

Assessing Mitochondrial Activity in Embryos from Heat-Stressed Ova

A Thesis Presented for the Master of Science Degree

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DEDICATION

This thesis is dedicated to my grandmother, Gilbert Medlin, aka 'Shorty'. Who with unconditional love and compassion has always been my biggest fan and toughest critic all in hopes to watch me prosper and achieve nothing less than my heart's desires. She is a generous woman of strength, kindness, faith, and love who has been more than a dear grandmother, but a role model whom I strive to follow.

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ABSTRACT

Elevated ambient temperatures negatively impacts pregnancy rates in agriculturally important females. An oocyte undergoing meiotic maturation exposed to a physiologically-relevant heat stress results in alterations of developmentally important processes and reportedly decreases subsequent developmental potential after fertilization. To address problems of reduced competence after exposure to heat stress for the first 12 h of meiotic maturation (**hIVM**), Payton (2009) assessed the transcriptome profiles of oocytes and associated cumulus cells by microarray analysis and reported heat-induced perturbations at the molecular level in the oocyte. Specifically, 20 transcripts involved in mitochondrial function exhibited alterations in relative abundance of heat-stressed oocytes which coincided with significantly more ATP content at 24 hIVM compared to non-heat-stressed controls (Payton et al., unpublished). These findings of altered transcripts and elevated ATP production suggest heat-induced perturbations of mitochondrial function. To further discern whether these heat-induced alterations in mitochondria persists in subsequent embryos after fertilization, the present study measured ATP content in oocytes matured at 38.5°C for 24 h or 41°C for the first 12 h followed by 38.5°C for the remaining 12 h, resultant cleavage, and blastocyst-stage embryos. Results demonstrated a significant increase in ATP content in heat-stressed matured oocytes ($P = 0.0148$) and of resultant 8-to-16-cell ($P = 0.0323$) stage embryos but was not evident in blastocyst stage embryos. Here we provide information that heat stress alters mitochondrial function in oocytes which persists following fertilization, which may ultimately contribute to a reduced developmental potential of embryos from heat-stressed oocytes. A second objective

was to validate microarray findings via qPCR of: 1) electron transport chain transcripts, COQ3 and NDUFC2, in the oocyte and 2) IHH of the Hedgehog Pathway in the surrounding cumulus cells. Results agreed with the previous microarray data (Payton, 2009) reporting heat-induced decreases of NDUFC2 ($P \leq 0.002$) and COQ3 ($P = 0.02$) in oocytes and heat-induced increases of IHH ($P = 0.007$) in cumulus cells. While functional significance of these findings remains unclear, heat-induced perturbations at the molecular level in the oocyte are evident and may provide explanation for reductions seen in developmental competence of resultant embryos.

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION	1
CHAPTER 2 REVIEW OF LITERATURE.....	5
Introduction	5
Oocyte Maturation and Heat Stress Effects	5
ATP Production	9
Metabolic Substrates and ATP Production.....	11
Quiet Embryo Hypothesis	12
ATP Content in Oocytes.....	13
ATP Content of Early Embryos	15
Heat Stress Exposure and Effect on ATP Content.....	17
Heat Stress Effects on Mitochondrial Function in Oocytes and Embryos	19
Heat Stress Effects on Mitochondrial Function in Other Cell Types.....	21
Mitochondria and Developmental Competence	22
ATP Content and Developmental Competence	23
CHAPTER 3 Assessing Mitochondrial Activity in Embryos Derived from Heat-Stressed Ova.....	26
ABSTRACT	26
INTRODUCTION	27
MATERIALS AND METHODS	31
Materials	31
In Vitro Production of Embryos	32
Experimental Design and Endpoints.....	33
ATP Content Assay	36
Statistical Analysis	36
RESULTS	37
Embryo Development of Oocytes Matured at 38.5 or 41.0°C	37

Effects of Heat Stress on ATP Content.....	39
DISCUSSION.....	43
ACKNOWLEDGMENTS.....	50
CHAPTER 4 Relative Abundance of NDUFC2 and COQ3 in Bovine Oocytes, and IHH	
Transcript in Surrounding Cumulus after Heat Stress Exposure	51
ABSTRACT	51
INTRODUCTION	51
MATERIALS AND METHODS	55
Source of Oocyte Samples	55
Source of Cumulus Samples	55
Quantitative Polymerase Chain Reaction	59
Statistical Analyses.....	63
RESULTS	63
Relative Abundance of NDUFC2 & COQ3 Transcripts in Heat-Stressed Oocytes .	63
Relative Abundance of IHH Transcript in Cumulus from Heat-Stressed Oocytes...	66
DISCUSSION.....	66
ACKNOWLEDGMENTS.....	73
LIST OF REFERENCES	74
VITA	96

LIST OF FIGURES

Figure	Page
Figure 3.1. Schematic of heat-induced perturbations of transcripts of the electron transport chain (ETC) from the oocyte from microarray analysis	30
Figure 3.2. Schematic of experimental design and endpoints	34
Figure 3.3. ATP Content in Matured Oocytes and Cleavage and Blastocyst-Stage-Embryos	40
Figure 3.4. ATP Content in Blastocysts of Different Stages and Quality Scores from Control and Heat-Stressed Oocytes.....	41
Figure 3.5. Exponential decay of ATP content in control and heat-stressed matured oocytes, and in resultant 8-to-16-cell and blastocyst-stage embryos	42
Figure 4.1. Model of the electron transport chain and oxidative phosphorylation for ATP production, highlighting the multi-protein subcomplexes which contain NDUFC2 and COQ3	53
Figure 4.2. Schematic of experimental design for oocytes samples	56
Figure 4.3. Schematic of experimental design for cumulus samples	58
Figure 4.4. Relative abundance of NADH dehydrogenase (NDUFC2)	61
Figure 4.5. Relative abundance of Coenzyme Q3 homolog (COQ3)	62
Figure 4.6: Relative abundance of Indian hedgehog (IHH)	64

LIST OF TABLES

Table	Page
Table 3.1. Embryo development of developmentally competent oocytes	38
Table 4.1. Sequences of primers and annealing conditions used for quantitative PCR analyses	59

CHAPTER 1

INTRODUCTION

Elevated environmental temperatures negatively impact the dairy industry economically, costing between 800 and 900 million dollars annually (St-Pierre et al., 2003, Collier et al., 2006). Two large factors contributing to this deficit include the decrease in milk production (reviewed by West, 2003) and reduced fertility (reviewed by Rensis and Scaramuzzi, 2003). During hot summer, months pregnancy rates of lactating dairy cattle may decrease as much as 50% when compared to milder, cooler months (reviewed by Hansen and Arechiga, 1999). Heat-induced decreases in fertility are not limited to cattle but are evident in numerous other species including mice, rats, rabbits, sheep, and pigs (Austin and Braden, 1954, Dutt, 1963, Tompkins et al., 1967).

While many factors may contribute to reduced fertility heat-induced increases in hyperthermia may be most detrimental. Over time it has been reported that rectal temperatures of dairy cows may reach and even exceed 41.0°C (Seath and Miller, 1946, Monty and Wolff, 1974, Ealy et al., 1993). In both cattle and sheep, rectal temperatures have been negatively correlated with pregnancy rates, with a 1°C elevation in rectal temperature causing a 25% decrease in pregnancy rates (Ulberg and Burfening, 1967).

During summer, elevated ambient temperatures are not an isolated incident but are most problematic when occurring during estrus (i.e., when dominant follicle is housing the maturing ovum). For instance, Gwazdauskas et al. (1973) reported that elevated uterine and rectal temperatures at the time of

insemination negatively impacts conception rates thereafter. Similar results have been reported by others (Cavestany et al., 1985, Putney et al., 1989, Rutigliano et al., 2008). Putney et al. (1989) showed a major consequence of heat stress exposure during estrus was to reduce embryo quality. Heat-induced reductions in embryo development might be related to an altered maternal environment or to direct effects of elevated temperatures on the maturing oocyte. To assess the latter, numerous studies report that direct exposure of bovine oocytes to heat stress during in vitro maturation (which is coincident with estrus in the cow) repeatedly resulted in reduced blastocyst development (Edwards and Hansen, 1996, 1997, Roth and Hansen, 2004, Edwards et al., 2005, 2009, Schrock et al., 2007, Wang et al., 2009b, Payton et al., 2011), and are similar to reductions in blastocyst development reported in vivo (Putney et al., 1989).

