	Oocyte		Cumulus
	Poly(A)	Whole Transcriptome	Poly(A)
Stage	#	#	#
GV-stage	12,025	10,738	13,227
Control 24 hIVM	12,494	10,192	14,829
Heat Stress 24 hIVM	12,170	10,127	14,459

Statistical Analysis of Microarray Data

Statistical analyses were performed following the method used by Rowe et al. (2008). Prior to testing for differential expression, data were filtered to remove Affymetrix control probes (n = 133) and probes detected as marginal or absent on all arrays. Differential transcript abundance was tested for each of the three summarization methods using *limma* (v. 2.16.5, Smyth and Speed 2003; Smyth 2004). Only probes having a p-value < 0.01 and called present for at least half of the arrays were considered significant. The intersection of the lists produced by each summarization method resulted in an overall list of transcripts with differential abundance. This approach increased the stringency of the study, improved robustness to experimental noise (Gondro and van der Werf, 2008), and reduced the false discovery rate (FDR). To limit the lists of differentially-abundant transcripts, a fold change of 1.3 (average of individual fold changes from each summarization method) was set for oocytes, as changes of a similar magnitude have been reported to be highly statistically significant (Jin *et al.,* 2001; Wolfinger *et al.,* 2001) and to have functional significance (Wolfinger *et al.,* 2001; Zuccotti *et al.,* 2008). For cumulus cells, lists were limited by differences in relative abundance of at least 2–fold (averaged across all summarization methods) as utilized by others for cumulus/granulosa cells (Hamel *et al.,* 2008; Tesfaye *et al.,* 2009).

Correction for multiple testing (i.e., adjust p-values to control FDR, Benjamini and Hochberg 1995) was attempted despite the already rigorous criteria utilized to control FDR. Doing so resulted in extreme loss of statistical power for oocyte comparisons at 24 hIVM. Others have demonstrated that FDR inflates p-values when fold changes are small due to more even distribution of p-values between 0 and 1 rather than a high proportion around 0 tapering steeply to 1 (Pawitan *et al.,* 2005). Inspection of the p-value distributions indicated that FDR

correction measures would not work for 24 hIVM comparisons (see appendix Figures A3, A4, and A5). Thus, based upon stringent criteria utilized to obtain lists of differentially-abundant transcripts, no FDR correction method for multiple testing was necessary.

Three contrasts were performed to identify transcripts whose abundance may have been altered by heat stress application during the first 12 hIVM: 1) GV-stage versus 24 hIVM control (GV-C), 2) GV-stage versus 24 hIVM heat stress (GV-HS), and 3) 24 hIVM control versus HS (C-HS). Contrasts were carried out within a cell type (i.e., comparing oocytes to oocytes and cumulus to cumulus) and, for oocytes, within an amplification method (i.e., analyzed poly(A) separately from WT).

Functional Profiling

Annotation of differentially expressed probes was performed using an R annotation package from *Bos taurus* build 4.0. Control, undetected, and non-annotated probes were dropped from the analyses to avoid over-inflated p-values. Functional profiles for transcripts having differential abundance were derived for each of the Gene Ontology (GO, Ashburner *et al.,* 2000) categories (cellular component, molecular function, and biological process) by mapping from their Entrez identifier to their most specific GO term and these were used to span the GO tree structure and test for gene-enriched terms. Profiles for each category were also constructed for the differentially expressed transcripts for different tree depths (levels 2 through 5 where level 2 contains less specific terms than level 3 which is less specific than level 4, etc.). Significant GO terms ($P \leq 0.10$ based upon number of significant transcripts versus number expected of total number annotated transcripts within each GO term) were also identified. In addition, transcripts having differential abundance were subjected to KEGG biological pathway analysis (Kanehisa *et al.,* 2006).

RESULTS

Effects of heat stress during meiotic maturation on embryo development

Heat stress during the first 12 h of meiotic maturation did not alter recovery or lysis of putative zygotes (PZ) after removal of associated cumulus cells and spermatozoa (Table 5-2). Overall cleavage rates were similar for embryos from control or heat-stressed oocytes but the proportion of 8 to 16-cell embryos was reduced. Concomitant with this effect was an increase in the proportion of 2- and 4-cell embryos (Table 5-2). Heat stress during the first 12 hIVM reduced blastocyst rates after fertilization by more than 50% (Table 5-2). Reduced ability of heat-stressed oocytes to develop into blastocyst-stage embryos after IVF was not solely due to lower proportions of 8 to 16-cell embryos on day 3 post-IVF, as blastocyst reductions were still evident when expressed as a proportion of 8 to 16-cell embryos (46.1 vs. 32.2%; SEM = 4.1%, $P < 0.03$). Despite heat-induced reductions in blastocyst yield, blastocyst stage was not altered but quality was reduced, as evidenced by higher quality score values (Table 5-2).

Effects of heat stress during meiotic maturation on general features of RNA

The amount of total RNA in oocytes or cumulus did not change during maturation or in response to 41ºC for the first 12 hIVM (Table 5-3). Features of oocyte RNA including RIN value and ratios of rRNA and 18S/fast region were similar across IVM and after heat stress (Table 5-3). Ratio of 18S/inter region was lower at GV-stage than at 24 hIVM (Table 5-3). For cumulus RNA, RIN value and ratios of rRNA and 18S/fast region were lower after 24 hIVM (Table 5-3). No differences in 18S/inter region ratio for cumulus RNA were noted (Table 5-3). Direct comparison of RNA from oocytes to cumulus from the same stage and treatment (e.g., GV-stage oocytes compared to their surrounding cumulus) found that RNA from oocytes had

Table 5-2. Embryo development for oocytes matured at a physiologically-relevant elevated temperature (n = 7 replicates).

		Cleavage						Blastocyst Development			
		18 to 20 hpi*		70 to 74 hpi*				192 to 213 hpi*			
IVM temperature	Total COC	Recovered (%)	Lysed (%)	Cleaved (%)	2-cell** (%)	4-cell** (%)	8 to 16-cell** (%)	Blast/PZ (%)	Blast/Cleaved (%)	Stage	Quality
38.5ºC	610	98.36	0.93	73.79	5.42[b]	8.88[b]	85.64[a]	28.28[a]	39.51[a]	6.45	1.30[a]
41.0ºC***	597	96.09	1.27	68.74	12.04[a]	17.82[a]	70.07[b]	15.15[b]	22.49[b]	6.45	1.68[b]
	SEM	1.11	0.68	4.27	1.57	2.21	2.30	2.03	3.36	0.07	0.11
	P	0.15	0.56	0.14	0.01	0.02	0.0005	0.003	0.004	0.99	0.03

* Hours post insemination (after added sperm to matured oocytes)

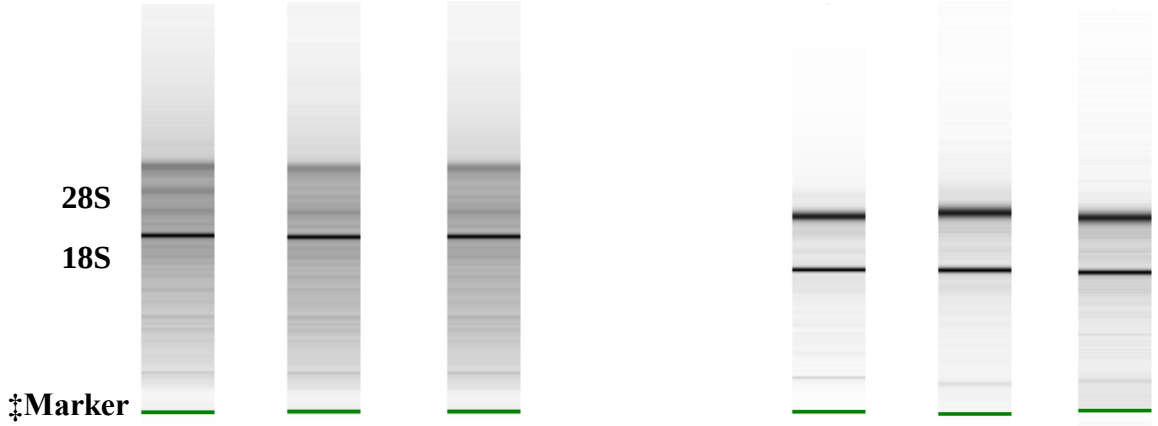
** Relative to proportion cleaved

*** Applied during the first 12 hIVM (38.5ºC thereafter)

[ab] Means different within a column

Table 5-3. Characteristics of total RNA from oocytes and surrounding cumulus cells.

	Oocyte					Cumulus				
Variables	GV-stage	24 hIVM	24 hIVM	SEM*	*P*	GV-stage	24 hIVM	24 hIVM	SEM*	*P*
IVM temp (ºC)	---	38.5	41.0	---	---	---	38.5	41.0	---	---
Total RNA**	3.49	3.15	2.97	0.43	0.69	87.66	80.35	75.87	10.14	0.71
rRNA ratio	0.72	0.76	0.68	0.03	0.27	1.76^{x}	1.41^{y}	1.43^{y}	0.10	0.05
RIN	3.90	3.49	3.30	0.21	0.11	8.38^{x}	7.60^{y}	7.65^{y}	0.13	0.002
18S/fast region ratio	0.17	0.21	0.16	0.02	0.07	0.98^{x}	0.56^{y}	0.64^{y}	0.04	< 0.0001
18S/inter region ratio	0.52^{b}	0.72^{a}	0.69^{a}	0.05	0.02	1.35	1.33	1.30	0.06	0.83



* Pooled standard error of the mean (SEM)

** ng/oocyte or ng/µl (cumulus)

[ab] Different superscripts within a row indicate significant differences ($P \leq 0.05$) for oocytes

[xy] Different superscripts within a row indicate significant differences ($P \leq 0.05$) for cumulus

‡Images are representative

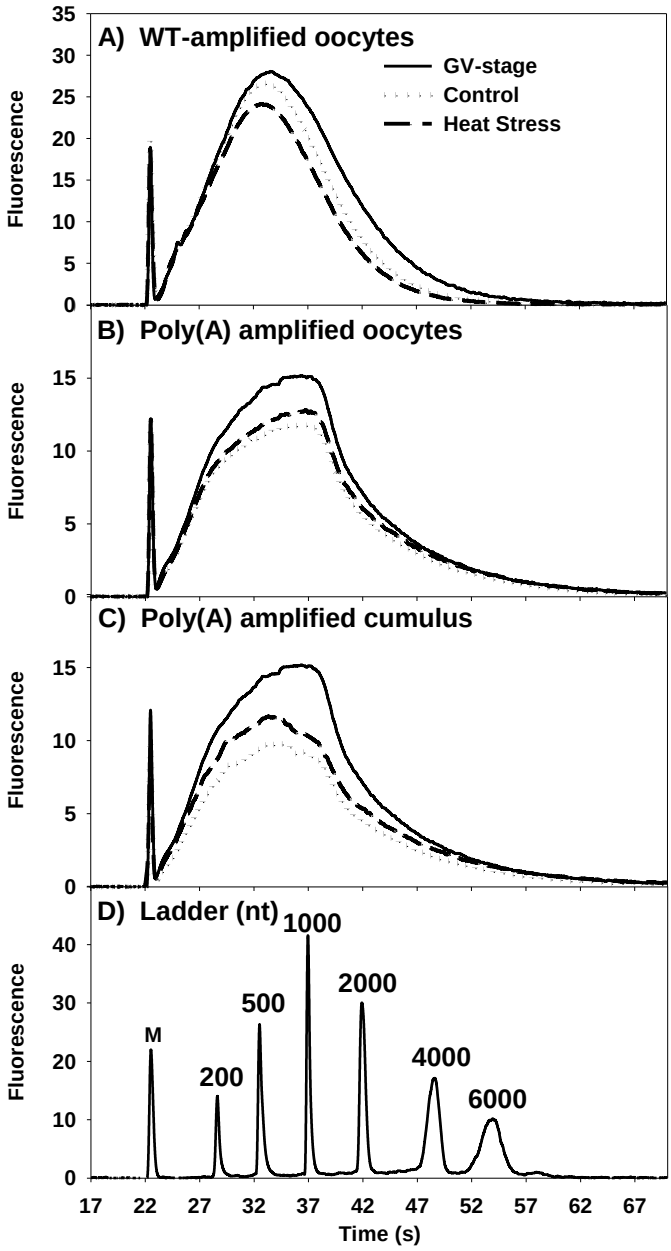
lower values for each endpoint compared to that of their surrounding cumulus ($P < 0.0001$). However, after total RNA isolation and amplification, no apparent differences in size distribution of amplified cDNA were noted regardless of cell type, IVM time (0 or 24 hIVM), or IVM temperature (Figure 5-2).

Relative abundance of oocyte transcripts after IVM and exposure to elevated temperature

Changes observed in polyadenylated transcripts

The first objective was to examine changes in polyadenylated transcripts during meiotic maturation and the potential impact of exposure to elevated temperature during the first 12 hIVM. During meiotic maturation at 38.5ºC (GV-C; i.e., under thermoneutral conditions), the abundance of 8,820 polyadenylated transcripts changed ($P < 0.01$ and fold change ≥ 1.3; Figure 5-3, panel A yellow circle) with 5,021 increasing and 3,799 decreasing. A small proportion of those with differential abundance were also stage-specific (104 present at GV-stage but absent at 24 hIVM; 84 absent at GV-stage but present at 24 hIVM). After culture at 41ºC for the first 12 hIVM, the abundance of 9,133 polyadenylated transcripts changed between GV-stage and matured oocytes (GV-HS; Figure 5-3, panel A orange circle), with 5,102 oocyte transcripts increased and 4,031 decreased during meiotic maturation. A subset of these was stage-specific with 147 present only at GV-stage and 112 present only after 24 hIVM. The majority of the changes occurring between the onset and end of meiotic maturation were similar regardless of IVM temperature; of the 8,476 transcript differences common between GV-C and GV-HS, 4,757 increased and 3,719 decreased during IVM (Figure 5-3, panel A, intersection of yellow and orange circles).

Figure 5-2. Amplified cDNA from total RNA of oocytes and cumulus cells during in vitro maturation and after heat stress exposure. A) Whole transcriptome (WT)-amplified oocyte cDNA at GV-stage (solid line) or after 24 hIVM at 38.5 (control; stippled line) or 41ºC for first 12 hIVM (heat stress; dashed line). B) Poly(A)-amplified oocyte cDNA. C) Poly(A)-amplified cumulus cDNA. D) A ladder is provided with nucleotide length of each peak for estimation of size distribution of cDNA products. M: marker, present for each sample, 25 nucleotides in length.


A) WT-amplified oocytes
GV-stage
Control
Heat Stress
B) Poly(A) amplified oocytes
C) Poly(A) amplified cumulus
D) Ladder (nt)
M
200
500
1000
2000
4000
6000
Fluorescence
Time (s)

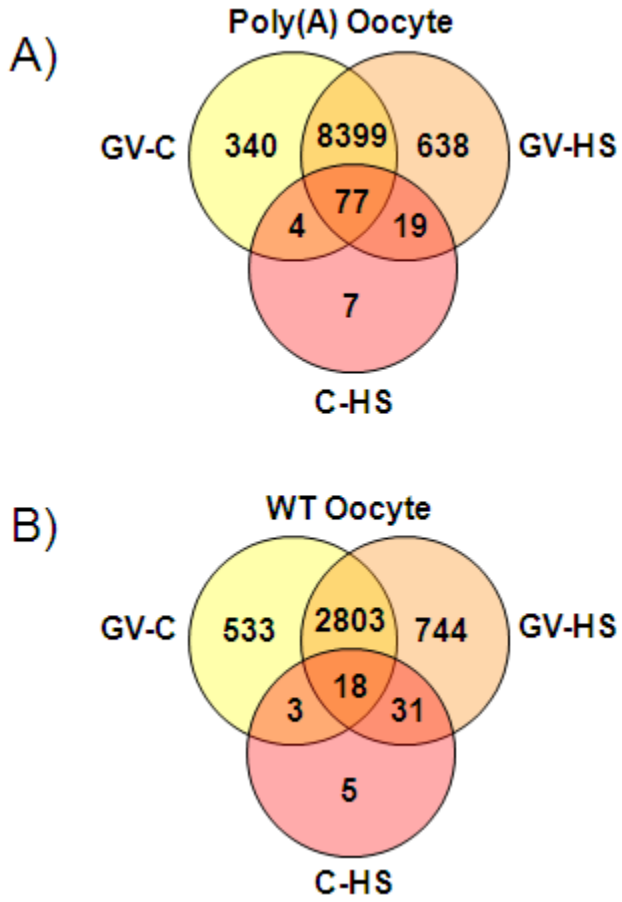


Figure 5-3. Venn diagrams of differentially abundant oocyte transcripts ($P < 0.01$; fold change $\geq$ 1.3). A) Poly(A)-amplified oocyte RNA for each contrast (GV-stage vs. 24 hIVM control (GV-C)= yellow circle, GV-stage vs. 24 hIVM HS (GV-HS)= orange circle, 24 hIVM control vs. HS (C-HS)= red circle), B) WT-amplified oocyte RNA.