Heat stress exposure during the first half of oocyte meiotic maturation has altered numerous developmental processes (See Chapter 2) to explain decreased developmental competence, but it does so without impact on the oocyte's ability to be penetrated and fertilized by a spermatozoon or undergo subsequent cleavage divisions in majority of reports in literature (Edwards and Hansen, 1996, 1997, Roth and Hansen, 2004, Edwards et al., 2005, 2009, Schrock et al., 2007, Wang et al., 2009b, Payton et al., 2011). However, Edwards et al. (2009) reported a reduction in resultant embryos from heat-stressed oocytes to undergo compaction, which would explain some decreases in blastocyst development.

Developmental competence of resultant embryos of heat-stressed oocytes was further assessed, by exposing compact morulae to 41°C for an additional 12 h, which further resulted in a decrease in blastocyst rates (Edwards et al., 2009). Consistent with this finding, Cartmill et al. (2001) and Rutigliano et al. (2008) demonstrated that cows exposed to elevated temperatures during estrus and artificial insemination were more likely to lose their pregnancies than nonheat-stressed cattle.

To identify molecular mechanisms underlying heat-induced reductions in blastocyst development, Payton (2009) examined transcriptome profiles of oocytes and associated cumulus after heat stress exposure during the first 12 h of meiotic maturation using a bovine genome microarray analysis. Heat stress exposure altered the relative abundance of numerous transcripts in oocytes and their surrounding cumulus cells (Payton, 2009). Of particular interest, Gene Ontology (**GO**) analysis indicated heat-induced reductions in the relative abundance of 13 transcripts important for mitochondrial function, specifically the electron transport chain (**ETC**, ATP producing machinery, Figure 3.1.). While functional significance of these findings remain unclear, subsequent efforts of Payton et al. (unpublished – preliminary data) reported increased ATP content in heat-stressed oocytes when examined at 24 hIVM compared to nonheat-stressed oocytes. Furthermore, preliminary data showed that numerically, ATP content was higher in blastocysts derived from heat-stressed oocytes compared to control which suggests enhanced mitochondrial function in the maturing oocyte may carry over to resultant embryos.

Increased mitochondrial function (i.e., increased ATP production) is concerning due to correlations seen with decreased developmental competence. For instance, Igosheva et al. (2010) reported increased mitochondrial membrane potential in oocytes and zygotes of obese mice, coincided with a decrease in blastocyst rates compared to leaner counterparts. Increased mitochondrial activity is problematic according to Leese et al. (2002, 2008) who have reported that a 'quiet' metabolism (i.e., less mitochondrial activity) is important for a more developmentally viable phenotype. Heat-induced alterations of mitochondrial function in the oocyte are intuitive to carry over to resultant embryos following fertilization, since oocytes contribute >99.9% of the ooplasm including mitochondria to consequential embryos.

The working hypothesis for this thesis is that heightened mitochondrial function of heat-stressed oocytes is inherited by resultant embryos and may persist in later stages of development, accounting for reduced blastocyst formation. To test this hypothesis, the main objective was to examine ATP content of cleavage and blastocyst-stage embryos from heat-stressed oocytes to assess the extent to which heat-induced alterations in mitochondrial function may persist. A secondary objective was to validate microarray data by further investigating a subset of transcripts in both oocyte and cumulus cells using qPCR.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

In an attempt to discern one of the underlying mechanisms whereby heat stress reduces developmental competence of the oocyte following fertilization by altering mitochondrial function, the following is a review of literature highlighting: oocyte meiotic maturation and the consequences of heat stress exposure on nuclear and cytoplasmic events, ATP production and content in oocytes and embryos including a review of mitochondrial function. Furthermore, what is known about heat-induced alterations of ATP content and mitochondrial function in oocytes, embryos, and other cells is addressed. And finally, the relationship between ATP production and mitochondrial function in oocytes and embryos and its use as an indicator of developmental competence is described thereafter.

Oocyte Maturation and Heat Stress Effects

Meiotic maturation includes nuclear and cytoplasmic events that must occur in a discrete spatial and temporal manner, in order for the oocyte to acquire the capacity to be fertilized and undergo subsequent development (reviewed by Barnes and Sirard, 2000). In addition, changes must occur at the molecular level to make these processes possible (reviewed by Sirard, 2001). An oocyte arrested in the dictyate stage of prophase I is initiated into meiotic maturation in vivo by a surge of luteinizing hormone (Kruip et al., 1983) or in vitro following removal from the follicle (Pincus and Enzmann, 1935). Nuclear

maturation includes germinal vesicle break down (**GVBD**), chromatin condensation and segregation, extrusion of the first polar body, undergoing metaphase I (**MI**), anaphase I (**AI**), and telophase I, then re-arresting in metaphase II (**MII**; reviewed by Sirard et al., 2006) until fertilization. Cytoplasmic maturation includes the rearrangement of cytoskeleton (actin filaments, microtubules, and intermediate filaments), spindle formation and translocation of cortical granules (Gallicano, 2001), plus the migration of mitochondria (Van Blerkom et al., 1990). Alongside the discrete events involved in oocyte meiotic maturation, companion cumulus cells are undergoing additional changes. Cumulus cells are intimately associated with the oocyte via cytoplasmic extensions through the zona pellucida (de Loos et al., 1991) and provide bi-directional communication to the oocyte. As maturation continues, a progressive loss of junctions occurs at 12 to 18 hIVM, coinciding with the end of GVBD (Hyttel et al., 1986). Furthermore, cumulus expansion occurs along with the secretion of hyaluronan (Chen et al., 1990).

Heat stress during meiotic maturation decreases embryonic development. Heat-induced reductions of in vivo and in vitro development are as great as 40 to 65% in bovine (Stott and Williams, 1962, Putney et al., 1989, Edwards and Hansen, 1996, Lawrence et al., 2004, Schrock et al., 2007). Heat-induced decreases in fertility are not limited to cattle but are evident in numerous other species including mice, rats, rabbits, sheep, and pigs (Austin and Braden, 1954, Dutt, 1963, Tompkins et al., 1967). Heat-induced alterations of processes in the oocyte may carry over to resultant embryos but is not contingent on the ability of

the oocyte to be penetrated and fertilized or to undergo subsequent cleavage divisions (Edwards and Hansen, 1996, 1997, Edwards et al., 2005, 2009, Schrock et al., 2007, Payton et al., 2011). However, there have been instances where the proportion of 8-to-16-cell embryos was reduced (Lawrence et al., 2004) and resulting blastocysts had fewer trophoblast cells (Ju et al., 2005) and reduced total cell counts (Lawrence et al., 2004, Ju et al., 2005), which suggests heat-induced alterations in developmental competence. Edwards et al. (2009) reported a heat-induced reduction in compaction which may explain some of the heat-induced reductions in blastocysts. Resultant embryos of heat-stressed oocytes were further assessed by exposing compact morulae to 41°C for an additional 12 h, which resulted in a decrease in blastocyst rates (Edwards et al., 2009). This result was unforeseen since data says compact morulae from 38.5°C are thermotolerant (Ealy et al., 1993, Edwards and Hansen, 1997). Although some embryos develop from heat-stressed oocytes, heat-induced alterations are often not evident until later in development. For instance, Cartmill and coworkers in 2001 demonstrated cattle exposed to heat-stressed conditions (Temperature Humidity Index > 72) near the time of breeding had decreased embryo survival soon after placental attachment. Furthermore, Rutigliano et al. (2008) reported dairy cows exposed to elevated temperatures during artificial insemination were more likely to lose their pregnancies than nonheat-stressed cattle.

While underlying mechanisms of heat stress exposure to reduce developmental competence remain unclear, effects are likely attributed to a multitude of different heat-induced factors to alter developmentally important

dynamics of the maturing cumulus-oocyte complex. For instance, exposure to an elevated physiologically-relevant temperature during the first 12 h of in vitro maturation (**hIVM**) caused nuclear maturation to hasten, causing oocytes to reach MII 4 to 6 h earlier than non-heat-stressed oocytes making the oocytes 'aged' (Edwards et al., 2005). To compensate for this "aging" Schrock et al. (2007) fertilized heat-stressed oocytes 4 to 6 h earlier and improved blastocyst development.

Coupled to acceleration of nuclear maturation, alterations at the cytoplasmic level are evident when heat stress exposure occurs during meiotic maturation. Perturbations include alterations, cortical granule translocation in heat-stressed oocytes (increased type III cortical granules; Payton et al., 2003), and in microtubule, microfilaments and spindle organization (Roth and Hansen, 2005). Furthermore, studies have reported an increase in reactive oxygen species (**ROS**; Payton, 2009), decreased glutathione content (Wang et al., 2009b), and a reduction in intracellular calcium concentrations (Tseng et al., 2009) in heat-stressed oocytes in comparison to control counterparts. Also, heat-stressed oocytes exhibit a 30 to 50% decrease of de novo protein synthesis (Edwards and Hansen, 1996). Additionally, exposure to physiologically-relevant heat stress conditions impacted the companion cumulus as well as altering processes within the oocyte. Heat stress application for the duration of maturation reportedly reduced cumulus expansion and the quantity of hyaluronic acid produced (GAG expression; Lenz et al., 1983), but the extent to which this is

happening in our model is unclear, since no to slight reduction in cumulus expansion has been visually detected.

While less is known about heat-induced perturbations occurring at the molecular level in the oocyte, using microarray analysis, Payton (2009) compared the transcriptome profiles of oocytes and their associated cumulus cells after maturation at 38.5°C for 24 h (controls) or 41°C for the first 12 h followed by 38.5°C for the remaining 12 h (heat stress). Numerous heat-induced differences in the relative abundance of various transcripts in both oocytes and associated cumulus cells were noted. A gene ontology analysis alluded to heat-induced differential expression of transcripts important for mitochondrial function. Specifically, transcripts involved with the electron transport chain (**ETC**), ATP producing machinery, exhibited a decrease in mRNA abundance. Subsequent efforts of Payton et al. (unpublished – preliminary data) reported that exposure of bovine oocytes to 41°C for the first 12 hIVM elevated ATP content at 24 hIVM. Although, ATP content was similar in germinal vesicle (**GV**)-stage and 12 hIVM (1.10 to 1.19 pmol/oocyte) regardless of heat stress application, but by 24 hIVM heat-stressed oocytes (2.01 pmol/oocyte) had significantly more ATP (1.65 pmol/oocyte) than nonheat-stressed oocytes.

ATP Production

Events of meiotic maturation, fertilization, and subsequent development thereafter require energy in the form of adenosine triphosphate (**ATP**), the universal unit of energy exchange (Johnson et al., 2003). Almost all ATP

produced and utilized by the oocyte and early embryo is derived from mitochondria via the electron transport chain and oxidative phosphorylation (**OXPPOS**) pathway (Leese, 1995, Van Blerkom et al., 1995, Dumollard et al., 2007). Although, mitochondria in human oocytes begin as immature and structurally underdeveloped with a spherical shape and few, short cristae they still are capable of OXPPOS to provide ATP to this metabolic active and unique cell (Van Blerkom, 2010). The ultrastructure of mitochondria changes throughout oocyte maturation and early embryonic development following fertilization. A murine model demonstrated that following fertilization to the 2-cell stage mitochondria appeared to have a dumb-bell shape but at the 4-cell to morulae-stage, mitochondria become more elongated with well-developed and defined transverse cristae (Stern, 1971).

The electron transport chain is located in the inner mitochondrial matrix and transfers electrons from reducing equivalents to oxygen to create an electrical and pH gradient, which then dissipates to create ATP via OXPPOS. Imbedded in the lipid bilayer of the inner membrane, the entire system consists of five multiprotein enzyme complexes and two electron carriers, coenzyme Q and cytochrome c (reviewed by Hatefi, 1985, and Smeitink et al., 2001). NADH carries electrons from carbon oxidations to Complex I or NADH Coenzyme Q oxidoreductase of the ETC (43 subunits). Electrons are carried via Coenzyme Q to Complex III or Coenzyme Q- cytochrome c oxidoreductase (11 subunits). Electrons are then carried by cytochrome c to the final electron acceptor, Complex IV or cytochrome c oxidase, where oxygen is reduced to water. Other

metabolites, like those from organic acid oxidations (i.e., FADH), enter coenzyme Q via Complex II or succinate-CoQ oxidoreductase (4 subunits). Throughout each transfer step, energy is lost and used by the subunits to transfer hydrogen from the matrix to the inner membrane space of the mitochondria. This proton gradient is used by Complex V or ATP Synthase (14 subunits) to ultimately generate ATP (reviewed by Hatefi, 1985, and Smeitink et al., 2001).

Although mitochondria have their own circular DNA, which is very compact and contains no introns, the majority of proteins in the subunits are encoded by the nuclear genome (reviewed by Hatefi, 1985, and Smeitink et al., 2001). Smeitink et al. noted in their review that only 7 of the 43 subunits are encoded by mitochondrial DNA (**mtDNA**) of complex I. Complex II is completely derived from the nuclear genome, while complex III has one of the 11 subunits encoded by the mtDNA. Complex IV consists of 3 subunits encoded by mtDNA out of 13 subunits, and complex V has two of its 14 subunits encoded by the mtDNA with all other subunits in each complex encoded by nuclear DNA. Therefore interplay between both genomes is needed for regulation of the OXPHOS system; however, the biogenesis regulatory mechanisms remain unknown (reviewed by Smeitink et al., 2001, Dumollard et al., 2009). However, a tight regulation of both genomes in response to physiological and metabolic conditions is necessary.

Metabolic Substrates and ATP Production

The production of ATP requires metabolic substrates to provide reducing equivalents for the transfer of electrons. Oocytes and subsequent embryonic

stages following fertilization have a changing pattern in preferred metabolic substrates for ATP production, including pyruvate, glucose, lactate, and glutamine (reviewed by Dumollard et al., 2009). For instance, pyruvate is the desired substrate for oxidation prior to the 16-cell stage, but glucose becomes the preferred metabolic metabolite in developmental stages succeeding thereafter of bovine embryos (Khurana and Niemann, 2000). Furthermore, in mice, glucose consumption increases at the blastocyst stage but only comprises 17% of ATP produced whereas 40% is still contributed by the oxidation of pyruvate (Houghton and Leese, 2004). Metabolic requirements are intimately associated with mitochondrial function, in order to properly utilize these different carbohydrate sources (i.e., glucose and pyruvate) for ATP production to support cellular activity (Cummins, 2004).

Quiet Embryo Hypothesis

Two of the most energy (ATP) consuming processes within a cell include protein synthesis and the ion pumping $\text{Na}^+\text{K}^+\text{ATPase}$ (Kelly, 1990). Since the maturing oocyte is translationally active and the blastocyst stage embryo requires ion pumps for the formation of the blastocoel, it is pertinent that ATP content is sufficient to meet these cellular energy demands (Leese et al., 2008), while maintaining a certain level of metabolic quiescence which is desired for oocytes and embryos (Leese et al., 2008b, Dumollard et al., 2009). Specifically, the quiet embryo hypothesis states that embryos possessing a quieter metabolic activity level exhibit a more viable phenotype to undergo further development (Leese,

2002, Leese et al., 2008a). Studies using human and mouse zygotes and embryos (cleavage-stage to expanded blastocyst-stage) and somatic cells, evaluated the net depletion and release of various nutrients (pyruvate, glucose, lactate, and amino acids) in addition to oxygen consumption. Based on results of studies it was demonstrated that the most viable embryos and cells were those with lowest overall metabolism, glycolytic rates, and amino acid turnover along with highest amounts of anti-oxidant enzymes and 5'AMP protein kinase (Leese, 2002, Leese et al., 2008a). Furthermore, Leese (2002) speculated early embryos exposed to stressful environments exhibit perturbations which may cause nutritional imbalances, oxidative stress, leading to defective genomic imprinting that may result in a less viable embryo.