As an additional test of the hypothesis that heat stress during meiotic maturation alters maternal stores of RNA that may account for some of the observed reductions in embryo development after fertilization, the polyadenylated transcriptomes of matured oocytes cultured at 38.5ºC for 24 hIVM or 41ºC for the first 12 hIVM (38.5ºC thereafter) were directly compared (C-HS). Exposure of maturing oocytes to 41ºC altered the relative abundance of 107 polyadenylated transcripts with 10 increasing and 97 decreasing in response to heat stress (Figure 5-3, panel A red circle). The majority of these (n = 77) were common between comparisons of GV-stage and matured oocytes (intersection of all 3 contrasts; Figure 5-3, panel A). An additional 4 and 19 transcripts were common between 24 hIVM comparison and GV-stage versus 24 hIVM comparisons (i.e., intersection of C-HS with either GV-C or GV-HS; Figure 5-3, panel A). Very few differentially-abundant transcripts were unique to the C-HS contrast (n = 7; Figure 5-3, panel A).

Changes observed in WT-amplified transcripts

Because not all oocyte transcripts have a poly(A) tail and those that do may undergo changes in adenylation status during meiotic maturation (reviewed by Bettegowda and Smith 2007), a second objective was to examine alterations in the abundance of WT-amplified oocyte transcripts after meiotic maturation, and the potential impact of heat stress during the first 12 hIVM on those transcripts. A total of 3,357 WT-amplified transcripts changed during meiotic maturation after culture at 38.5ºC (GV-C; Figure 5-3, panel B), with 1,378 increasing and 1,979 decreasing. Of these, 64 were stage-specific (61 only present at GV-stage, 3 only present after 24 hIVM). After culture at 41ºC for the first 12 hIVM (38.5ºC thereafter), 3,596 WT-amplified transcripts changed during maturation in heat-stressed oocytes (GV-HS; Figure 5-3, panel B) with 1,458 increasing and 2,138 decreasing. Within those having differential

abundance in matured oocytes previously exposed to heat stress, 65 were stage-specific (61 only in GV-stage oocytes and 4 only in matured oocytes). The majority of the changes occurring during IVM were the same, regardless of IVM temperature (n = 2,803; Figure 5-3, panel B, intersection of GV-C and GV-HS). Of these, 1,048 increased and 1,773 decreased during IVM.

As an additional test, RNA from matured oocytes cultured at 38.5ºC for 24 hIVM or 41ºC for the first 12 hIVM (38.5º thereafter) was directly compared after WT-amplification (C-HS) revealing differential abundance of 57 transcripts (14 increased, 43 decreased; Figure 5-3, panel B). Within these 57, 18 were common to GV-stage versus 24 hIVM comparisons (Figure 5-3, panel B). An additional 3 and 31 transcripts were common between C-HS and GV-C or GV-HS, respectively. The remaining 5 transcripts were only determined to be differentially-abundant by direct comparison of control and HS oocytes at 24 hIVM (Figure 5-3, panel B).

Overlap of differentially-abundant poly(A) and WT-amplified oocyte transcripts

To determine pattern of transcript changes occurring during meiotic maturation between the two amplification methods [i.e., WT versus poly(A)], lists of differentially-abundant WT or polyadenylated transcripts for each contrast (i.e., GV-C, GV-HS, and C-HS) were compared. A total of 2,784 transcripts changed during meiotic maturation at 38.5ºC (GV-C) regardless of RNA amplification method. Of these, 993 increased during IVM while 1,710 decreased. The direction of change in the remaining 81 transcripts was dependent upon amplification method. Specifically, 77 increased if WT-amplified but decreased if poly(A)-amplified; the remaining 4 increased if poly(A)-amplified but decreased if WT-amplified.

When comparing transcript changes during meiotic maturation after exposure to 41ºC for the first 12 hIVM (GV-HS), 2,864 were common between poly(A) and WT-amplified oocyte RNA, with 953 increasing and 1,777 decreasing. The direction of change in the remaining 134

transcripts was dependent upon how RNA was amplified with 122 decreasing if poly(A) amplified but increasing if WT-amplified and 12 increasing if poly(A) amplified but decreasing if WT-amplified. This suggests that the abundance of certain transcripts may be mediated by adenylation rather than degradation. Comparison of poly(A) and WT-amplified transcripts having differential abundance between control and HS oocytes at 24 hIVM revealed only 5 transcripts in common (Table 5-4).

Functional profiling of differentially abundant oocyte transcripts

As a next step, biological themes were extracted from differentially-abundant transcripts by annotation (*Bos taurus* build 4.0) and gene ontology (GO) and KEGG analyses. More than half of the polyadenylated oocyte transcripts detected as having differential abundance were annotated (5,575 of 8,820 for GV-C, 5,958 of 9,133 for GV-HS, and 91 of 107 for C-HS). A similar proportion of WT-amplified transcripts were annotated for comparisons before and after meiotic maturation (2,620 of 3,357 for GV-C and 2,822 of 3,596 for GV-HS). However, comparison of control and HS oocytes at 24 hIVM (C-HS) had too few of the differentially-abundant WT-amplified transcripts annotated, precluding GO and KEGG analyses. Annotated transcripts for all other comparisons were subjected to GO and KEGG analyses.

Comparison of GV-stage oocytes to those that have undergone maturation at 38.5ºC (GV-C for poly(A) transcripts) revealed numerous biological themes occurring during meiotic maturation including those related to regulation of transcription and translation, cell killing, antioxidant activity, and chemoattractant activity among others (Figure 5-4). These terms were virtually identical to those found for the comparison of GV-stage oocytes to 24 hIVM oocytes exposed to heat stress for the first 12 hIVM (GV-HS; data not shown), although the

Table 5-4. Overlap between heat-induced differences in oocyte transcripts after poly(A) or whole transcriptome amplification.*

		Average Fold Change	
Gene Symbol	Gene Name	Poly(A)	WT
RNF139	Ring finger protein 139	1.53	1.43
FAM114A1	Family with sequence similarity 114, member A1	1.42	1.38
LOC615685	UPF0452 protein C7orf41 homolog	-1.79	-1.49
---	Unknown, Grade E annotation	-1.76	-2.07
---	Unknown, Grade E annotation	1.38	1.59

*Negative fold changes increased in heat-stressed oocytes, positive values decreased in heat-stressed oocytes

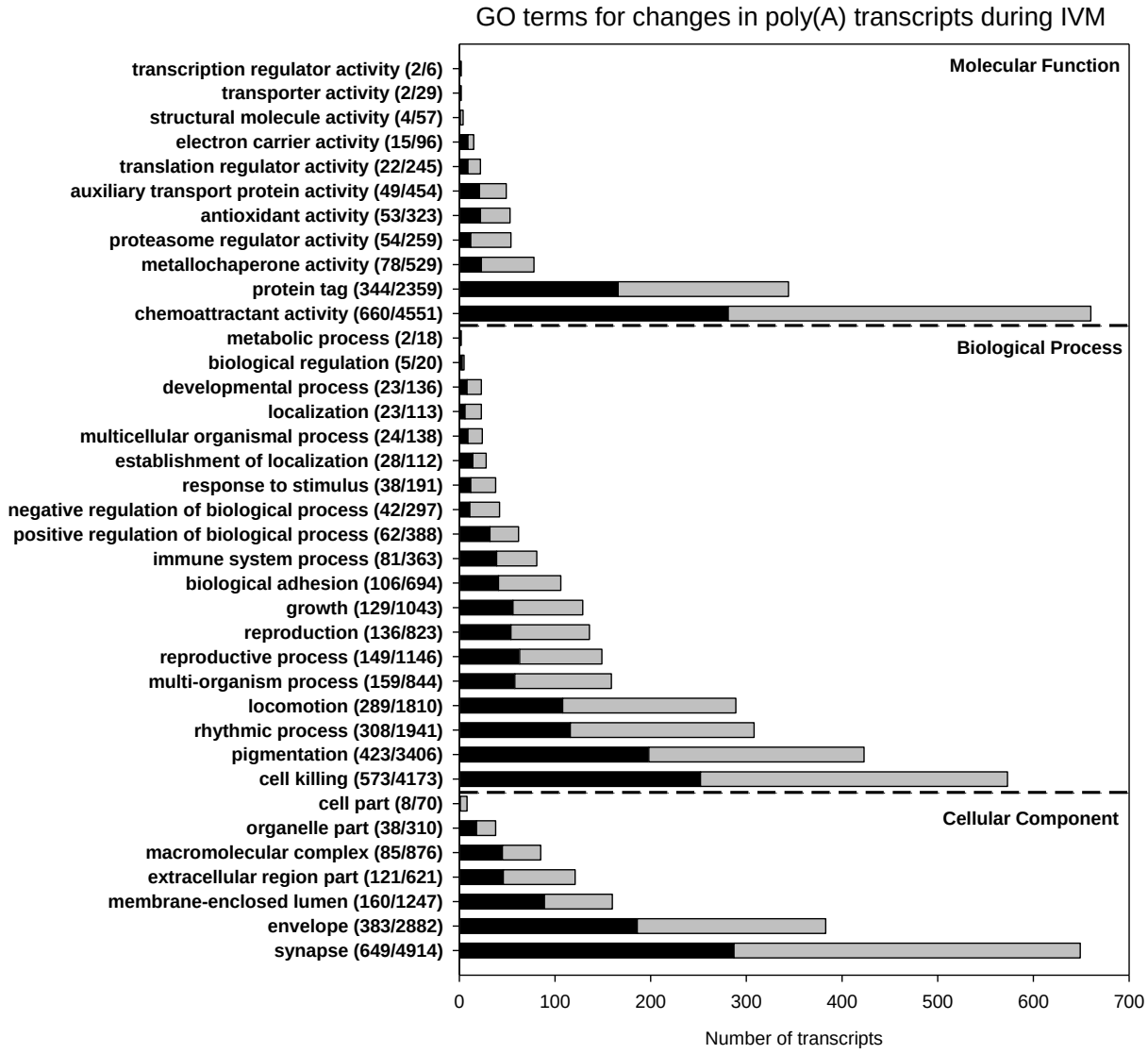


Figure 5-4. Biological themes (level 2 GO terms for molecular functions, biological processes, and cellular components) for polyadenylated transcript changes occurring in oocytes from GV-stage to 24 hIVM after culture at 38.5ºC (GV-C). Black indicates transcripts reduced after meiotic maturation while grey represents transcripts increased after meiotic maturation. Numbers in parentheses after each GO term indicate the number of transcripts having differential abundance out of the total number annotated for that GO term.

number of poly(A) transcripts representing each GO term varied. A number of highly significant (i.e., over-represented based upon number of transcripts expected to be differentially-abundant as a proportion of the number annotated for a given GO term) biological themes were identified to occur in oocytes from GV-stage to 24 hIVM (Table 5-5: molecular functions, Table 5-6: biological processes, and Table 5-7: cellular components). Several of these GO terms were common regardless of IVM temperature (GV-C or GV-HS) or RNA amplification method (poly(A) or WT; Tables 5-5 to 5-7) such as those related to translation (translation regulator activity, translation, structural constituent of ribosome) and energy production (NADH dehydrogenase activity, electron transport chain, ATP synthesis coupled proton transport). A number of significant KEGG pathways for various metabolic processes were identified to occur in oocytes (Table 5-8) including oxidative phosphorylation, fatty acid metabolism, and ubiquitin-mediated proteolysis.

Direct comparison of oocytes matured at 38.5 or 41ºC (for the first 12 hIVM; C-HS) revealed only one over-represented molecular function: pyrophosphatase activity (7 significant of 338 annotated; P = 0.07). However, three biological processes, all involved in mitochondrial function, were identified [ubiquinone biosynthetic process (2 significant of 8 annotated; P = 0.0008), ATP synthesis coupled proton transport (2 significant of 46 annotated; P = 0.03), and oxidation reduction (6 significant of 341 annotated; P = 0.03)]. Specific mitochondrial transcripts involved are listed in Table 5-9. Over-represented cellular component GO terms were ribonucleoprotein complex (6 significant of 329 annotated; P = 0.03) and mitochondrial respiratory chain (2 significant of 57 annotated; P = 0.05); specific transcripts are also listed in Table 5-9. Several KEGG pathways, all involved in mitochondrial function, were over-represented in heat-stressed oocytes compared to 24 hIVM controls [ubiquinone biosynthesis (2

Table 5-5. Top molecular function GO terms* from comparison of GV-stage to 24 hIVM oocytes.

		GV-stage versus Control 24 hIVM (GV-C)						GV-stage versus HS 24 hIVM (GV-HS)					
		Poly(A)-amplified			WT-amplified			Poly(A)-amplified			WT-amplified		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Structural constituent of ribosome	151	63.75	89	<0.0001	29.83	61	<0.0001	65.71	99	<0.0001	32.19	65	<0.0001
Hydrogen ion transmembrane transporter activity	70	29.55	44	0.0004	13.92	30	<0.0001	30.46	45	0.0004	15.02	30	<0.0001
NADH dehydrogenase (ubiquinone) activity	17	7.18	14	0.0009	3.38	9	0.002	7.40	14	0.001	3.65	7	0.05
Translation regulator activity	69	29.13	42	0.002	13.72	24	0.008	30.03	43	0.004	14.81	25	0.009
C-acyltransferase activity	5	2.11	5	0.01	---	---	---	2.18	5	0.02	---	---	---
ATP-dependent helicase activity	44	18.58	26	0.02	8.75	14	0.04	19.15	27	0.01	---	---	---
DNA-dependent ATPase activity	10	4.22	8	0.02	1.99	5	0.03	4.35	8	0.02	---	---	---
Peroxidase activity	25	10.55	16	0.02	---	---	---	10.88	16	0.03	---	---	---
Ubiquitin-protein ligase activity	45	19.00	26	0.03	---	---	---	---	---	---	---	---	---
RNA binding	339	143.12	161	0.03	66.82	88	0.002	147.53	167	0.02	72.10	96	0.001
Nucleoside-triphosphatase activity	315	132.99	164	0.03	---	---	---	137.08	168	0.03	66.09	87	0.07
ATP binding	731	308.61	333	0.03	141.78	159	0.05	318.12	335	0.10	152.99	173	0.03
Unfolded protein binding	52	21.95	29	0.03	10.14	18	0.007	22.63	31	0.01	10.94	20	0.003
Carbon-oxygen lyase activity	26	10.98	16	0.04	---	---	---	11.31	17	0.02	---	---	---
NAD or NADH binding	21	8.87	13	0.05	---	---	---	---	---	---	4.29	8	0.05
Thiolester hydrolase activity	45	---	---	---	---	---	---	19.58	27	0.02	---	---	---
Peroxiredoxin activity	9	---	---	---	1.79	6	0.003	---	---	---	1.93	6	0.004
Cation-transporting ATPase activity	26	---	---	---	5.17	10	0.02	---	---	---	---	---	---
Small conjugating protein ligase activity	57	---	---	---	11.33	18	0.02	---	---	---	12.23	20	0.01
Peptidyl-prolyl cis-trans isomerase activity	27	---	---	---	5.37	10	0.03	---	---	---	5.79	11	0.02
4 iron, 4 sulfur cluster binding	14	---	---	---	2.78	6	0.04	---	---	---	3.00	7	0.02
ATPase activity, coupled	117	---	---	---	---	---	---	---	---	---	25.10	38	0.02
DNA-directed RNA polymerase activity	33	---	---	---	---	---	---	---	---	---	7.08	12	0.04

*Some transcripts may be represented by multiple GO terms. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-6. Top biological process GO terms* from comparison of GV-stage to 24 hIVM oocytes.