ATP Content in Oocytes

The oocyte contributes 99.9% of the cytoplasm containing organelles such as mitochondria to the resultant embryo; therefore, the metabolic status (i.e., mitochondrial activity and ATP production) of the oocyte intuitively impacts subsequent embryonic development (Krisher, 2004). ATP production and consumption changes with the demands of the oocyte. Few immature oocytes show mitochondrial activity (Tarazona et al., 2006) likely due to the mass majority of immature mitochondria (Wilding et al., 2001, Stojkovic et al., 2001, Tamassia et al., 2004). Due to the immature state of mitochondria in an oocyte, energy has been reported to be passively received from surrounding cumulus cells (Tanghe et al., 2002, Fair, 2003). It is evident that ATP content increases from GV-stage

(immature) oocytes to MII (mature) oocytes. Payton et al. (unpublished) reported an increase in ATP content from 1.1 pmol to 1.65 pmol during in vitro maturation of the bovine oocyte. Other studies of in vitro matured bovine oocytes observed an increase in ATP content from 1.8 pmol and 1.2 pmol to 2.5 pmol and 2.0 pmol in GV to MII oocytes, respectively (Stojkovic et al., 2001, Iwata et al., 2011). Regardless of the cytoplasmic appearance (1 (homogeneously granulated ooplasm) through 6 (homogeneous black ooplasm)) of abattoir-derived bovine oocytes ATP content increased when measured immediately after their collection (ranging from 0.53 pmol to 0.95 pmol) to after maturation (ranging from 0.70 pmol to 1.39 pmol; Nagano et al., 2006a).

While ATP content increased between 0 h and 24 hIVM, amount of ATP actually varies during IVM depending on meiotic stage of bovine oocytes. Nagano et al. (2006a) examined ATP content and stage of maternal chromatin at 0, 3, 6, 9, 12, 17, and 22 hIVM. Almost all oocytes were at GV-stage between 0 and 3 hIVM but at 6 hIVM more than half had undergone GVBD and showed a decrease in ATP content (~0.07 pmol to ~0.04 pmol; the lowest observed during maturation). At 9 h most oocytes underwent chromosomal condensation and progressed to MI and at 12 h all oocytes reached MI stage coinciding with an increase in ATP content (~0.07 pmol). At 17 to 22 hIVM, 80% of oocytes were at the AI to MII stage and ATP content was at its highest level (~1.0 pmol; Nagano et al., 2006a). In contrast, in vivo matured bovine oocytes have little to no change (2.5 pmol to 2.4 pmol) in ATP content during maturation (Tamassia et al., 2004),

but could be a result of in vivo derived (ovum pick-up) compared to abattoir derived of the previous studies.

The increase in ATP content over in vitro maturation is believed to provide energy to the matured oocyte about to undergo the events of fertilization and subsequent stages of development (Krisher and Bavister, 1998). For instance, mitochondrial aggregates around the nucleus and clusters around the endoplasmic reticulum are thought to provide immediate energy for pronuclear formation (Wilding et al., 2001) and calcium release following penetration of the spermatozoon (Van Blerkom et al., 2003).

ATP Content of Early Embryos

ATP production changes following the events of fertilization and subsequent development to adapt to cellular demands (reviewed by Dumollard et al., 2009). In human cleavage stage embryos, Slotte et al. (1990) reported ATP content remained almost constant or slightly decreased from fertilization to the 4-cell stage (1.93 pmol to 1.73 pmol) followed by a significant increase at the 8-to 16-cell stage (2.76 pmol). Subsequently, a decrease in ATP content was observed in compact morulae (2.36 pmol) and early blastocyst stage (2.08 pmol) followed by a slight increase at expanded blastocyst stage (2.26 pmol; Slotte et al., 1990). In contrast, Spielmann et al. (1984) and Ginsberg and Hillman (1973) reported a continuous decrease in ATP content from the 1-cell zygote stage to the late blastocyst stage in mouse models. A decrease in ATP content over embryo development was observed in an in vivo study of day 7 bovine

blastocysts. Thompson (1996) demonstrated a significant decrease in oxygen consumption and ATP production in normal to expanding blastocysts stages. Using batch analysis on expanded blastocyst Rieger (1997) reported 0.75 pmol ATP per bovine blastocyst in comparison to 0.08 pmol ATP per ovine blastocyst (Rozell et al., 1992) and 2.26 pmol ATP per human expanded blastocysts (Slotte et al., 1990).

In addition to ATP content, mitochondrial membrane potential ($\Delta\Psi$; reflective of hydrogen ions pumped across inner membrane during ETC and OXPHOS, the driving force of ATP production) is an excellent indicator of mitochondrial activity levels. Acton et al. (2004) reported that $\Delta\Psi$ changes throughout preimplantation development. For instance, Jc-1 staining of mouse zygotes, 2-, 4-, 8-cells, compact morulae and blastocyst stage embryos indicated that mitochondrial activity was low in pre-compaction stages, but metabolic parameters increased thereafter. This finding is interesting since previous reports indicated ATP content to decrease further in development (Spielmann et al., 1984; Ginsberg and Hillman, 1973). A possible explanation may be that since there is no mitochondrial replication until blastocyst development (Pikó and Taylor, 1987) the initial population of mitochondria must be rationed and therefore the metabolic activity of fewer mitochondria per cell must increase to meet the rising demands of cellular activity.

Heat Stress Exposure and Effect on ATP Content

Little has been done to examine consequences of heat stress on ATP production in preimplantation development and although heat stress exposure during the first 12 h of meiotic maturation increased ATP production in oocytes at 24 hIVM, it was not the case in heat-stressed zygotes or embryos. Rivera and Hansen (2001), exposed bovine oocytes during fertilization and at the 1- and 2-cell stages to heat stress conditions and disrupted early embryonic development (i.e., decreased blastocyst rates). However, it went without any impact on ATP content in two-cell embryos (28 hours post insemination; **hpi**) after exposure to 41.0°C for 6 h which did not differ from control counterparts (0.30 pmol/embryo and 0.26 pmol/embryo respectively; Rivera et al., 2004).

Experimental efforts have shown that hyperthermic conditions may alter ATP production in other cell types. Exposure to hyperthermia has been reported to induce conformational changes to intermembrane protein particles (reviewed by Streffer, 1985) which would potentially be problematic for proteins associated with the ETC and OXPHOS systems. For instance, in HeLa cells heat stress exposure causes an inactivation or even loss of Na⁺K⁺ATPase from the cell membrane (Burdon et al., 1984). Data also suggests that cells and tissues of mice exposed to whole body elevated temperatures of 40° and 41°C results have in an increase of energy usage (measured by O₂ consumption) and therefore metabolic turnover rates are enhanced (Streffer, 1982). Therefore exposure to elevated temperatures may alter mitochondrial function by changing the production of ATP or initiating permeability changes of the inner mitochondrial

membrane which may cause an uncoupling of oxidative phosphorylation systems causing the proton electrochemical gradient to dissipate (Finkel and Holbrook, 2000). For instance, melanoma cells showed an increase in ATP production even though a decrease in absolute quantity of ATP was noticed (i.e., ATP turnover more rapid) after exposure to 37°C. Specifically, ATP levels decreased while ³H-adenine incorporation into ATP increased suggesting that the energy demand in cells exposed to hyperthermic conditions increased as well (Mirtsch et al., 1984). Moreover, cellular ATP has been correlated with cell survival in L5178Y cells treated with hyperthermia, reporting that an increase in ATP content results in higher survival rates (Shinohara et al., 1993). However, Lunec and Cresswell (1983) reported a decrease in ATP content in murine lymphoma cells following exposure to 44°C (considered extreme conditions) for 10 to 60 min, whereas no change occurred in Ehrlich ascites cells under the same conditions (i.e., depended on cell type). Dependent upon cell lines of HeLa cells, exposure to elevated temperatures causes either no change or a decrease in ATP content (Shinohara et al., 1987). Furthermore, Chinese hamster ovary fibroblasts (HA-1 cells) exhibit a decrease in ATP production over time when exposed to elevated temperatures under starvation conditions (Calderwood, 1987) alluding to the effect of heat to inactivate processes involved in the uptake and metabolism of nutrients. Whether heat-stress is directly altering the production of ATP is unclear. But if heat-induced decreases in ATP content of cells is considered, then data may indicate that ATP turnover is increased in these cells (reviewed by

Streffer, 1985). Unlike other somatic cells, a transcriptionally quiescent oocyte may not be utilizing all of the additional ATP in response to hyperthermia.

Heat Stress Effects on Mitochondrial Function in Oocytes and Embryos

The increase in ATP content in matured oocytes at 24 hIVM after exposure to a physiologically-relevant temperature of 41°C for the first half of meiotic maturation is suggestive of an increase in mitochondrial activity. Heat stress may modify membrane integrity by altering the structure of membrane associated proteins (Streffer, 1982) and since the ETC and OXPHOS systems reside within the inner mitochondrial membrane, the multi-protein complexes may be compromised.

Reactive oxygen species (**ROS**) are normal free-radical by-products of OXPHOS (Brookes et al., 2004, Al-Gubory et al., 2010). As well as altering the machinery that produces ATP, the effect of heat stress may exacerbate the production of free radicals and alter the concentration of antioxidants (Payton et al., unpublished). Payton et al. (unpublished) reported an increase in mitochondrial ROS after exposure to 41°C after 6 h incubation. Nabenishi et al. (2011) reported increased ROS in mature bovine oocytes following exposure to elevated temperatures during maturation compared to control oocytes and oocytes exposed to elevated temperatures with the addition of cysteine (a low molecular weight thiol compound that stimulates glutathione synthesis). Although ROS have been associated with numerous important physiological events like ovulation (Miyazaki et al., 1991), sperm capacitation (Bize et al., 1991), and

implantation (Manes and Lai, 1995) they can still cause oxidative stress and cellular injury if not properly neutralized (reviewed by Dumollard et al., 2009). Loven (1988) reviewed the production of free radicals in response to heat stress and states that the damage caused by ROS include lipid peroxidation, causing a disruption of the cytoskeleton and calcium metabolism, both important aspects of the maturing ovum. The damage caused by oxidative stress is usually compensated for naturally by the neutralization from glutathione (**GSH**) and other antioxidants. A lower level of cellular GSH is suggestive of heat-induced increases in ROS generation. Whereas the application of heat stress applied during the first 12 hIVM increased total GSH, a decrease in GSH content was observed at 24 hIVM after a 12 h recovery period under thermal neutral conditions compared to control counterparts (Payton et al., unpublished). This is consistent with work of Nabenishi and coworkers (2011), who reported a reduction in GSH in mature bovine oocytes with and without cysteine supplementation following exposure to elevated temperatures during maturation compared to controls. Arechiga et al. (1995) also reported a decrease in the antioxidant GSH in murine embryos exposed to heat-stressed conditions.

Collectively, increased ATP content, increased ROS generation, and a decrease in GSH in heat-stressed oocytes at 24 hIVM support the theory that heat stress caused an increase in mitochondrial function. However, Soto and Smith (2009) reported a reduced mitochondrial membrane potential in bovine oocytes exposed to 41°C for the entire duration of meiotic maturation, indicating a decrease in mitochondrial activity. This contradictory finding may be explained

by the extended (>12 h) duration of exposure to elevated temperatures. Exposing bovine oocytes to heat stress >12 h has dramatic effects (inhibits maturation, etc, Nabenishi et al., 2011) which likely are not physiologically-relevant. The later study exposed oocytes for the duration of maturation compared to just partial seen in previous studies. Regardless, data provides evidence that heat stress perturbs mitochondrial function in the oocyte.

Heat Stress Effects on Mitochondrial Function in Other Cell Types

Numerous cell types exhibit perturbed mitochondrial activity after exposure to increased temperatures. A significant increase in the activity of mitochondrial respiratory complexes in the ETC and OXPHOS systems was reported in rat cardiac tissue after exposure to heat stress (41.8 to 42.0°C) conditions when compared to control (37.0 to 38.7°C) counterparts measured by mitochondrial oxygen consumption (Sammur et al., 2001). In addition, Willis et al. (2000) demonstrated rat liver mitochondria exhibited increased activity of Q10 of pyruvate carboxylase (ATP/ADP ratio are important determinants of activity) between 37 and 43°C. However, the metabolic and mitochondrial behavior (i.e., ATP content) of cloned microglia was reduced 40% of basal levels due to exposure to 45°C for 20 minutes, but 24 h proceeding exposure, the microglia recovered to control ATP levels (de Gannes et al., 1998).

Heat stress alters not only ATP content in various cell types but perturbations are evident within protein structures and activity levels. Hyperthermia modifies functional properties of polypeptide complexes of the ETC

in Ehrlich ascites tumor mitochondria (Floridi et al., 1987). Floridi et al. (1987) reported isolated mitochondria from tumor cells exposed to 41°C for one hour expressed a decrease rate of oxygen consumption, a reduction of electron transport rate in all energy-conserving stages, and also a decline in the oxidoreduction state of respiratory carriers. Although ATP synthesis wasn't inhibited, overall mitochondrial function exhibited heat-induced impairments (Floridi et al., 1987).

Mitochondria and Developmental Competence

For subsequent development to occur after fertilization, the distribution of mitochondria to the daughter blastomeres must be sufficient to produce adequate ATP to meet cellular demands since mtDNA replication does not begin until blastocyst stage (Pikó and Taylor, 1987, Smith et al., 2004) or until after implantation (Larsson et al., 1998). Symmetrical divisions occur in the 2-cell stage embryo but asymmetrical formation (due to gradients of their distribution) of blastomeres occur thereafter (Tarazona et al., 2006). Before compaction (between the 2- and 10- cell stage) of in vitro produced human embryos, blastomeres may cease development (no longer undergo cleavage) or even undergo lysis due to an inadequate energy supply (Van Blerkom et al., 2000). Van Blerkom and coworkers (2000) reported that the quantity of mitochondria and content of ATP differed between apoptotic blastomeres and those considered 'healthy' (able to undergo subsequent cleavage). Asymmetrical distribution of mitochondria at the pronuclear stage caused an uneven

distribution of mitochondria in resultant daughter blastomeres which were unable to undergo further cytokinesis due to a decrease in mitochondrial content and ATP production (Van Blerkom, 2010).

Contrary to previous observations, Iwata et al. (2011) reported no correlation between mtDNA copy number and ATP content of matured oocytes, which suggests activity of mitochondria rather than number is a more precise indicator of viability. Tarazona et al. (2006) evaluated mitochondrial activity, using Jc-1 fluorescent detection, to determine mitochondrial membrane potentials. It was reported that matured oocytes exhibited a high level of activity (76 to 100%). Furthermore, the majority of competent embryos (hpi and stage matched) displayed a mid-range (31 to 50%; fluorescent intensity) of activity level. Of embryos considered to be incompetent 90% exhibited a much lower level (1 to 15%) of mitochondrial activity throughout various stages of early embryonic development, while only 6% of embryos deemed incompetent had a maximum activity level of 16 to 30% (Tarazona et al., 2006). Data are suggestive that a certain mid-range level of mitochondrial activity is ideal for developmental competence, and agree with the quiet embryo hypothesis proposed by Leese and coworkers (2002, 2008).

ATP Content and Developmental Competence

The most developmentally competent oocytes and early embryos that have the highest potential of establishing a pregnancy (i.e., increased embryo development and quality) contain levels of ATP between 1.8 and 2.2 pmol in

human MII oocytes and pronuclear zygotes (Van Blerkom et al., 1995, Stojkovic et al., 2001, Van Blerkom, 2004a, 2004b). Zeng et al. (2007) also indicated that human MII oocytes should have a cytoplasmic ATP concentration ranging from 1.7 to 2.0 pmol. These studies have correlated levels of ATP content with “normal” pre-implantation development (ability to develop into blastocyst stage embryos). In addition, low ATP content suggest oocyte aging due to depressed mitochondrial function (Fissore et al., 2002) and aged oocytes are associated with a reduced developmental competence (Ward et al., 2002, Agung et al., 2006). On the other hand, ATP levels that are too high also predict poor development potential (Nagano et al., 2006) and abnormal fertilizations (Iwata et al., 2011).

Abattoir-derived oocytes are evaluated prior to maturation to only include those that have a dark evenly granulated ooplasm and tight compact multiple layers of cumulus cells to undergo maturation and IVF (Nagano et al., 2006a). Evaluating cytoplasmic morphologies of bovine oocytes using five different classifications with regards to coloration and granular distribution coincident with ATP content which revealed that groups 2 (brown in color with homogenous appearance and a dark zone around the periphery), 3 (brown in color with heterogeneous cytoplasm containing dark clusters), and 5 (pale coloration with heterogeneous cytoplasm containing dark clusters) contained a moderate level of ATP (Nagano et al., 2006a). Along with moderate ATP levels, another study reported oocytes of groups 2, 3 and 5 had good developmental competence (blastocyst rates: 24.4, 29.4, and 24%, respectively; Nagano et al., 2006b).