		GV-stage versus Control 24 hIVM (GV-C)						GV-stage versus HS 24 hIVM (GV-HS)					
		Poly(A)-amplified			WT-amplified			Poly(A)-amplified			WT-amplified		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Translation	290	123.12	161	<0.0001	59.05	104	<0.0001	126.81	173	<0.0001	63.44	107	<0.0001
Cellular respiration	21	8.92	17	0.0004	---	---	---	9.18	17	0.0006	---	---	---
Lymphocyte homeostasis	7	2.97	7	0.002	---	---	---	3.06	7	0.003	---	---	---
Mitosis	71	30.14	41	0.006	---	---	---	31.05	43	0.003	---	---	---
Electron transport chain	89	37.78	52	0.008	18.04	37	<0.0001	38.92	55	0.003	19.38	35	0.0002
Response to oxidative stress	42	17.83	26	0.008	8.82	13	0.09	18.37	28	0.002	---	---	---
ATP synthesis coupled proton transport	46	19.53	28	0.009	9.84	18	0.005	20.12	28	0.01	10.57	18	0.01
DNA metabolic process	213	90.43	107	0.01	---	---	---	93.14	113	0.003	---	---	---
Protein transport	400	169.82	195	0.02	---	---	---	174.92	206	0.008	---	---	---
Hair follicle development	12	5.09	9	0.02	---	---	---	5.25	9	0.03	---	---	---
ER to Golgi vesicle-mediated transport	16	6.79	11	0.03	---	---	---	7.00	11	0.04	---	---	---
Nuclear export	20	8.49	13	0.04	---	---	---	8.75	13	0.05	4.19	9	0.01
Mitochondrial ATP synthesis coupled electron transport	9	3.82	7	0.04	---	---	---	3.94	7	0.04	---	---	---
Germ cell development	26	11.04	16	0.04	---	---	---	11.37	16	0.05	---	---	---
Response to DNA damage stimulus	121	51.37	62	0.04	---	---	---	52.91	64	0.03	---	---	---
rRNA processing	47	19.95	26	0.05	---	---	---	20.55	30	0.004	---	---	---
Protein import into mitochondrion	6	2.55	5	0.05	---	---	---	2.62	5	0.06	---	---	---
Lymphocyte apoptosis	6	2.55	5	0.05	---	---	---	2.62	5	0.06	---	---	---
Microtubule-based process	94	39.91	44	0.05	---	---	---	41.11	45	0.08	---	---	---
Cellular response to stimulus	149	63.26	75	0.07	---	---	---	---	---	---	---	---	---
RNA processing	244	103.59	119	0.07	---	---	---	106.70	127	0.05	52.65	79	<0.0001
Protein folding	105	44.58	51	0.08	20.50	31	0.006	45.92	55	0.02	22.03	36	0.0009
Cell division	112	47.55	55	0.09	---	---	---	48.98	58	0.05	---	---	---
Protein export from nucleus	8	---	---	---	1.64	6	0.001	---	---	---	---	---	---
Endosome transport	13	---	---	---	2.67	7	0.008	---	---	---	2.86	7	0.01
Glycosaminoglycan metabolic process	8	---	---	---	1.64	5	0.01	---	---	---	1.76	4	0.08
Ribosonucleoside monophosphate biosynthetic process	14	---	---	---	2.87	7	0.01	---	---	---	3.08	7	0.02
mRNA processing	137	---	---	---	28.09	39	0.02	---	---	---	---	---	---
RNA splicing	106	---	---	---	21.73	33	0.02	---	---	---	---	---	---
Nucleobase biosynthetic process	6	---	---	---	1.23	4	0.02	---	---	---	1.32	4	0.02
Tricarboxylic acid cycle	18	---	---	---	3.69	8	0.02	---	---	---	3.96	7	0.08
Glutamine metabolic process	9	---	---	---	1.85	5	0.02	---	---	---	---	---	---
Ribonucleoprotein complex biogenesis	111	---	---	---	22.76	32	0.02	---	---	---	---	---	---

		GV-stage versus Control 24 hIVM (GV-C)						GV-stage versus HS 24 hIVM (GV-HS)					
		Poly(A)-amplified			WT-amplified			Poly(A)-amplified			WT-amplified		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Nucleotide-excision repair	13	---	---	---	2.67	6	0.03	---	---	---	2.86	6	0.05
Double-strand break repair	13	---	---	---	2.67	6	0.03	---	---	---	2.86	8	0.002
Pyrimidine ribonucleotide metabolic process	9	---	---	---	1.85	3	0.04	---	---	---	1.98	4	0.05
Ribosome biogenesis	73	---	---	---	---	---	---	---	---	---	16.08	25	0.01
Intracellular protein transport	204	---	---	---	---	---	---	---	---	---	44.94	62	0.02
Proton transport	53	---	---	---	10.87	21	0.06	---	---	---	11.67	22	0.03
In utero embryonic development	66	---	---	---	13.53	19	0.06	---	---	---	14.54	21	0.03
Monoamine transport	7	---	---	---	---	---	---	---	---	---	1.54	4	0.05
DNA replication	97	---	---	---	---	---	---	---	---	---	21.37	.0	0.05

*Some transcripts may be represented by multiple GO terms. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-7. Top cellular component GO terms* from comparison of GV-stage to 24 hIVM oocytes.

		GV-stage versus Control 24 hIVM (GV-C)						GV-stage versus HS 24 hIVM (GV-HS)					
		Poly(A)-amplified			WT-amplified			Poly(A)-amplified			WT-amplified		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Mitochondrial envelope	265	113.13	159	<0.0001	54.65	91	0.01	---	---	---	58.14	98	0.0008
Intracellular membrane-bounded organelle	3260	1391.66	1533	<0.0001	---	---	---	1433.16	1587	<0.0001	714.24	821	<0.0001
Cytoplasm	3097	1322.08	1466	<0.0001	---	---	---	1361.50	1521	<0.0001	---	---	---
Cell part	5942	2536.58	2605	<0.0001	1223.27	1268	<0.0001	2612.21	2688	<0.0001	1301.21	1350	<0.0001
Proton-transporting ATP synthase complex	20	8.54	16	0.0008	---	---	---	8.79	15	0.005	---	---	---
Centrosome	63	26.89	38	0.003	13.29	19	0.06	27.70	39	0.003	---	---	---
cis-Golgi network	6	2.56	6	0.006				2.64	6	0.007	---	---	---
Microtubule associated complex	38	16.22	24	0.009	---	---	---	16.71	23	0.03	---	---	---
Proton-transporting two-sector ATPase complex, proton-transporting domain	24	10.25	16	0.02	5.49	12	0.004	10.55	15	0.05	5.39	9	0.07
Mitochondrial large ribosomal subunit	15	6.40	11	0.02	3.17	10	0.0002	---	---	---	3.37	9	0.002
Microtubule	75	32.02	41	0.02	15.40	22	0.04	32.97	41	0.04	16.39	23	0.05
Large ribosomal subunit	23	9.82	17	0.04	4.85	14	0.07	10.11	18	0.008	---	---	---
Endoplasmic reticulum membrane	209	89.22	101	0.05	---	---	---	91.88	102	0.09	---	---	---
Chromosome	155	66.17	76	0.06	---	---	---	68.14	80	0.02	---	---	---
Membrane-bounded organelle	3262	1392.51	1534	0.06	671.88	778	<0.0001	---	---	---	714.69	822	0.07
Organelle envelope lumen	15	---	---	---	3.17	6	0.08	6.59	11	0.02	3.37	7	0.03
Exosome (RNase complex)	7	---	---	---	---	---	---	3.08	6	0.03	---	---	---
Macromolecular complex	1105	---	---	---	---	---	---	485.78	556	0.04	---	---	---
Mitochondrial membrane part	76	---	---	---	16.04	40	<0.0001	---	---	---	17.06	39	<0.0001
Ribonucleoprotein complex	320	---	---	---	67.53	111	<0.0001	---	---	---	71.83	124	<0.0001
Cytosolic part	35	---	---	---	7.39	16	0.0009	---	---	---	---	---	---
Clathrin coat of coated pit	5	---	---	---	1.06	4	0.008	---	---	---	1.12	4	0.01
Replication fork	17	---	---	---	3.59	8	0.02	---	---	---	3.82	8	0.02
Nucleolus	117	---	---	---	24.69	34	0.02	---	---	---	26.26	39	0.004
Cytosol	191	---	---	---	40.30	56	0.10	---	---	---	42.87	63	0.0004
Clathrin-coat of trans-Golgi network vesicle	6	---	---	---	---	---	---	---	---	---	1.35	4	0.03
Vacuolar membrane	33	---	---	---	---	---	---	---	---	---	7.41	12	0.05

*Some transcripts may be represented by multiple GO terms. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-8. Significant biological (KEGG) pathways* during IVM for polyadenylated oocyte transcripts.

		GV-stage vs. Control 24 hIVM (GV-C)			GV-stage vs. HS 24 hIVM (GV-HS)		
KEGG pathways	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Oxidative phosphorylation	97	55.43	81	<0.0001	57.05	83	<0.0001
Parkinson's disease	97	55.43	78	<0.0001	57.05	80	<0.0001
Alzheimer's disease	118	67.44	90	<0.0001	69.41	92	<0.0001
Fatty acid elongation in mitochondria	10	5.71	10	0.004	5.88	10	0.005
Selenoamino acid metabolism	13	7.43	12	0.007	7.65	12	0.01
B cell receptor signaling pathway	41	23.43	31	0.01	---	---	---
Fatty acid metabolism	32	18.29	25	0.01	18.82	25	0.02
Ubiquitin mediated proteolysis	101	57.72	68	0.02	59.41	70	0.02
Nucleotide excision repair	35	20.00	26	0.03	20.59	26	0.04
Citrate cycle (TCA) cycle	26	14.86	20	0.03	15.29	20	0.04
Benzoate degradation via CoA ligation	6	3.43	6	0.03	3.53	6	0.04
Valine, leucine & isoleucine degradation	34	19.43	25	0.04	---	---	---
Apoptosis	56	32.00	39	0.04	---	---	---
DNA replication	31	17.72	23	0.04	---	---	---
Methionine metabolism	16	9.14	13	0.04	---	---	---
Aminosugars metabolism	16	9.14	13	0.04	---	---	---
Adipocytokine signaling pathway	44	25.15	31	0.05	---	---	---
Ribosome	67	---	---	---	39.41	54	0.0001
Proteasome	38	---	---	---	22.35	29	0.02
Folate biosynthesis	14	---	---	---	8.23	12	0.03

*Some transcripts may be represented by multiple KEGG pathways. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each KEGG pathway (annotated).

Table 5-9. Heat-induced reductions in polyadenylated oocyte transcripts (C-HS) related to mitochondrial function and spindle stability.

Function & Gene Name	**Average Fold Change***
Electron Transport Chain	
Coenzyme Q5 homolog, methyltransferase (S. cerevisiae)	1.6
Coenzyme Q3 homolog, methyltransferase (S. cerevisiae)	1.5
NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5 kDa	1.5
ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	1.4
Alcohol dehydrogenase, iron containing, 1	1.5
NADH dehydrogenase (ubiquinone) 1 subcomplex unknown, 1, 6 kDa	1.3
Mitochondrial translation	
Mitochondrial ribosomal protein S17	1.8
Mitochondrial ribosomal protein L2	1.4
Mitochondrial ribosomal protein S36	1.3
Response to reactive oxygen species	
Thioredoxin-like 4A	1.8
Spindle stability	
Beta tubulin 2A	1.4
Proline/serine-rich coiled-coil 1 (a.k.a. DDA3)	1.7

*Positive fold changes reduced in heat-stressed oocytes

significant of 5 possible; P = 0.0006), oxidative phosphorylation (4 significant of 97; P = 0.007), and Parkinson's disease (3 significant of 97; P = 0.04), encompassing transcripts listed in Table 5-9.

Heat-induced alterations in polyadenylated cumulus transcripts during oocyte maturation

A third objective was to determine how polyadenylated transcript abundance in the cumulus cells surrounding maturing oocytes changes after maturation and how direct application of heat stress during the first 12 hIVM might alter transcript levels. During meiotic maturation of oocytes at 38.5ºC (GV-C), 2,572 cumulus transcripts changed with 1,556 increasing and 1,016 decreasing (Figure 5-5). A small proportion of these were stage-specific, with 26 present only in non-expanded cumulus from GV-stage oocytes (absent after 24 hIVM) and 168 present only after cumulus expansion at 24 hIVM (absent at onset of IVM). The relative abundance of slightly fewer transcripts (n = 2,303) was altered during the 24 h maturation period when cumulus were heat-stressed for the first 12 hIVM (GV-HS; Figure 5-5). There were 27 present in unexpanded cumulus (i.e., those from GV-stage oocytes) that were absent in expanded cumulus previously exposed to heat stress and 131 only present after 24 hIVM. The majority of changes occurring in cumulus transcripts after 24 hIVM were similar regardless of IVM temperature in that 2,098 (Figure 5-5; 1,270 increased, 840 decreased) differentially-abundant transcripts changed in the same direction for GV-C and GV-HS comparisons.

Comparison of expanded cumulus from control versus heat-stressed oocytes after 24 hIVM (C-HS) found 25 transcripts different by at least 2-fold (Figure 5-5). Specifically, 12 transcripts were increased and 13 were decreased. Of the transcripts determined to be differentially-abundant in expanded cumulus at 24 hIVM, 12 were common among

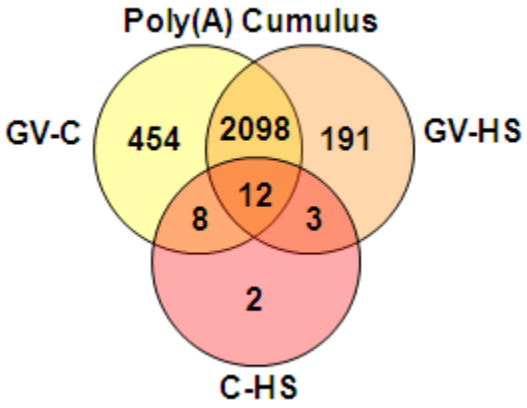


Figure 5-5. Venn diagram of differentially abundant polyadenylated cumulus transcripts ($P < 0.01$; fold change ≥ 2). Each contrast is represented by a different circle: GV-stage vs. 24 hIVM control (GV-C)= yellow circle, GV-stage vs. 24 hIVM HS (GV-HS)= orange circle, 24 hIVM control vs. HS (C-HS)= red circle.

comparisons of cumulus from GV-stage oocytes to 24 hIVM oocytes (i.e., intersection of all 3 comparisons; Figure 5-5). An additional 8 were found to be different only between GV-C and C-HS comparisons while 3 were determined to be different only for GV-HS and C-HS comparisons (Figure 5-5).

Functional profiling of differentially abundant polyadenylated cumulus transcripts

Biological themes were extracted from differentially-abundant cumulus transcripts by annotation in conjunction with gene ontology (GO) and KEGG analyses. More than half of the transcripts determined to be differentially expressed in cumulus cells were annotated (1,606 of 2,572 for GV-C, 1,440 of 2,304 for GV-HS, and 24 of 25 for C-HS). Numerous biological themes were identified to change in cumulus as they underwent expansion during maturation including those related to extracellular region, chemoattractant activity, and cell killing (Figure 5-6). When cumulus were exposed to 41ºC for 12 h during IVM (GV-HS), results were largely unchanged (data not shown). A number of highly significant processes were identified to occur as cumulus underwent expansion during IVM (Table 5-10: molecular functions, Table 5-11: biological processes, and Table 5-12: cellular components). Most of these GO terms were common regardless of IVM temperature (i.e., similar between GV-C and GV-HS comparisons; Figure 5-6; Tables 5-10 to 5-12). Several biological (KEGG) pathways were identified as over-represented in cumulus cells during meiotic maturation of oocytes (Table 5-13) and were consistent with increased cellular replication and extracellular matrix-mediated expansion known to occur for cumulus cells during meiotic maturation of oocytes.