Stojkovic et al. (2001) evaluated ATP content between different bovine oocyte classifications and reported category 1 and 2 had significantly more ATP than category 3 and 4 oocytes and an increased ability to undergo development to the blastocyst stage. Oocytes in category 4 had significantly lower ATP levels compared to all other categories coincident with a reduced proportion making it to the blastocyst stage. Not only did category 1 and 2 oocytes have higher ATP and a better rate of blastocyst development, but also significantly more cell numbers at expanded blastocyst stage (Stojkovic et al., 2001). Selection criteria of oocytes for the in vitro procedure of bovine embryos relies heavily on oocyte morphology (coloration) as well as the presence of tightly compacted cumulus cells and data suggests that ATP content in addition to oocyte morphology is correlated with development potential following in vitro fertilization.

CHAPTER 3

Assessing Mitochondrial Activity in Embryos Derived from Heat-Stressed Ova

ABSTRACT

Unpublished, preliminary results of Payton et al. showed that heat-stressed oocytes have higher ATP content than nonheat-stressed oocytes. Since the oocyte contributes >99.9% of ooplasm containing mitochondria to the resultant embryo, it was hypothesized that this perturbation may persist in early stage embryos. Potential for this to occur is concerning as an increase in ATP content is indicative of increased mitochondrial activity which has been correlated with reductions in development. To assess the extent to which heightened mitochondrial function (i.e., increased ATP content) carries over to embryos from heat-stressed oocytes, ATP content was assessed in cleavage and blastocyst-stage embryos from oocytes that were matured at 38.5°C for 24 h or 41°C for the first 12 h followed by 38.5°C. Heat-induced increases in ATP content are evident in matured oocytes which persisted into early cleavage-stage embryos. However, increased ATP content is not evident in embryos from heat-stressed oocytes by the blastocyst-stage. ATP content exhibited exponential decay from 0 hours post insemination (**hpi**) to 204 hpi, furthermore, poorer quality blastocyst had significantly less ATP than blastocysts deemed good in quality. The extent to which heat-induced alterations of oocytes persist into early embryos to decrease developmental competences is unclear.

INTRODUCTION

Heat stress is economically important to the dairy industry, costing between 800 and 900 million dollars annually (St-Pierre et al., 2003, Collier et al., 2006). This problem is largely due to heat-induced reductions in fertility (Rensis and Scaramuzzi, 2003) related to rectal temperatures that may reach or even exceed 41.0°C (Seath and Miller, 1946, Monty and Wolff, 1974, Ealy et al., 1993). Hyperthermia is problematic during estrus (i.e., a time period when a dominant follicle houses the maturing ovum) as it increases the incidence of early embryonic death (Putney et al., 1989). Reductions in early embryonic development may be due to hyperthermia induced changes in maternal environment (reviewed by Rensis and Scaramuzzi, 2003) or due to direct effects of elevated temperature on the oocyte. For instance, in vitro studies which expose bovine oocytes directly to heat stress during oocyte maturation, have shown repeatedly that heat stress exposure reduces embryo development (Edwards and Hansen, 1996, 1997, Roth and Hansen, 2004, Edwards et al., 2005, 2009, Schrock et al., 2007, Wang et al., 2009b, Payton et al., 2011), in a manner similar to what occurs in vivo (Putney et al., 1989).

Edwards et al. (2009) showed that embryos from heat-stressed oocytes are less developmentally competent than those from nonheat-stressed oocytes. In particular, it was shown that embryos from heat-stressed oocytes were more susceptible to an additional heat stress than those from nonheat-stressed oocytes. Consistent with this finding, Cartmill et al. (2001) and Rutigliano et al. (2008) demonstrated that exposure to elevated temperatures during estrus and

artificial insemination (respectively) were more likely to lose their pregnancies than nonheat-stressed cattle.

Oocyte maturation includes nuclear and cytoplasmic events that must occur in a specific spatial and temporal manner in order for the oocyte to acquire the capacity to be fertilized and undergo subsequent development (reviewed by Barnes and Sirard, 2000). Several of these developmentally-important processes are altered when oocytes are exposed to elevated temperatures during maturation (coinciding with estrus in the cow). For instance, exposure to an elevated physiologically-relevant temperature during the first 12 h of in vitro maturation (**hIVM**) hastens nuclear maturation (Edwards et al., 2005); in extreme cases it may actually inhibit nuclear maturation (Roth and Hansen, 2005, Nabenishi et al., 2011). Heat-induced alterations at the cytoplasmic level are also evident, and may include decreased de novo protein synthesis (Edwards and Hansen, 1996), altered cortical granule translocation (Edwards et al., 2005), decreased glutathione content (Wang et al., 2009a) and calcium release (Tseng et al., 2009), and modifications of cytoskeletal components (Roth and Hansen, 2005).

As a first step towards identifying molecular mechanisms that may be underlying heat-induced reductions in embryo development, Payton (2009) examined the transcriptome profiles of oocytes following heat stress exposure during meiotic maturation. Total RNA was isolated from oocytes after 24 hIVM at 38.5 or 41.0°C for the first 12 h followed by 38.5°C for bovine genome microarray analysis. Heat-induced differences in the relative abundance of numerous

transcripts were detected with ~20 transcripts having importance in mitochondrial function (Payton, 2009). The majority of these transcripts (n = 13) are known to be involved with electron transport chain (**ETC**), located in the inner mitochondrial membrane and responsible for ATP production (Smeitink et al., 2001; Figure 3.1); impact of heat stress was to decrease relative abundance. While functional significance of these findings remain unclear, in a separate study Payton et al. (unpublished – preliminary data) showed increased ATP content at 24 hIVM in oocytes exposed to heat stress for the first 12 hIVM compared to nonheat-stressed oocytes.

Heat-induced increases in mitochondrial activity (i.e., increased ATP content) may explain some of the reductions in development of heat-stressed oocytes. Others have reported that increased mitochondrial membrane potential (representative of mitochondrial status) in oocytes from obese mice coincides with a reduction in subsequent blastocyst development compared to leaner mice (Igosheva et al., 2010). Since the oocyte contributes not only half of the genetic material but >99.9% of cytoplasm containing organelles including all mitochondria to the resultant embryo, persistence of heat-induced perturbations into early embryo development is intuitive.

It was hypothesized that elevated mitochondrial function of heat-stressed oocytes may be inherited by the zygote and persist through early cleavage stage divisions after fertilization; thus possibly explaining why embryos from oocytes

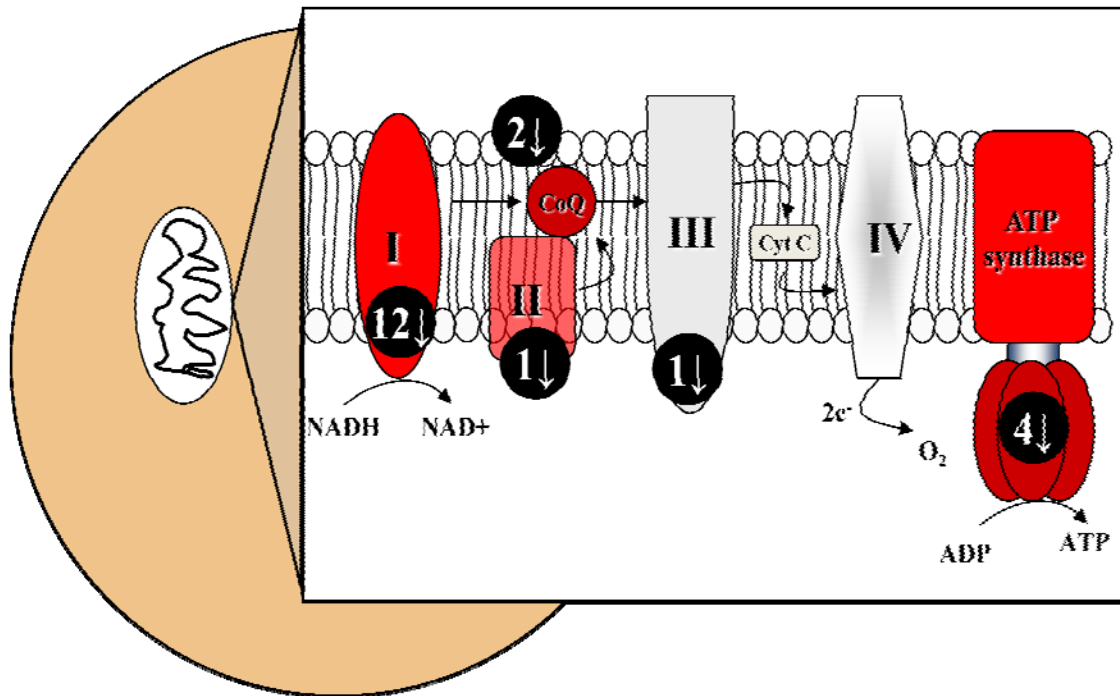


Figure 3.1: Schematic of heat-induced perturbations of transcripts of the electron transport chain (**ETC**) from the oocyte from microarray analysis. Multi-protein complexes with transcripts reduced are shown in red with the number of transcripts affected indicated in each circle (adapted from Payton, 2009).