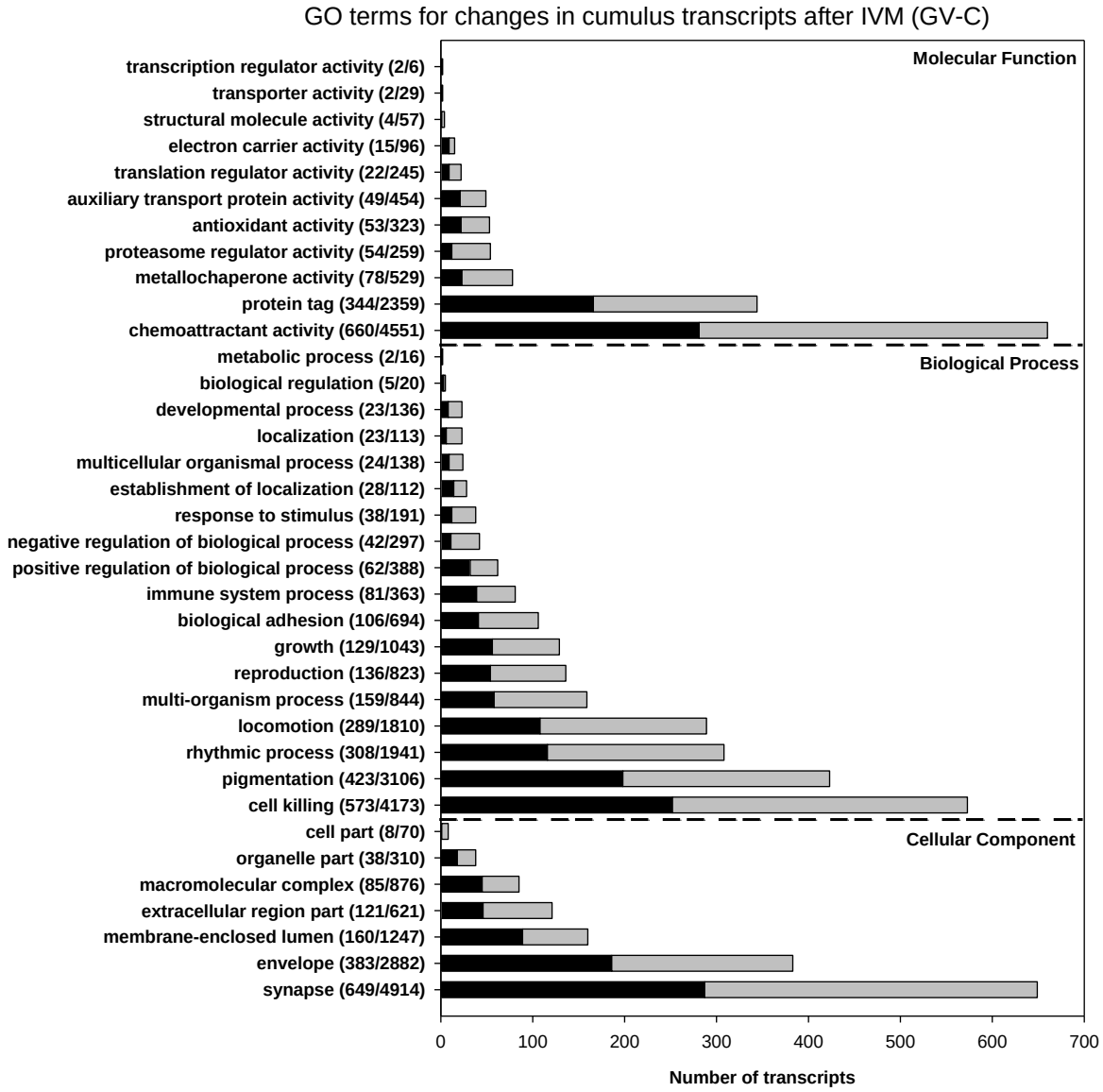


Figure 5-6**.** Biological themes (level 2 GO terms for molecular functions, biological processes, and cellular components) for polyadenylated transcript changes occurring in cumulus cells from GV-stage (unexpanded) versus matured oocytes (expanded) after culture at 38.5ºC (GV-C). Black indicates transcripts reduced after meiotic maturation while grey represents transcripts increased after meiotic maturation. Numbers in parentheses after each GO term indicate the number of transcripts having differential abundance out of the total number annotated for that GO term.

Table 5-10. Top molecular function GO terms* from comparison of cumulus cells from GV-stage or 24 hIVM oocytes.

		GV vs. Control 24 hIVM (GV-C)			GV vs. HS 24 hIVM (GV-HS)		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Growth factor activity	74	8.24	18	0.001	7.40	12	0.06
Steroid dehydrogenase activity	9	1.00	5	0.001	0.90	5	0.0009
Transcription repressor activity	48	5.34	13	0.002	4.80	10	0.02
Insulin-like growth factor binding	19	2.11	7	0.003	1.90	6	0.009
Actin binding	132	14.69	25	0.005	13.21	19	0.07
Endopeptidase inhibitor activity	88	9.79	18	0.007	---	---	---
Phospholipase inhibitor activity	5	0.56	3	0.01	0.50	3	0.009
Cyclin-dependent protein kinase regulator activity	9	1.00	4	0.01	0.90	3	0.05
Calcium ion binding	357	39.74	53	0.02	35.71	52	0.003
Serine-type peptidase activity	69	7.68	14	0.02	---	---	---
Hyaluronic acid binding	10	1.11	4	0.02	---	---	---
Calcium-dependent phospholipid binding	10	1.11	4	0.02	1.00	4	0.01
Protein tyrosine kinase activity	63	7.01	13	0.02	---	---	---
Protein serine/threonine kinase inhibitor activity	6	0.67	3	0.02	---	---	---
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	47	5.23	10	0.03	4.70	10	0.02
Thrombin receptor activity	7	0.78	3	0.03	0.70	3	0.03
Steroid hormone receptor binding	7	0.78	3	0.03	0.70	3	0.03
Sugar binding	49	5.45	10	0.04	4.90	9	0.05
Hormone activity	43	4.79	9	0.04	---	---	---
Receptor signaling protein activity	37	4.12	8	0.05	3.70	7	0.07
Guanylate cyclase activity	8	0.89	3	0.05	---	---	---
Retinoid binding	8	0.89	3	0.05	0.80	3	0.04
Glutathione transferase activity	19	2.11	5	0.05	---	---	---
Protease inhibitor activity	91	---	---	---	9.10	19	0.001
Polysaccharide binding	47	---	---	---	4.70	12	0.002
Oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor	12	---	---	---	1.20	4	0.03
Microtubule motor activity	26	---	---	---	2.60	6	0.04
Steroid hormone receptor activity	34	3.78	7	0.08	3.40	7	0.05
Purine ribonucleotide binding	998	---	---	---	99.84	116	0.05

*Some transcripts may be represented by multiple GO terms. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-11. Top biological process GO terms* from comparison of cumulus cells from GV-stage or 24 hIVM oocytes.

		GV vs. Control 24 hIVM (GV-C)			GV vs. HS 24 hIVM (GV-HS)		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Cell growth	41	4.65	17	<0.0001	4.24	14	<0.0001
Blood vessel morphogenesis	99	11.23	25	<0.0001	10.25	24	<0.0001
Mitosis	70	7.94	19	0.0002	7.25	18	0.0002
Negative regulation of cellular biosynthetic process	116	13.15	25	0.001	12.01	19	0.03
Negative regulation of macromolecule biosynthetic process	109	12.36	23	0.002	11.28	18	0.03
Neurite development	66	7.48	16	0.002	6.83	15	0.003
DNA replication initiation	10	1.13	5	0.003	1.04	4	0.01
Negative regulation of gene expression	104	11.79	23	0.003	---	---	---
Regulation of cell proliferation	166	18.82	33	0.004	17.18	28	0.03
Negative regulation of cell motion	11	1.25	5	0.005	1.14	5	0.003
Regulation of protein complex assembly	32	3.63	9	0.005	---	---	---
Cell morphogenesis involved in neuron differentiation	54	6.12	13	0.006	5.59	13	0.003
Regulation of hormone levels	44	4.99	11	0.006	---	---	---
Steroid biosynthetic process	32	3.63	10	0.009	3.31	9	0.02
Branching morphogenesis of a tube	33	3.74	9	0.009	3.42	9	0.005
Response to hypoxia	28	3.18	8	0.01	2.90	7	0.02
Actin filament polymerization	27	3.06	8	0.01	---	---	---
Smooth muscle cell migration	5	0.57	3	0.01	0.52	2	0.09
Multicellular organismal catabolic process	5	0.57	3	0.01	0.52	3	0.009
Blood vessel endothelial cell migration	9	1.02	4	0.01	---	---	---
Negative regulation of developmental process	153	17.35	28	0.01	---	---	---
Cell division	111	12.59	21	0.02	11.49	21	0.006
Developmental maturation	50	5.67	11	0.02	5.18	11	0.01
Pinocytosis	6	0.68	3	0.02	---	---	---
Midbrain development	6	0.68	3	0.02	0.62	3	0.02
Lymphocyte apoptosis	6	0.68	3	0.02	0.62	4	0.001
Positive regulation of apoptosis	84	9.53	16	0.02	---	---	---
Multi-organism process	126	14.29	23	0.03	---	---	---
Regulation of DNA replication	21	2.38	6	0.03	---	---	---
Lung development	45	5.10	10	0.03	4.66	9	0.04
Neuropeptide signaling pathway	27	3.06	7	0.03	---	---	---
Response to toxin	11	1.25	4	0.03	1.14	3	0.10
Positive regulation of endocytosis	11	1.25	4	0.03	1.14	4	0.02
Gonad development	28	3.18	7	0.03	2.90	7	0.02
Adrenal gland development	7	0.79	3	0.04	---	---	---
cGMP biosynthetic process	12	1.36	4	0.04	---	---	---
DNA fragmentation during apoptosis	12	1.36	4	0.04	1.24	5	0.005
Regulation of neuron differentiation	23	2.61	6	0.04	2.38	7	0.007
Extracellular matrix organization	36	4.08	8	0.05	3.73	7	0.07
Sensory perception of mechanical stimulus	18	2.04	5	0.05	---	---	---
Hair follicle development	13	1.47	4	0.05	---	---	---
Collagen metabolic process	13	1.47	4	0.05	---	---	---
Acute-phase response	8	0.91	3	0.05	0.83	3	0.04
Response to estrogen stimulus	8	0.91	3	0.05	0.83	3	0.04
Protein amino acid phosphorylation	354	40.14	51	0.05	---	---	---
Negative regulation of survival gene product expression	5	---	---	---	0.52	3	0.009
T cell homeostasis	6	---	---	---	0.62	3	0.02
Embryonic limb morphogenesis	23	---	---	---	2.38	6	0.03
Response to antibiotic	7	---	---	---	0.72	3	0.03
Ovulation cycle process	18	---	---	---	1.86	5	0.03
Regulation of anatomical structure morphogenesis	108	---	---	---	11.18	26	0.03
Regulation of mitochondrial membrane potential	8	---	---	---	0.83	3	0.04

*Some transcripts may be represented by multiple GO terms.

Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-12. Top cellular component GO terms* from comparison of cumulus cells from GV-stage or 24 hIVM oocytes.

		GV vs. Control 24 hIVM (GV-C)			GV vs. HS 24 hIVM (GV-HS)		
GO term	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Extracellular region	611	66.71	121	<0.0001	60.31	111	<0.0001
Extracellular space	139	15.18	24	0.02	---	---	---
Microsome	64	6.99	13	0.02	6.32	10	0.10
Replication fork	17	1.86	5	0.03	---	---	---
Nucleosome	29	3.17	7	0.03	2.86	6	0.06
Sarcolemma	25	2.73	6	0.05	2.47	5	0.09
Lamellipodium	19	2.07	5	0.05	---	---	---
Proteinaceous extracellular matrix	129	14.08	23	0.05	---	---	---
Mitochondrial outer membrane	35	3.82	7	0.08	3.45	9	0.006
Microfibril	6	---	---	---	0.59	3	0.02
Myofibril	35	3.82	7	0.08	3.45	8	0.02
Basement membrane	29	---	---	---	2.86	7	0.02
Apical part of cell	35	---	---	---	3.45	7	0.05
Vesicle membrane	58	---	---	---	5.73	9	0.05

*Some transcripts may be represented by multiple GO terms. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each GO term (annotated).

Table 5-13. KEGG pathways* over-represented in cumulus cells before or after IVM.

		GV vs. Control 24 hIVM (GV-C)			GV vs. HS 24 hIVM (GV-HS)		
KEGG pathways	**Annotated**	**Expected**	**Significant**	**P**	**Expected**	**Significant**	**P**
Cell cycle	84	14.98	30	<0.0001	13.21	25	0.0007
ECM-receptor interaction	47	8.38	18	<0.0001	7.39	18	0.0001
Regulation of actin cytoskeleton	104	18.55	31	0.002	16.36	24	0.03
Arachidonic acid metabolism	26	4.64	10	0.01	4.09	8	0.04
Focal adhesion	118	21.04	30	0.02	18.56	27	0.02
PPAR signaling pathway	50	8.92	15	0.02	7.86	14	0.02
DNA replication	32	5.71	10	0.05	---	---	---
Androgen and estrogen metabolism	12	2.14	5	0.05	1.89	5	0.03
p53 signaling pathway	41	7.31	12	0.05	6.45	13	0.008
Glycine, serine, & threonine metabolism	29	---	---	---	4.56	9	0.03
Bladder cancer	29	---	---	---	4.56	9	0.03

*Some transcripts may be represented by multiple KEGG pathways. Significance was determined by the number of differentially-abundant transcripts observed (significant) in relation to the number expected to be different by random chance (expected) and the number annotated for each KEGG pathway (annotated).

Only one over-represented molecular function was identified by comparison of cumulus cells from control and HS oocytes at 24 hIVM (C-HS)—growth factor activity (2 significant of 74 annotated (P = 0.01). However, several biological processes were over-represented including defense response (4 significant of 144 annotated; P = 0.0002), nitric oxide biosynthetic process (2 significant of 21 annotated; P = 0.001), neuropeptide signaling pathway (2 significant of 27 annotated; P = 0.002), response to hypoxia (2 significant of 28 annotated; P = 0.002), negative regulation of cell differentiation (2 significant of 52 annotated; P = 0.006), anatomical structure development (6 significant of 724 annotated; P = 0.04), and metal ion transport (2 significant of 165 annotated; P = 0.05). Cellular component terms included perinuclear region of cytoplasm (2 significant of 37 annotated; P = 0.003), extracellular region (7 significant of 611 annotated; P = 0.03), and Golgi membrane (2 significant of 151 annotated; P = 0.05). Furthermore, KEGG pathway analysis revealed that hedgehog signaling was over-represented in cumulus from oocytes exposed to elevated temperature during the first 12 hIVM compared to 24 hIVM controls (2 significant of 20 possible, P = 0.002).

DISCUSSION

To our knowledge, this is the first study to examine the effects of elevated temperature on maternal transcripts during meiotic maturation as well as heat-induced transcript alterations in surrounding cumulus using microarray technology. Inclusion of oocytes and cumulus immediately after removal from the follicle provided the ability to compare the changes in transcripts after culture at 38.5ºC versus 41ºC for the first 12 hIVM. In addition, the experimental design allowed for the unique opportunity to compare the differences in relative

abundance of transcripts detected when all oocyte RNA was amplified (WT) versus only those transcripts having poly(A) tails.

While this is the second study to examine transcripts in bovine oocytes before and after meiotic maturation using the Affymetrix platform (Fair *et al.,* 2007), it substantially adds to those findings by providing information regarding stage-specific transcripts as well as those whose abundance is regulated by changes in adenylation status. No gene lists were provided by Fair et al. (2007), making it impossible to assess these findings in the context of others also using the bovine Affymetrix array. While studies comparing GV-stage oocytes to those after meiotic maturation have been performed in other species (Cui *et al.,* 2007; Su *et al.,* 2007; Wells and Patrizio 2008), none have presented stage-specific transcripts. Thus, identification of transcripts present only before or after meiotic maturation is a novel aspect of the study presented herein. Presence of some transcripts only in matured oocytes is difficult to explain, as oocytes are largely transcriptionally-silent upon meiotic resumption. A brief period of transcription prior to breakdown of the GV (Rodman and Bachvarova 1976; Memili *et al.,* 1998; Lodde *et al.,* 2008) could be responsible for observation of transcripts in matured oocytes that were absent at GV-stage. Another explanation would be increased or decreased polyadenylation of specific transcripts during meiotic maturation (reviewed by Bachvarova 1992).

Comparison of the complete mRNA transcriptome (WT-amplified) to polyadenylated is a novel aspect of this study. The vast majority of RNA amplification methods rely upon the presence of a poly(A) tail for a transcript to be amplified. However, it is well-established that maternal transcripts within the oocyte are stored for lengthy periods of time (Jahn *et al.,* 1976), some with short or no poly(A) tails (reviewed by Kashiwabara *et al.,* 2008). With this in mind,

the entire oocyte transcriptome, not only the portion having adenylation at the 3' end of transcripts, was of interest and was the impetus for WT amplification.

The abundance of a small subset of transcripts was dependent upon amplification method [i.e., increased if amplified by one method (e.g., WT) but decreased if amplified by the other (e.g., poly(A) primed)]. While previous microarray analysis of murine oocytes using a WT amplification approach identified transcripts that were degraded as oocytes progressed from GV-stage to MII, differences related to presence or absence of a poly(A) tail were not examined (Su *et al.*, 2007). Our findings suggest that the abundance of certain transcripts may be regulated by adenylation rather than degradation. For example, GAPDH increased if RNA was amplified by 3'poly(A) and internal primers (WT) but decreased if only 3'-poly(A) amplified (data not shown). A previous report examining housekeeping genes in bovine oocytes by qPCR determined that GAPDH undergoes deadenylation from GV-stage to MII (Bettegowda *et al.*, 2006). Because most other transcripts listed have not been characterized on a global (i.e., WT) versus polyadenylated level, these are novel findings. Several transcripts with amplification-dependent differential abundance are known to be important for meiotic maturation and fertilization such as CDC6, a necessary factor for DNA replication (Lemaitre *et al.*, 2004) and Zwilch, a protein involved in chromosome segregation (Karess 2005) such as occurs during meiosis.

When differentially-abundant WT and polyadenylated transcripts from control versus heat-stressed oocytes (C-HS) were compared, only 5 were common (Table 5-4). Ring finger protein 139 (RNF139) is a target of post-transcriptional regulation in pachytene spermatocytes of mice (Cho *et al.*, 2005) while FAM114A1 has been implicated in Stargardt disease (Ala *et al.*, 2008), a form of macular degeneration (Koenekoop *et al.*, 2007). Neither RNF139 or

FAM114A1 have any reported function in oocytes or reproduction. None of the remaining 3 have any known function (Table 5-4). This provides the opportunity to further characterize these transcripts and their importance in oocyte maturation and subsequent development.

Finding so little overlap between poly(A)- and WT-amplified transcripts was interesting since the RNA used for each amplification was from the same initial oocyte pool (i.e., was identical) and suggests that results will vary depending upon how the RNA is amplified before hybridization. There are several potential reasons for different results to have occurred. Comparison of hybridization intensities for individual genes after different amplification methods revealed that transcripts for some genes are predominantly polyadenylated (Figure 5-7, panel A) while others have varying levels of polyadenylated transcripts compared to overall transcript abundance (Figure 5-7, panel B). Transcripts for GDF9 decreased during IVM if poly(A)-amplified but were similar throughout IVM when WT-amplified. This suggests that GDF9 mRNA may be regulated by changes in polyadenylation status rather than changes in the overall abundance of transcripts (i.e., by degradation). Other studies have indicated that translation in oocytes is largely controlled by changes in polyadenylation status (reviewed by Piccioni *et al.,* 2005). The importance of polyadenylation during oocyte maturation is highlighted by the finding that inhibition of cyclin B1 polyadenylation prevents germinal vesicle breakdown and subsequent progression through meiosis (Traverso *et al.,* 2005). The length of poly(A) tails on specific transcripts has also been correlated with developmental potential, with higher levels of polyadenylation associated with increased embryo development (Brevini-Gandolfi *et al.,* 1999). Another potential explanation of differences lies in the design of the microarray. While the Affymetrix Bovine array offered the most genome coverage at the time of analysis, probe design is biased towards the 3' end of the transcript near where the poly(A) tail

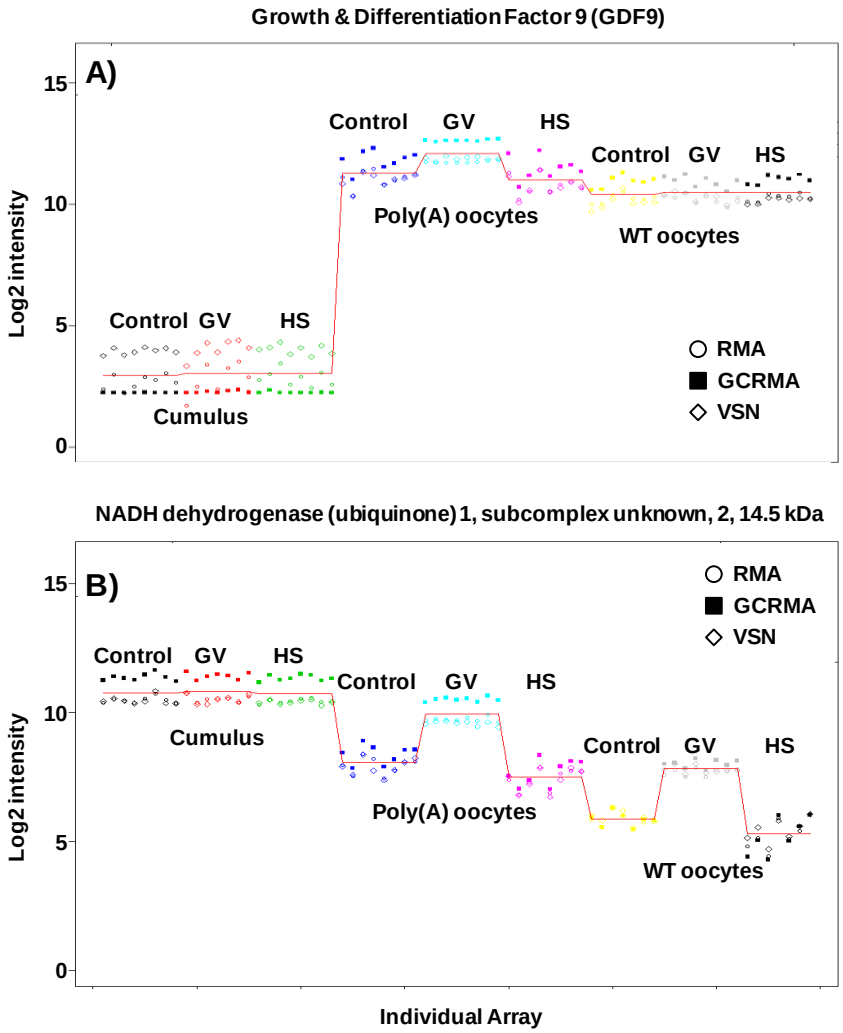


Figure 5-7. Log_2 intensity plots to illustrate differences in transcript abundance depending upon amplification method and cell type. Within each plot from left to right are cumulus, poly(A)-amplified oocytes, and WT-amplified oocytes. Within each cell type and treatment are the intensity values for a specific transcript (y-axis) on each individual array (x-axis) for each summarization method (as indicated in legend on plot). A) Intensity plot for GDF9. This transcript is absent in cumulus cells but present in oocytes. B) Intensity plot for NADH dehydrogenase.

would be for polyadenylated transcripts. This may result in an increased ability of polyadenylated transcripts to hybridize. Different amounts of input RNA were also utilized for amplification (25 ng for poly(A) vs. 10 ng for WT); this was necessary to maintain linear amplification within each methodology. However, direct comparison of WT versus polyadenylated transcripts was still informative, in general terms, of differential regulation of gene subsets by degradation versus deadenylation.

The numerous changes in oocyte transcripts from GV-stage to 24 hIVM likely reflect the dynamic events occurring during meiotic maturation. In this regard, transcripts involved in transcriptional regulation (USF2, MYB, IRF1, POLD2, GTF2F1, NR2F2, AHR), chromatin modification (SMARCAL1), apoptosis (CD97, CLU, ZDHHC16, CTNNBL1, DNASE2, BCAP31, PSEN1), protein folding (HSPA1B, CCT3), signal transduction (GNAS1, SLC25A3, GABARAP, SLC2A1, VDAC3, CLTA, COPZ1, CLIC4), and electron transport (MAOA, UQCRC2, TXN2, NDUFB9, SDHB) identified in matured bovine oocytes by Misirlioglu et al. (2006) were also detected in oocytes at 24 hIVM herein. Subsequent qPCR analysis validated findings for transcripts such as SMARCAL1 (Misirlioglu *et al.*, 2006). In addition, transcripts important for processes such as oxidative phosphorylation, protein synthesis, and protein folding were present during maturation of murine oocytes (Su *et al.*, 2007), in agreement with findings reported here. Agreement of our work with other bovine and murine oocyte studies indicates not only the conservation of molecular changes underlying meiotic maturation, but also provides validation of the methodologies and analyses employed herein.

A key premise of this study was that heat-induced alterations in maternal transcripts may have consequences on subsequent embryo development. The samples used for arrays had heat-induced reductions in blastocyst development of at least 25%. This maximized our ability to

correlate developmental potential with heat-induced alterations in maternal transcripts since differences between control and HS oocytes and cumulus at 24 hIVM were subtle (majority of fold changes for oocytes at 24 hIVM < 1.5). However, several lines of evidence suggest that small fold changes are to be expected. Proteomic analysis of control and heat-stressed oocytes using 2-D DIGE revealed small (< 1.6) fold changes in protein abundance (West-Rispoli *et al.*, 2006). Small fold changes have been associated with large functional differences; a 1.4 fold decrease in Stella transcripts within MII murine oocytes was associated with embryo arrest at the 2-cell stage (Zuccotti *et al.*, 2008).

Multiple polyadenylated transcripts associated with various mitochondrial functions (Table 5-9) were reduced in heat-stressed oocytes compared to 24 hIVM controls. Furthermore, the only KEGG pathways altered by heat stress involved mitochondria. Heat-induced alterations in cytoplasmic processes during meiotic maturation (e.g., cortical granule translocation to the oolemma) have been reported (Edwards *et al.*, 2005), leaving open the possibility for similar perturbations in mitochondria. Others have demonstrated that healthy mitochondria are necessary for normal oocyte maturation (Takeuchi *et al.*, 2005) and that mitochondrial dysfunction in oocytes is detrimental for subsequent embryo development (Thouas *et al.*, 2004) and pregnancy (Thouas *et al.*, 2006). This is not unexpected given that embryos inherit > 99% of cytoplasm and half of genome from the oocyte (Smith and Alcivar 1993).

The culmination of changes occurring in mitochondrial transcripts is illustrated in Figure 5-8, whereby reduced transcripts for electron transport chain components may alter the ability of heat-stressed oocytes to produce energy (i.e., ATP). This is supported by reduced mitochondrial membrane potential in oocytes exposed to 41ºC for the duration of meiotic maturation (Soto and Smith 2009) and preliminary results suggesting that ATP levels in heat-stressed oocytes may be

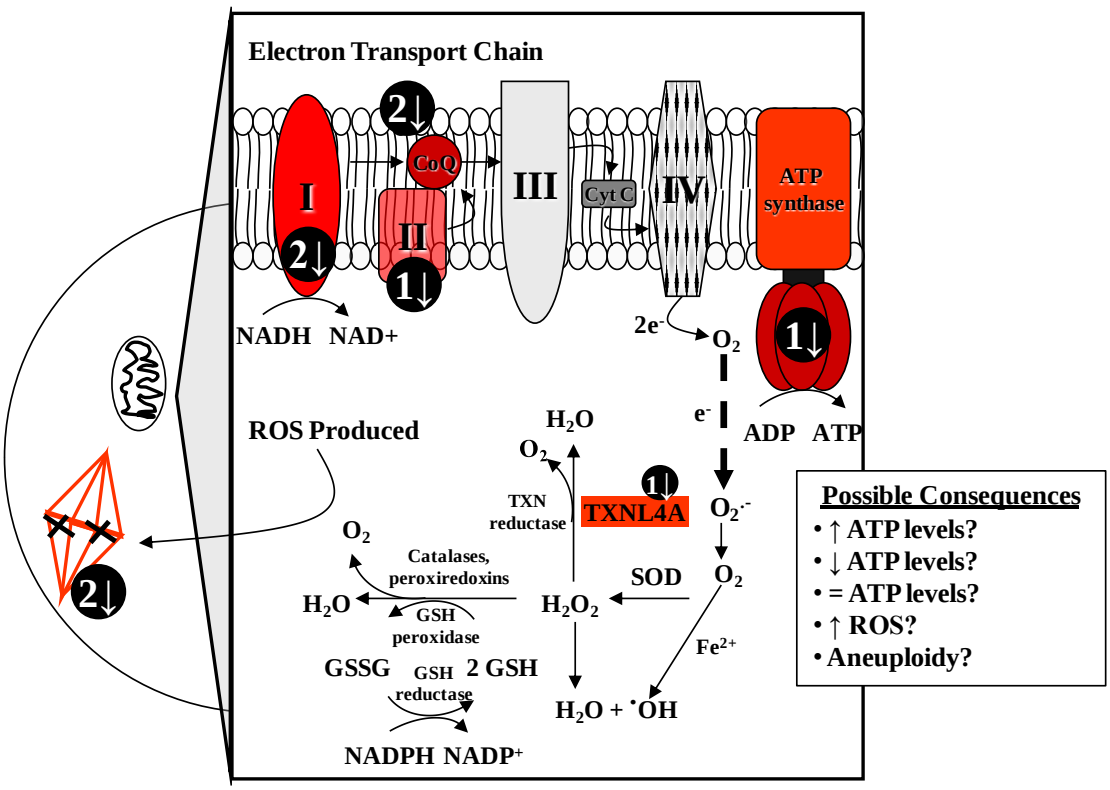


Figure 5-8. Proposed model of heat-induced alterations in mitochondrial-related transcripts and spindle formation with possible functional consequences. Components with transcripts reduced by heat stress are shown in red with the number of transcripts affected indicated in each circle.

increased at 24 hIVM (unpublished), indicative of more mitochondrial activity after exposure to elevated temperature. Higher ATP levels in the oocyte are correlated with better embryo development and quality (Stojkovic *et al.,* 2001) as well as improved pregnancy rates (Van Blerkom *et al.,* 1995). Low ATP is characteristic of oocyte aging (Fissore *et al.,* 2002). Oocytes with increased mitochondrial activity (i.e., producing more ATP) have reduced developmental potential after fertilization (Torner *et al.,* 2008) similar to that observed with heat stress and oocyte aging. It is unclear how reductions in mitochondrial transcripts observed in oocytes after exposure to physiological elevated temperature may affect mitochondrial proteins. However, heat-induced perturbation of transcripts necessary for energy production via oxidative phosphorylation may alter the capacity of embryos derived from heat-stressed oocytes to undergo a normal developmental regimen, ultimately resulting in reduced blastocyst development.