exposed to heat stress during meiotic maturation are developmentally impaired. To test this hypothesis, the main objective was to examine ATP content of cleavage and blastocyst-stage embryos from heat-stressed oocytes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Standard fetal bovine serum (**S-FBS**) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA) while premium fetal bovine serum (**P-FBS**) and gentamicin were purchased from BioWhittaker (Walkersville, MD). Medium-199 (**M199**) containing Earle's salts was purchased from Invitrogen (Division of Life Technologies; Carlsbad, CA, USA) while M199 with Hank's salts was purchased from Mediatech (Manassas, VA, USA). Penicillin-streptomycin was purchased from Millipore (Billerica, MA, USA). Oocyte maturation medium (**OMM**) consisted of M199 (with Earle's salts), 10% P-FBS, 50 µg/mL gentamicin, 5 µg/mL FSH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine (Schrock et al., 2007). Modified Tyrodes' Albumin Lactate Pyruvate media (TALP; HEPES-TALP, IVF-TALP, and Sperm-TALP) was prepared as per Parrish et al. (1988). Potassium simplex optimized medium was prepared as previously described (Biggers et al., 2000) but modified to contain 0.5% BSA, 10 mM glycine, 1 mM L-glutamine, 1x nonessential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin (**KSOM**). Follicle stimulating hormone was obtained

from Bioniche (Belleville, Ontario, Canada). Frozen semen was generously provided by Dr. Mitch Hockett (Kingsmill Dairy Farm; Liberty, NC).

In Vitro Production of Embryos

Procedures describing in vitro production of embryos have been previously described by Edwards et al. (2005) with minor modifications. Briefly, cumulus-oocyte complexes (**COCs**) were obtained from antral follicles (~3 to 8 mm) of abattoir-derived ovaries. Only COCs with a dark, evenly granulated cytoplasm and multi-layer, compact cumulus were matured in cohorts of approximately 40 to 45 in 500 μ L OMM for 24 h at 38.5°C or 12 h at 41°C and 12 h at 38.5°C in 5.5% CO₂ and ambient O₂ (approximately 21%) in a humidified environment. Oocytes presumed mature underwent in vitro fertilization (**IVF**) at 24 hIVM. Percoll-prepared frozen-thawed semen was assessed for concentration and motility via computer assisted sperm analysis (**CASA**; Hamilton Thorne Biosciences; Beverly, MA, USA) and 500,000 motile sperm/mL was added to COCs in 500 μ L IVF-TALP containing 12.5 μ M penicillamine, 25 μ M hypotaurine, and 1.25 μ M epinephrine. Semen from 2 Holstein bulls was combined before use for all replicates of IVF.

After a 16 to 18 h co-incubation with sperm, presumptive zygotes (**PZs**) were denuded of associated cumulus cells and spermatozoa by vortexing for 4.5 minutes in HEPES-TALP containing 0.3 mg/mL of hyaluronidase. Groups of approximately 20 PZs were cultured in 500 μ L KSOM at 38.5°C in a 5.5% CO₂ and 7% O₂ in a humidified environment. At approximately 70 to 72 hours post

insemination (**hpi**), subsets of PZs were evaluated for their ability to divide into more than one-cell (i.e., cleavage assessment). Afterwards, essential amino acids (1x final concentration) was added to medium containing embryos and allowed to continue in development at 38.5°C in a 5.5% CO₂ and 7% O₂ humidified environment. At 204 to 216 hpi, blastocyst stage embryos were assessed and assigned stage (early (5), normal (6), expanded (7), or hatched (8)) and quality scores (good (1), fair (2), poor (3)) per International Embryo Transfer Society standards (Robertson and Nelson, 1998).

Experimental Design and Endpoints

Experimental design schematic is provided in Figure 3.2. In general, ATP content was examined in control and heat stress oocytes after maturation for 24 h at 38.5°C (control) or 41.0°C (first 12 h only, remaining 12 h at 38.5°C; heat stress). Embryos resulting after performing IVF of control and heat-stressed oocytes were also analyzed for ATP content. To this end, cleavage stage embryos (2-, 4-, and 8-to-16-cells) were removed from culture at 70 to 72 hpi while resultant blastocyst stages were removed at 204 to 216 hpi. This experiment was replicated on 12 different occasions with a total of 302 and 290 control and heat-stressed oocytes, 419 and 450 cleavage-stage embryos, and 210 and 144 blastocyst stage embryos from control and heat-stressed oocytes.