Byproducts of oxidative phosphorylation are reactive oxygen species (ROS; Figure 5-8). Several lines of evidence suggest that heat stress increases ROS levels, possibly via disruption of the electron transport chain. Heat stress of bovine embryos has been demonstrated to increase ROS production (Sakatani *et al.,* 2004; Sakatani *et al.,* 2008). Use of the antioxidant retinol while culturing oocytes at elevated temperature during maturation improved blastocyst development (Lawrence *et al.,* 2004), suggestive of heat-induced increases in oocyte ROS. Reduced abundance of polyadenylated thioredoxin-like 4A transcripts observed for heat-stressed oocytes suggests response to ROS. Addition of thioredoxin to embryo culture medium immediately following fertilization improved morula and blastocyst development in mice (Tokura *et al.,* 1993), indicating its importance for *in vitro* development and further indicating the potential for heat stress of oocytes to induce ROS.

Any heat-induced mitochondrial dysfunction may also impact microtubule-containing structures within the oocyte. Mitochondria-derived ATP is necessary for proper spindle morphology (Zhang *et al.,* 2006). Reductions in tubulin and microtubule-associated transcripts within heat-stressed oocytes (Table 5-9) are concerning due to implications for spindle stability, which was altered in maturing bovine oocytes after direct heat stress application (Roth and Hansen 2005). Proline/serine-rich coiled-coil, better known as DDA3, is involved in microtubule polymerization (Hsieh *et al.,* 2007) and helps control spindle dynamics and chromosome movement by regulating microtubule depolymerases (Jang *et al.,* 2008). If reduced transcript abundance is indicative of perturbed protein levels, integrity of meiotic and mitotic spindles in heat-stressed oocytes and subsequent embryos may be compromised, potentially resulting in altered ploidy (Figure 5-8). While the heat stress model employed in this study allows for progression to metaphase II at a rate similar to non-stressed controls (Edwards *et al.,* 2005), the possibility for anomalies such as aneuploidy exist. Heat stress of mice during meiotic maturation produced a higher rate of aneuploidy concomitant with increased embryo death before and after implantation compared to non-stressed controls (Baumgartner and Chrisman 1988). Developmental stages at which embryonic loss occurs in cattle are similar to those reported for mice (Putney *et al.,* 1988; Chebel *et al.,* 2004). Thus, heat-induced cytogenetic changes in bovine oocytes may be occurring to elicit some of the reductions in fertility of heat-stressed cattle.

While much of the research regarding improvements in fertility focuses on oocytes and embryos, cumulus cells surrounding the oocyte are an emerging area of interest. Transcripts in cumulus may be informative markers of oocyte competence as gene expression in surrounding cumulus of oocytes matured *in vitro* differed from that of those matured *in vivo* (Tesfaye *et al.,*

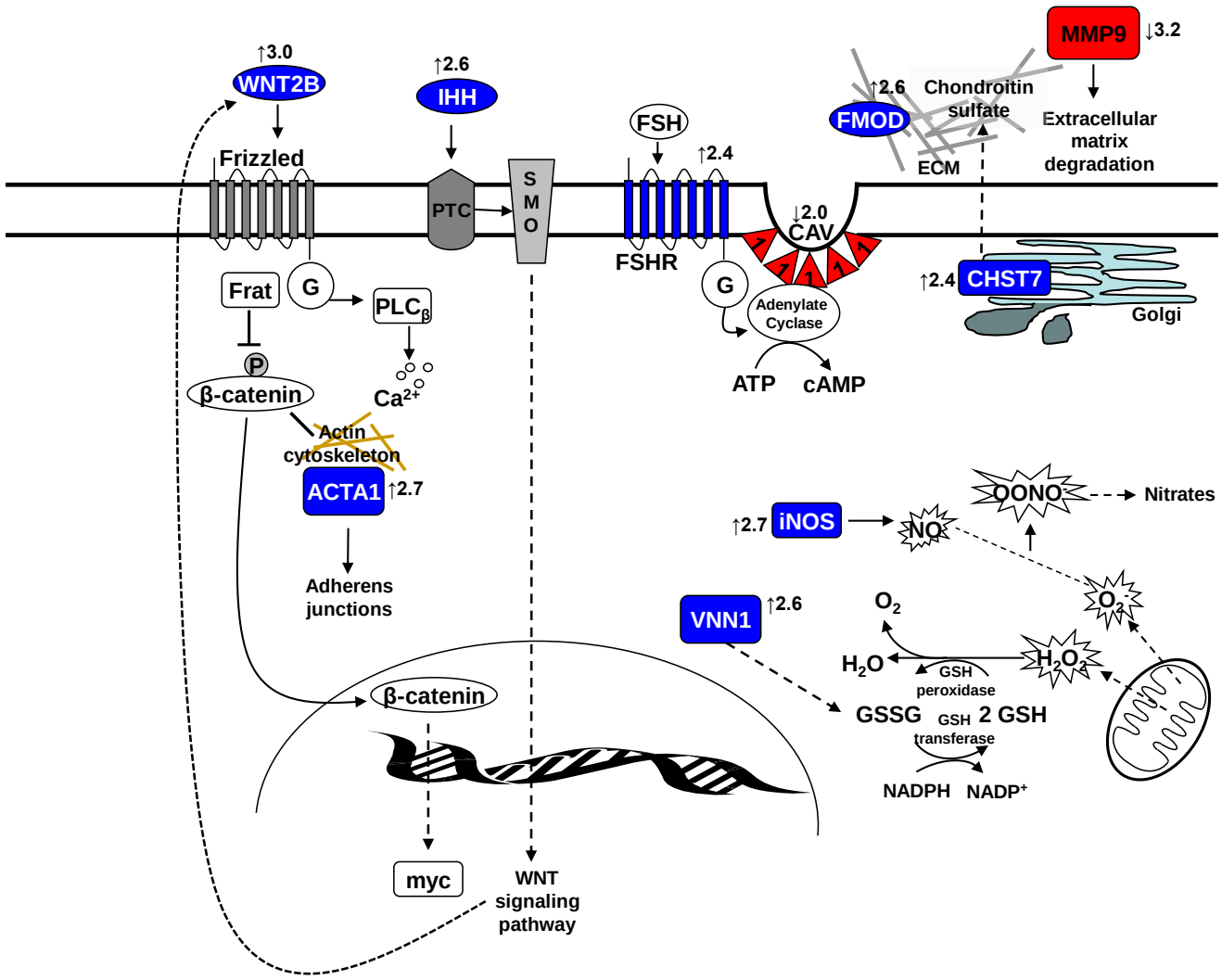
2009); *in vivo* matured oocytes have higher rates of embryo development than those matured *in vitro* (Rizos *et al.,* 2002). The ability to biopsy cumulus cells without disrupting the oocyte provides the ability to identify cumulus markers of oocyte competence. Doing so would be advantageous not only for mitigation of heat-induced reductions in oocyte developmental competence, but also to ameliorate reduced fertility resulting from other causes.

Transcriptional changes occurring in cumulus during oocyte maturation were consistent with phenotypic changes associated with expansion. Transcripts for extracellular matrix constituents like matrix metallopeptidases, collagen, and plasminogen activators were present in expanded cumulus but not in the unexpanded cumulus mass at the onset of oocyte maturation. Other transcripts important for cell growth such as insulin-like growth factors were also increased in expanded cumulus. Several of the transcripts changing in cumulus cells during oocyte maturation have been reported by others. For example, several putative cumulus markers of oocyte competence including inhibin βA (INHBA), and betacellulin (BTC) were reduced after 24 hIVM while hyaluronan synthase 2 (HAS2), CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), and prostaglandin-endoperoxidase synthase 2 (PTGS2) were increased after maturation (Assidi *et al.,* 2008). Of particular interest was the greater than 70 fold increase in TNFAIP6 transcripts, regardless of IVM temperature. This gene is involved in ECM stabilization (Nagyova *et al.,* 2008) and has been suggested to be a cumulus marker of oocyte quality (Tesfaye *et al.,* 2009).

The abundance of a number of cumulus transcripts was altered by exposure to elevated temperature during the first 12 hIVM (C-HS), despite a 12 h recovery period following heat stress. Putative functional changes related to the altered transcript abundance are summarized in Figure 5-9. Heat-induced alterations in cumulus function at 24 hIVM are intriguing as it is this

expanded cellular mass that sperm must first interact with to ultimately penetrate and fertilize the oocyte. Although no perturbations in fertilization due to heat stress during the first 12 hIVM have been observed by our laboratory (Edwards et al., unpublished), heat-induced alterations in the cumulus extracellular matrix (ECM; i.e., hyaluronan production) have been reported by others (Lenz *et al.,* 1983). Interestingly, cumulus expansion is mediated, in large part, by follicle stimulating hormone (Eppig 1979a, b) whose receptor (FSHR) had increased transcript abundance in heat stressed cumulus. Others have reported reduced FSHR transcripts in cumulus of *in vivo* matured murine oocytes (Hernandez-Gonzalez *et al.,* 2006), which have high developmental potential, in contrast to the reduced embryonic development observed for heat-stressed COCs. While it is unclear whether FSHR protein levels were also altered, reduced transcript levels may indicate that heat-stressed cumulus are becoming more like mural granulosa cells, making them less able to respond to FSH signals that mediate cumulus expansion. Several other cumulus transcripts alterations indicate that the ECM may be perturbed. Transcripts for matrix metalloproteinase (MMP) 9, an enzyme responsible for ECM degradation (Goldman and Shalev 2004), were reduced by exposure to elevated temperature. Higher MMP9 activity in follicular fluid has been associated with increased establishment of pregnancy in women (Lee *et al.,* 2005). Other ECM-related transcripts having higher abundance in heat-stressed cumulus were fibromodulin (FMOD), a major constituent of the ECM that may be broken down by MMPs (Figure 5-9). Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7 (CHST7) transcripts were also increased in heat-stressed cumulus which may alter the synthesis of chondroitin sulfate (reviewed by Kusche-Gullberg and Kjellen 2003), a ECM glycoaminoglycan (Figure 5-9).

Figure 5-9. Potential heat-induced alterations in pathways within cumulus cells at 24 hIVM. Red indicates heat-induced reductions in transcripts while blue indicates increased transcript abundance. WNT2B- wingless-type MMTV integration site family, member B; Frat- frequently rearranged in advanced T-cell lymphoma; myc- myelocytomatosis; PLC- phospholipase C; IHH- Indian hedgehog homolog (Drosophila); PTC- patched; SMO- smoothened; ACTA1- actin, alpha 1, skeletal muscle; FSHR- follicle stimulating hormone receptor; CAV1- caveolin 1; VNN1- vanin 1; FMOD- fibromodulin; ECM- extracellular matrix; MMP9- matrix metallopeptidase 9 (gelatinase B); CHST7- carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7; iNOS- nitric oxide synthase 2, inducible; GSH- reduced glutathione; GSSG- oxidized glutathione; NO; nitric oxide; O_2^-- superoxide; H_2O_2- hydrogen peroxide; $OONO^-$- peroxynitite


↑3.0
WNT2B
Frizzled
↑2.6
IHH
PTC
SMO
FSH
↑2.4
FSHR
↓2.0
CAV
MMP9
↓3.2
↑2.6
FMOD
Chondroitin sulfate
ECM
Extracellular matrix degradation
↑2.4
CHST7
Golgi
Frat
G
PLCβ
P
β-catenin
Ca2+
Actin cytoskeleton
ACTA1
↑2.7
Adherens junctions
G
Adenylate Cyclase
ATP
cAMP
↑2.7
iNOS
NO
OONO-
Nitrates
O2-
VNN1
↑2.6
O2
H2O
GSH peroxidase
H2O2
GSSG
GSH transferase
2 GSH
NADPH
NADP+
β-catenin
myc
WNT signaling pathway

Transcripts involved in antioxidant response were perturbed in cumulus exposed to elevated temperature. Transcripts for the inducible form of nitric oxide synthase (NOS2A/iNOS) were increased in cumulus after heat stress, which could alter the abundance of nitric oxide (NO; Figure 5-8), a ROS. Nitric oxide is capable of reacting with superoxide anion to form peroxynitrite ($ONOO^-$) which is broken down into nitrates (reviewed by Hughes 2008). Increased nitrate levels in bovine oocytes have been coincident with reduced developmental potential (Matta *et al.,* 2009). Addition of an iNOS inhibitor to oocyte maturation medium reduced cumulus expansion and subsequent embryo development (Matta *et al.,* 2009), suggesting that lower NO levels in COCs improves embryo development. An additional ROS-related factor, vanin 1 (VNN1), had increased transcript abundance in heat-stressed cumulus. Knockout mice null for VNN1 have less glutathione transferase and glutathione peroxidase activity (Di Leandro *et al.,* 2008). If similar reductions in enzyme activities are occurring in cumulus after heat stress exposure, ability to respond to ROS would be diminished (Figure 5-9).

The only significantly overrepresented KEGG pathway in expanded cumulus at 24 hIVM as affected by heat stress (C-HS) was for hedgehog signaling and involved two transcripts, Indian hedgehog (IHH) and WNT2B (Figure 5-9). The abundance of IHH transcripts in cumulus has been reported to decrease during maturation (Hernandez-Gonzalez *et al.,* 2006), in contrast to the increase observed in heat-stressed cumulus. WNT2B, transcripts for which were increased almost 3 fold in cumulus after heat stress, is necessary for β-catenin signaling leading to formation of adherens junctions (reviewed by Hartsock and Nelson 2008). Cytoskeletal actin is also necessary for adherens junctions (Hartsock and Nelson 2008); actin A1 (ACTA1) transcripts were increased in heat-stressed cumulus. A recent report confirmed the presence of proteins

involved in the WNT/β-catenin pathway within human cumulus cells (Wang *et al.,* 2009), indicating that this signaling mechanism is in place in mammalian cumulus cells. The finding of reduced transcripts for caveolin 1 (CAV1) after heat stress exposure casts additional doubt on the ability of cumulus to undergo normal cell signaling. Caveolin 1 is critical for caveolae-mediated membrane receptor endocytosis (reviewed by Lajoie and Nabi 2007). In addition, the actin cytoskeleton is required for certain pathways signaled through caveolae (Lajoie and Nabi 2007) and may further perturb cell signaling capacity.

A high number of experimental replicates (n = 8) was performed, exceeding the number used by others for similar studies (n = 2, Misirlioglu et al., 2006; n = 5, Fair et al., 2007; n = 3, Kues et al., 2008; n = 3, Katz-Jaffe et al., 2009), in lieu of downstream validation of transcript levels (e.g., by qPCR analysis). Others have forgone validation of RNA levels with as few as 5 replicates (Fair *et al.,* 2007). Regardless, statistical power is increased as the number of replicates increase (Wolfinger *et al.,* 2001), as demonstrated with 6 experimental replicates for microarray analysis of flies (Jin *et al.,* 2001). Increased replication allows for sound conclusions based upon array data rather than a secondary analysis. However, biological characterization of findings is important and experiments are currently underway to determine whether differences observed at the transcript level herein are informative of differences at the protein level. Doing so is an important next step as transcript abundance does not always correlate well with changes in protein levels (Griffin et al., 2002).

In summary, heat-induced transcript alterations were observed in both oocytes and cumulus corresponding to several biological themes (Figure 5-10). Given the importance of transcripts for translation, heat-induced perturbations of transcript abundance could explain, in

part, the reductions in protein synthesis (Edwards and Hansen 1996, 1997) and individual proteins (West-Rispoli et al., 2006) reported previously. Experiments are ongoing to determine the extent to which heat-induced transcript alterations in oocytes and cumulus are correlated with functional differences that may explain, in part, reduced developmental potential of heat-stressed oocytes. These may prove useful as markers of oocyte competence that could be used to develop therapeutic strategies to improve fertility during seasonal periods of heat stress.

ACKNOWLEDGMENTS

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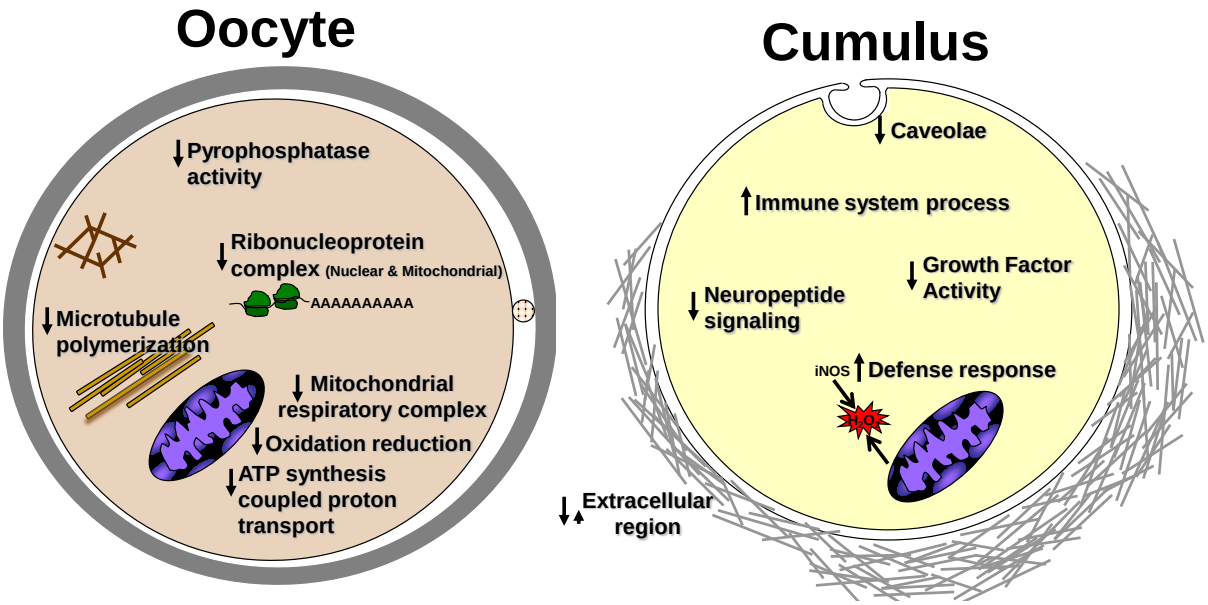


Figure 5-10. Proposed model summarizing some of the biological themes associated with transcript alterations in matured oocytes and their surrounding cumulus after exposure to 41ºC during the first 12 hIVM (C-HS).

CHAPTER 6: General Discussion & Conclusions

The hypothesis underlying the research described herein was that exposure to a physiologically-relevant elevated temperature may alter oocyte and cumulus RNA to account for some of the observed reductions in embryo development after fertilization. To test this hypothesis, a series of experiments were performed to determine the effects of heat stress on maternal pools of RNA within maturing bovine oocytes and their surrounding cumulus cells. The first research efforts utilized microcapillary electrophoresis to examine oocyte, cumulus, and sperm RNA during maturation in the absence of heat stress as a first important step toward analysis of transcriptome profiles. Next, the effects of heat stress on total RNA, polyadenylated RNA, size distribution (electrophoretic profiles) of RNA, and individual transcripts known to be important for meiotic maturation and response to heat stress were examined in oocytes and surrounding cumulus during maturation, and resultant embryos after fertilization. The final research chapter ascertained the effects of exposure to elevated temperature before and after meiotic maturation on the transcriptomes of oocytes and their surrounding cumulus vestment using microarray.

The results from these experimental efforts were informative in many respects. Specifically, the size distribution of RNA molecules, RIN values, and ratios of rRNA, 18S/fast region, and 18S/inter region differed for bovine oocytes compared to cumulus cells. These findings were conserved across several other mammalian species. Although total RNA from oocytes appeared quite different from that of cumulus, the integrity of the polyadenylated RNA populations was similar after amplification. Subsequent efforts found no impact of heat stress during the first 12 h of meiotic maturation to alter these same endpoints in oocytes or cumulus.

However, exposure to elevated temperature may perturb the abundance of polyadenylated RNA in oocytes during meiotic maturation. The relative abundance of transcripts for 18S, 28S, HSP70, cyclin B1, GDF9, BMP15, and PAP was not altered after heat stress exposure. Protein products of these transcripts have known importance for oocyte maturation and response to heat stress. Lack of a heat stress effect on these transcripts suggested that the impact of heat stress to alter oocyte RNA, if any, was subtle. Microarray analysis of the transcriptomes of oocytes and surrounding cumulus before and after meiotic maturation revealed thousands of transcripts that changed, some in a stage-specific and amplification-dependent manner. Direct application of elevated temperature for the first 12 hIVM impacted a small proportion of the transcripts present in oocytes and cumulus cells and indicated possible perturbations in oocyte mitochondrial function as well as potential heat-induced changes in intracellular signaling in cumulus.

Prior to the findings presented in this dissertation, it was well-documented that direct exposure of maturing oocytes to physiologically-relevant elevated temperatures resulted in reduced embryo development after fertilization (Edwards and Hansen 1996, 1997; Lawrence *et al.,* 2004; Roth and Hansen 2004a, b; Edwards *et al.,* 2005; Schrock *et al.,* 2007; Edwards *et al.,* 2009). Heat-induced reductions in development were coincident with alterations in the oocyte (Figure 6-1) including accelerated nuclear and cytoplasmic maturation (Edwards *et al.,* 2005) and decreased *de novo* protein synthesis (Edwards and Hansen 1996). However, nothing was known regarding the impact of heat stress on maternal pools of RNA despite reductions in *de novo* protein synthesis (Edwards and Hansen 1996) and alterations of specific proteins after physiological heat stress exposure (West-Rispoli *et al.,* 2006).

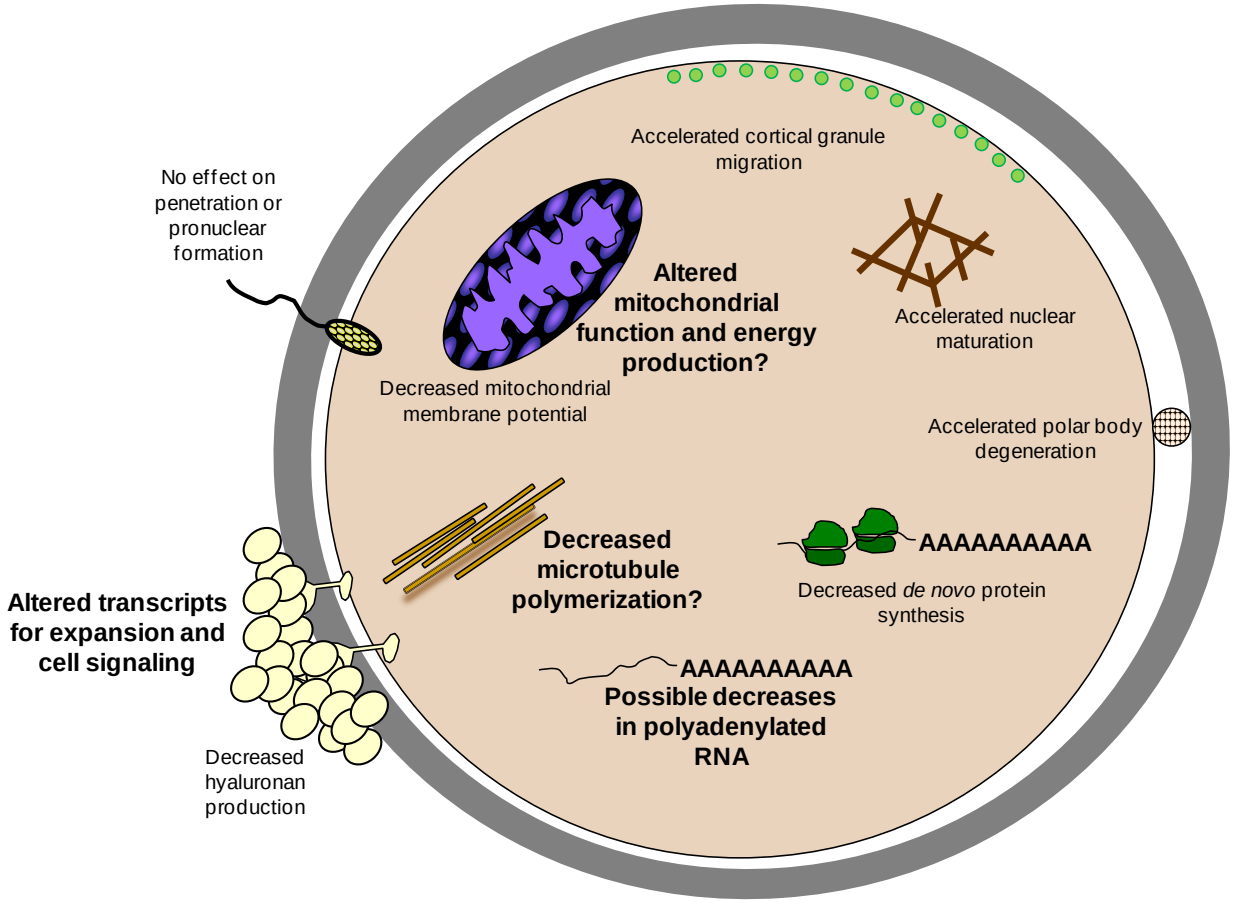


Figure 6-1. Proposed model summarizing the effects direct heat stress application during meiotic maturation on the oocyte and its surrounding cumulus cells. These heat-induced perturbations are coincident with and may explain a portion of reduced development of heat-stressed oocytes after fertilization. Knowledge generated through experimental efforts described in this dissertation is bolded.

The data generated from the experimental efforts described herein add to current knowledge regarding heat stress by implicating alterations in oocyte mitochondrial function with heat-induced reductions in embryo development (Figure 6-1). Mitochondrial membrane potential was reduced in bovine oocytes after exposure to 41ºC for the entire maturation period (Soto and Smith 2009). Indirect evidence indicates elevated temperature exposure may induce production of reactive oxygen species (ROS) as treatment of bovine oocytes with retinol during and after culture at 41ºC improved embryo development after fertilization (Lawrence et al., 2004). Others have demonstrated increased ROS within heat-stressed bovine embryos (Sakatani et al., 2004; Sakatani et al., 2008). These phenotypic data support the altered mitochondrial transcripts observed herein.

Several transcript alterations were observed for cumulus cells after 41ºC exposure for 12 h. Most of these were involved in cell signaling and extracellular matrix production. This is novel information as only one other study examined any effects of elevated temperature on cumulus (Lenz *et al.*, 1983). Lenz et al. (1983) reported reduced hyaluronan production by cumulus cells after culture at 41ºC for the entire maturation period. Synthesis of hyaluronan, one of the primary matrix proteins necessary for cumulus expansion as the oocyte undergoes meiotic maturation, is stimulated by FSH (Salustri et al., 1990). Transcripts for FSH receptor were perturbed in heat-stressed cumulus cells which could impair FSH-induced cumulus expansion. Reduced ability of cumulus to undergo expansion could be problematic during *in vitro* fertilization as the sperm must navigate the expanded cumulus mass to penetrate and fertilize the oocyte. Thus, any heat-induced dysfunction in cumulus could be partly responsible for reduced embryo development of heat-stressed oocytes.

As a direct result of the studies described in this dissertation, more is known regarding the molecular impact of heat stress on oocytes and their surrounding cumulus. Current experiments are underway to determine whether observed transcript alterations correlate with functional differences in protein levels and activities. Identification of biological factors that may be involved in heat-induced infertility could lead to development of therapeutic strategies to mitigate the negative effects of heat stress on oocytes and their surrounding cumulus cells, ultimately improving reproductive efficiency.

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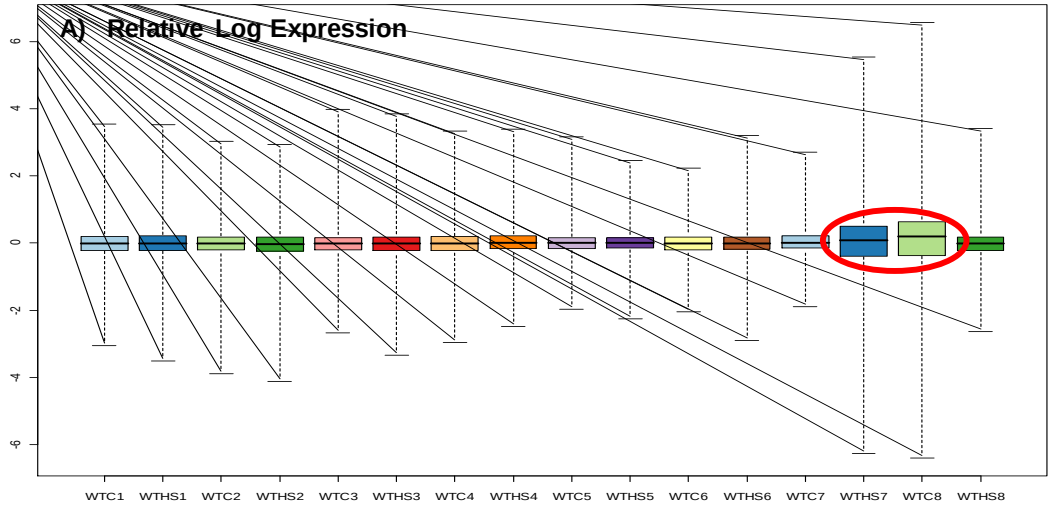
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APPENDIX

Figure A1. Pseudo-images of the 8 arrays of RNA from surrounding cumulus of GV-stage oocytes. The outlier array (circled in red) was deleted from the analysis due to the spatial defect in the lower right area of the chip


GVcum-hi-1.CEL
GVcum-hi-2.CEL
GVcum-hi-3.CEL
GVcum-hi-4.CEL
GVcum-hi-5.CEL
GVcum-hi-6.CEL
GVcum-hi-7.CEL
GVcum-hi-8.CEL

Figure A2. Relative log expression (A) and normalized unscaled standard error (B) plots for WT-amplified oocyte RNA at 24 hIVM. Outlier samples (circled in red) were deleted from the analysis due to having almost doubled values for expression and error.


A) Relative Log Expression
6
4
2
0
-2
-4
-6
WTC1
WTHS1
WTC2
WTHS2
WTC3
WTHS3
WTC4
WTHS4
WTC5
WTHS5
WTC6
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WTC7
WTHS7
WTC8
WTHS8

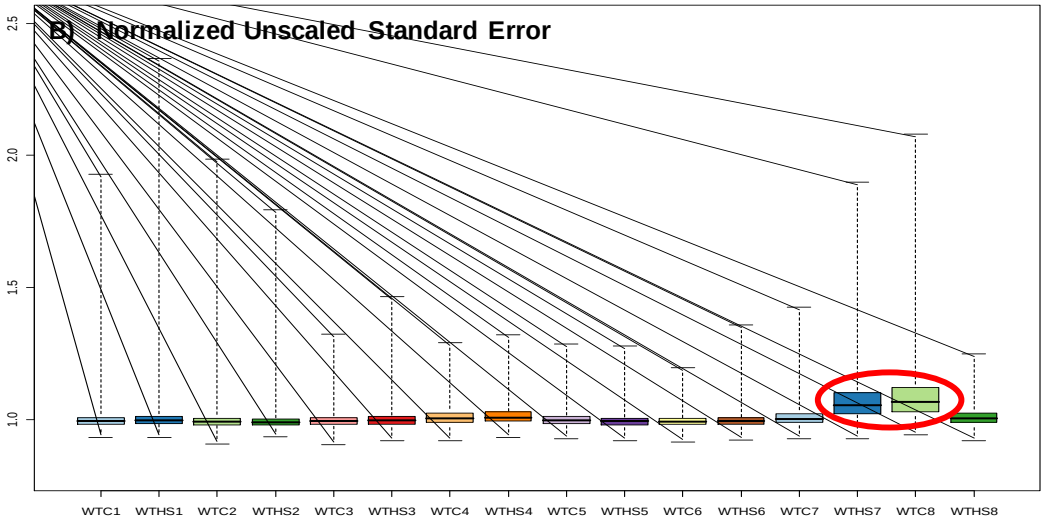
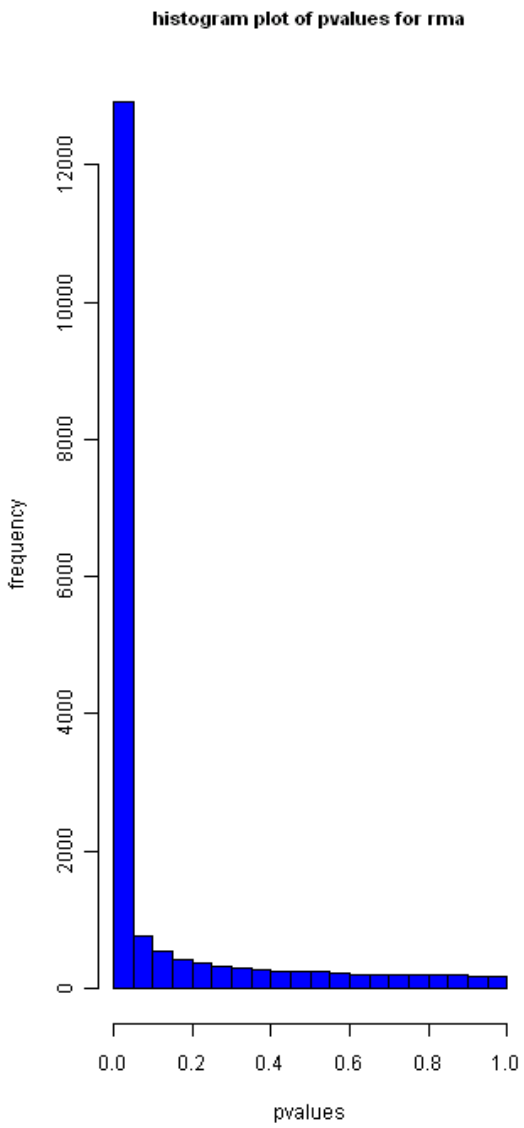
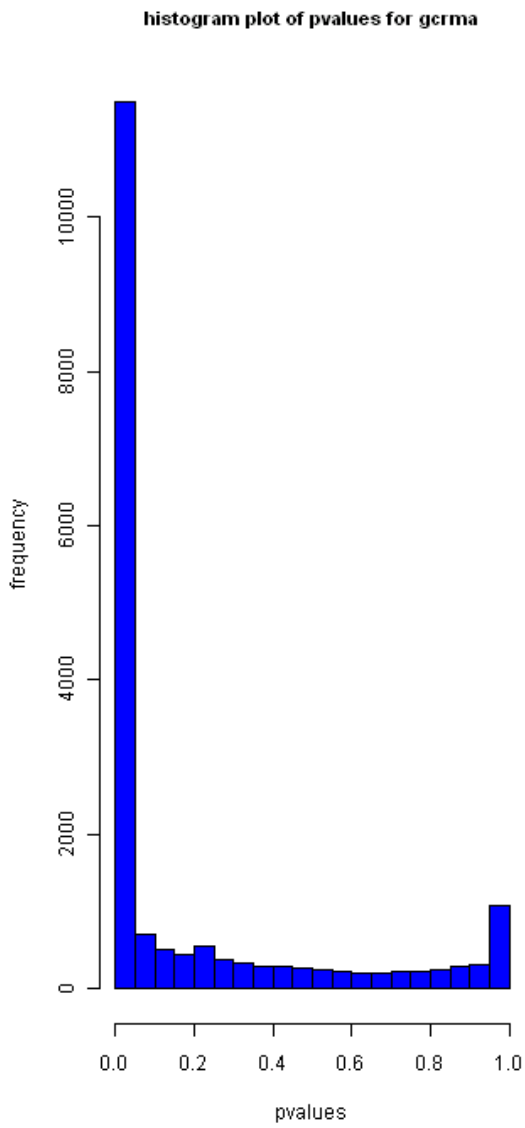

B) Normalized Unscaled Standard Error
2.5
2.0
1.5
1.0
WTC1
WTHS1
WTC2
WTHS2
WTC3
WTHS3
WTC4
WTHS4
WTC5
WTHS5
WTC6
WTHS6
WTC7
WTHS7
WTC8
WTHS8

Figure A3. Histogram showing the frequency of p-values (ranging from 0 to 1) for comparison of GV-stage and control 24 hIVM polyadenylated oocyte transcripts. From left to right are the values for RMA, GCRMA, and VSN summarization methods, respectively. Situations like these where small p-values greatly outnumber larger p-values are ideal for false discovery rate (FDR) correction (Benjamini and Hochberg 1995).


histogram plot of pvalues for rma
frequency
0
2000
4000
6000
8000
10000
12000
0.0
0.2
0.4
0.6
0.8
1.0
pvalues


histogram plot of pvalues for gcrma
frequency
0
2000
4000
6000
8000
10000
0.0
0.2
0.4
0.6
0.8
1.0
pvalues

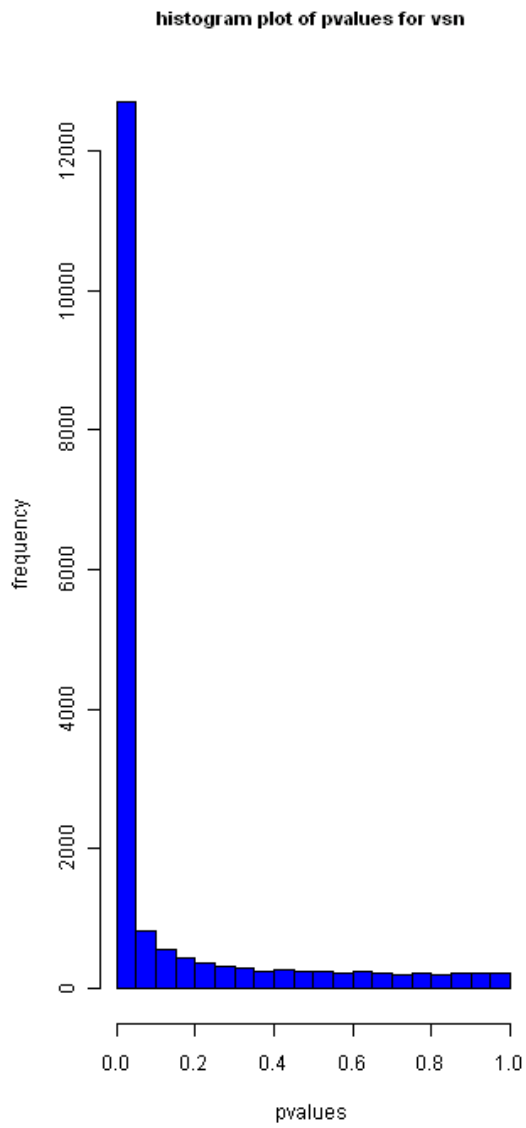

histogram plot of pvalues for vsn
frequency
0
2000
4000
6000
8000
10000
12000
0.0
0.2
0.4
0.6
0.8
1.0
pvalues

Figure A4. Histogram showing the frequency of p-values (ranging from 0 to 1) for comparison of 24 hIVM polyadenylated oocyte transcripts from control and heat-stressed oocytes. From left to right are the values for RMA, GCRMA, and VSN summarization methods, respectively. When p-values are distributed relatively equally between 0 and 1, FDR adjustments do not work and result in a tremendous loss of statistical power (Pawitan *et al.*, 2005). Note the drastic difference between these histograms and those shown in Figure A3.

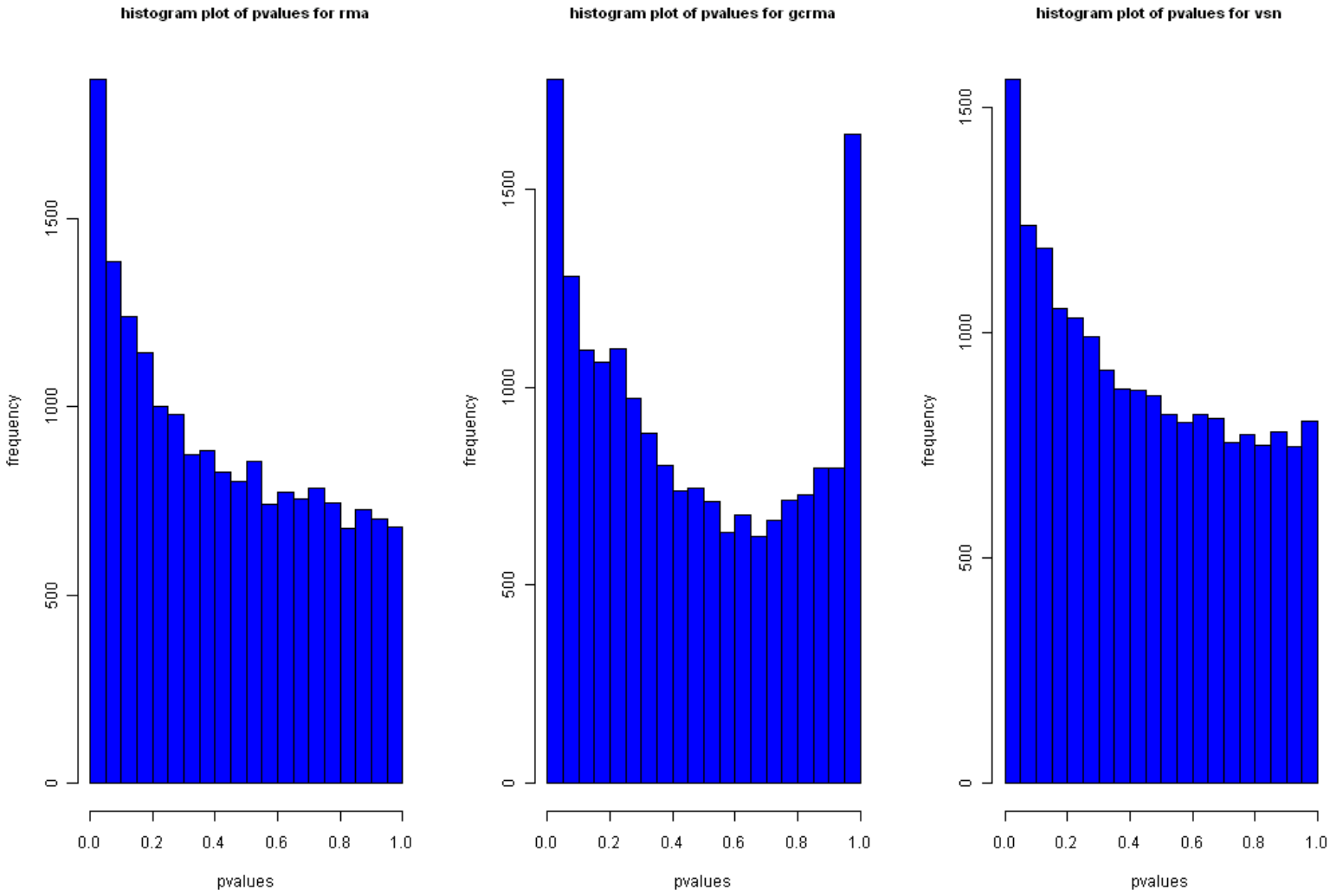
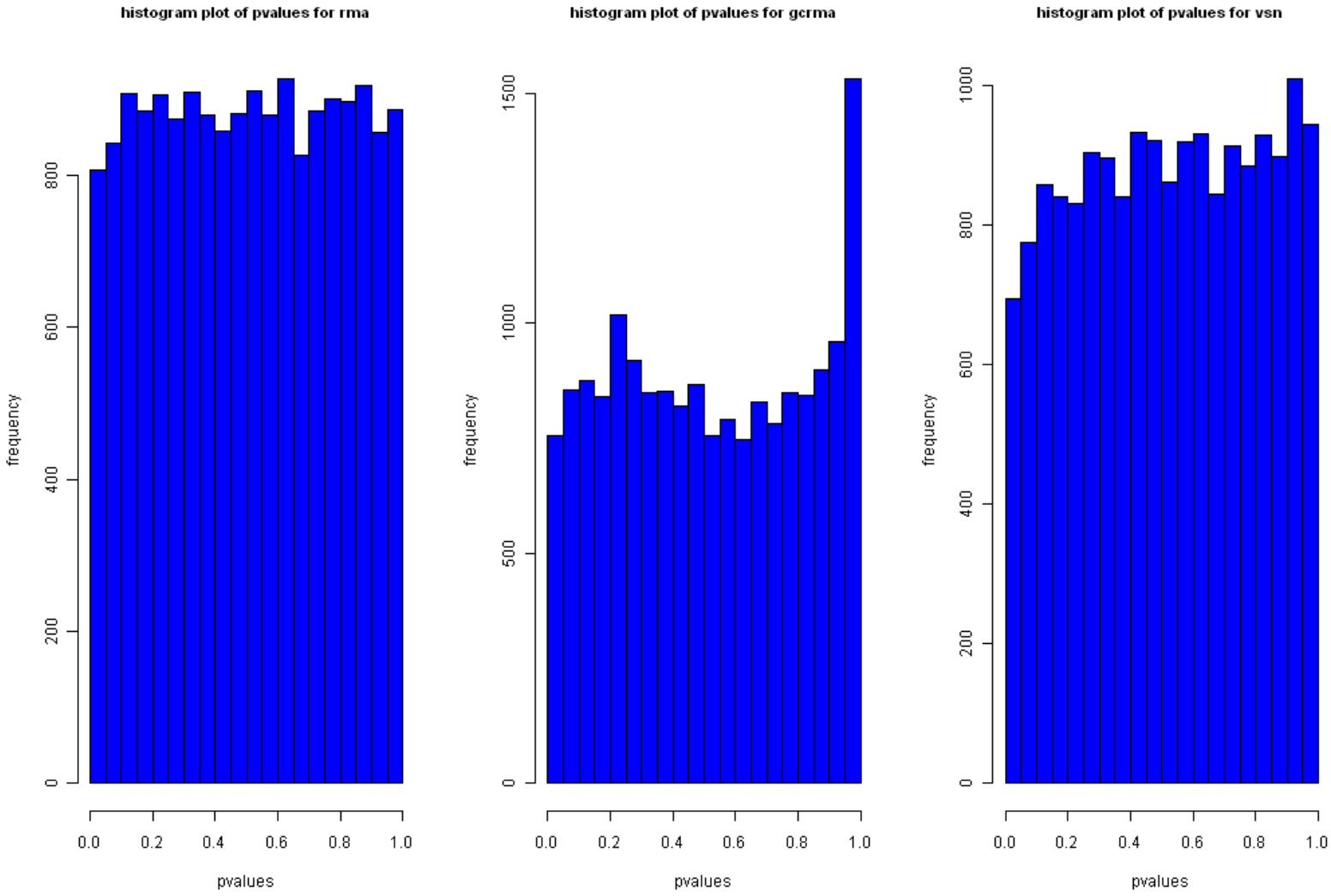

histogram plot of pvalues for rma
histogram plot of pvalues for gcrma
histogram plot of pvalues for vsn
frequency
pvalues
0
500
1000
1500
0.0
0.2
0.4
0.6
0.8
1.0

Figure A5. Histogram showing the frequency of p-values (ranging from 0 to 1) for comparison of 24 hIVM WT-amplified oocyte transcripts from control and heat-stressed oocytes. From left to right are the values for RMA, GCRMA, and VSN summarization methods, respectively. The relatively equal distribution of p-values between 0 and 1 indicates that FDR corrections would not work (Pawitan *et al.,* 2005). Note the drastic difference between these histograms and those shown in Figures A3 and A4.


histogram plot of pvalues for rma
frequency
0
200
400
600
800
0.0
0.2
0.4
0.6
0.8
1.0
pvalues
histogram plot of pvalues for gcrma
frequency
0
500
1000
1500
0.0
0.2
0.4
0.6
0.8
1.0
pvalues
histogram plot of pvalues for vsn
frequency
0
200
400
600
800
1000
0.0
0.2
0.4
0.6
0.8
1.0
pvalues

VITA

Rebecca Ruth (Frazor) Payton was born March 23, 1979 in Nashville, TN. Raised in Goodlettsville and Hendersonville, she graduated from Beech Senior High School in 1997. She attended the University of Tennessee, Knoxville, graduating with a B.S. in Agriculture in May 2001. She married Jason Scott Payton in July 2001 and, because her blood runneth orange, continued her education with a M.S. in Animal Science. Her love for animals and desire to help those in most peril by using reproductive biology as a means of endangered species preservation led her to further her education with doctoral studies. She is currently awaiting a better job market to pursue a career in conservation of endangered species.