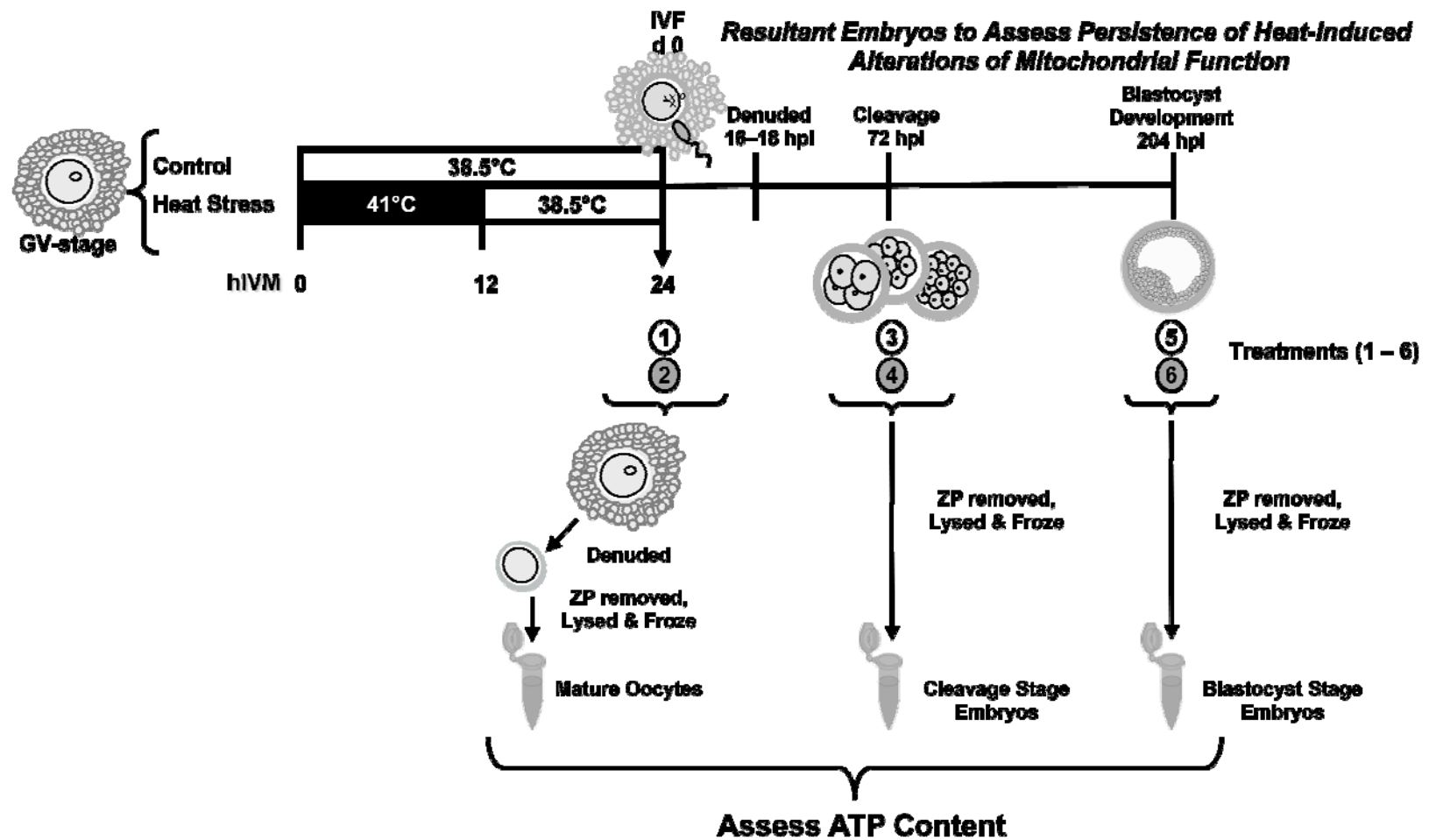


Figure 3.2. Schematic of experimental design and endpoints. Soon after removal from ovarian follicles, cumulus-oocyte complexes (**COCs**) were randomly allotted for maturation at 38.5°C for 24 h (indicated by white circles; control), while others were matured at 41.0°C for first 12 h followed by 38.5°C for the remainder of maturation (indicated by gray circles; heat-stressed). At 24 hIVM, subsets of both control and heat-stressed oocytes were removed from culture, denuded of associated cumulus cells, zona pellucidas removed via pronase, and lysed individually before storage at -80°C until ATP assay (Treatments 1 & 2). Remaining subsets of COCs underwent in vitro fertilization (**IVF**). Presumptive zygotes (**PZs**) were denuded 16 to 18 hours after IVF. A subset of cleavage stage embryos (2, 4, 8-to-16-cell) were collected at ~72 hpi and processed for ATP content individually (Treatments 3 & 4). Remaining embryos underwent further culture until ~204 hpi, when blastocyst stage embryos were individually processed for ATP content (Treatments 5 & 6)

ATP Content Assay

ATP content in oocytes and resultant embryos after IVF was determined using a quantitative bioluminescence assay (ATP determination kit; Invitrogen) as per manufacturer's instructions, using a tube-based luminometer (Berthold, Huntsville, AL, USA; 3 s hold & 10 s read). The luciferin-luciferase reaction is a terminal assay that uses ATP and oxygen to give off light in direct proportion of ATP available, thus allowing quantification of ATP content within a cell. Total amount of ATP was determined using a standard curve ranging from 0 to 5 pmol.

Before being processed for ATP content, oocytes were denuded of associated cumulus cells. Thereafter, cumulus-free oocytes were visually inspected using a stereomicroscope to ensure complete removal of cumulus. Denuded oocytes, cleavage and blastocyst-stage embryos were pronased (0.5%) to remove zona pellucida. Oocytes and embryos were then individually transferred in 1 μ L HEPES-PVA (HEPES-TL containing 0.3% polyvinyl alcohol) to a 0.5 mL microcentrifuge tube, lysed with addition of 9 μ L H₂O and stored at -80°C (Figure 3.2).

Statistical Analysis

Development and ATP content data were analyzed as a randomized block design (**RBD**) blocking on collection date using SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). The main effect of treatment (control and heat stress) and stage (matured oocyte, 2-, 4-, 8-to-16-cell, blastocyst) were analyzed using generalized linear mixed models (PROC GLIMMIX). Random effects include replicate and

replicate x treatment. Treatment differences were determined using an F-protected least significant difference test and reported as least square means $\pm$ standard error of the difference (**SED**) using the inverse link option. Pre-planned pairwise comparisons within a stage were also made.

To determine if heat stress exposure during meiotic maturation altered ATP content throughout embryo development, ATP data were analyzed using PROC GLIMMIX (SAS 9.2) with a log transformation in the X variable (hour). A Kenward-Roger degrees of freedom adjustment was used. To investigate a relationship, if any, of ATP content in oocytes and rate of blastocyst development, PROC GLIMMIX was used by calculating Nagelkerke R^2 value by conversion of -2 log-likelihood.

RESULTS

Embryo Development of Oocytes Matured at 38.5 or 41.0°C

Exposure to heat stress during first the 12 h of maturation did not affect the proportion of oocytes recovered ($P = 0.16$) or lysed ($P = 0.8$) after denuding of associated cumulus (Table 3.1). Despite overall cleavage rates being similar, there was a decrease in the proportion of 8-to-16-cell embryos from heat-stressed oocytes compared to those from nonheat-stressed oocytes ($P = 0.02$; Table 3.1). This effect coincided with an increase in the proportion of 4-cell embryos from heat-stressed oocytes ($P = 0.009$; Table 3.1). Exposure to heat stress for the first 12 of meiotic maturation decreased blastocyst development

Table 3.1. Embryo development of developmentally competent oocytes matured at 38.5 or 41°C (n = 14 replicates)

IVM Temperature (°C)	Total COC ¹	Cleavage (72 hpi)							Blastocyst (204 to 216 hpi)			
		Rcd (%) ²	Lysed (%) ³	No. PZ ⁵	Cleaved (%)	2-Cell ⁴ (%)	4-Cell ⁴ (%)	8 to 16- Cell ⁴ (%)	No. PZ ⁶	Percent of PZ	Stage	Quality
38.5	1999	94.74	1.341	750	70.05	8.718	22.02 ^B	71.00 ^A	856	29.73 ^A	6.8036	1.5085
41.0*	2032	93.50	1.247	789	69.87	9.224	27.08 ^A	63.50 ^B	853	19.91 ^B	6.8912	1.5911
SEM		0.9419	0.2887		2.583	1.435	1.376	2.4185		2.264	0.09437	0.07001
P-Value		0.1651	0.8011		0.9404	0.7765	0.0090	0.0221		0.0005	0.4942	0.3472

Abbreviations: hpi = hours post insemination; IVM = in vitro maturation; Rcd = presumptive zygotes recovered after removal of cumulus; PZ = presumptive zygote

¹Cumulus-oocyte complex that underwent IVM

²PZ recovered after denuding as a proportion of total number of COCs matured

³PZ without an intact plasma membrane after denuding as a proportion of recovered

⁴Relative to proportion of embryos cleaved

⁵Number of embryos evaluated at 72 hpi

⁶Number of embryos evaluated 204 hpi

*First 12 h of maturation followed by 38.5°C thereafter

^{A,B} Within a column differs

rates (Table 3.1). On average, blastocyst development from heat-stressed oocytes was 33% less than that of controls. Of the blastocysts that developed, heat stress had no impact on stage ($P = 0.494$) or quality ($P = 0.347$) scores (Table 3.1).

Effects of Heat Stress on ATP Content

At 24 hIVM, ATP content was higher in oocytes exposed to 41°C during the first 12 hIVM compared to nonheat-stressed oocytes ($P = 0.0148$; Figure 3.3). When examining cleavage-stage embryos ATP content was higher in 8-to-16-cell stage embryos from heat-stressed oocytes ($P = 0.0323$; Figure 3.3). However, by the blastocyst stage ATP content was similar in embryos from control and heat-stressed oocytes (Figure 3.4).

Independent of IVM temperature, blastocysts assigned a quality score 3 (poor; 0.3632 ± 0.03773) had less ATP content than blastocysts assigned a quality score 1 (good; $0.4610 \pm .02708$; $P < 0.05$) or quality score of 2 (fair; $0.4158 \pm .03113$). Regression analysis was performed using data derived from matured oocytes through the blastocyst stages following fertilization to better clarify developmental changes in ATP content (data included 8-to-16-cell stage embryo (72 hpi) and expanded and hatched (7 and 8, respectively; 204 hpi). There was an IVM temperature x stage interaction ($P = 0.0385$; Figure 3.5), where heat-stress treatment significantly increased the slope and intercept ($P < 0.0001$ and $P < 0.0001$, respectively) of the exponential decay in ATP content compared to nonheat stressed. Additional efforts showed no correlation was

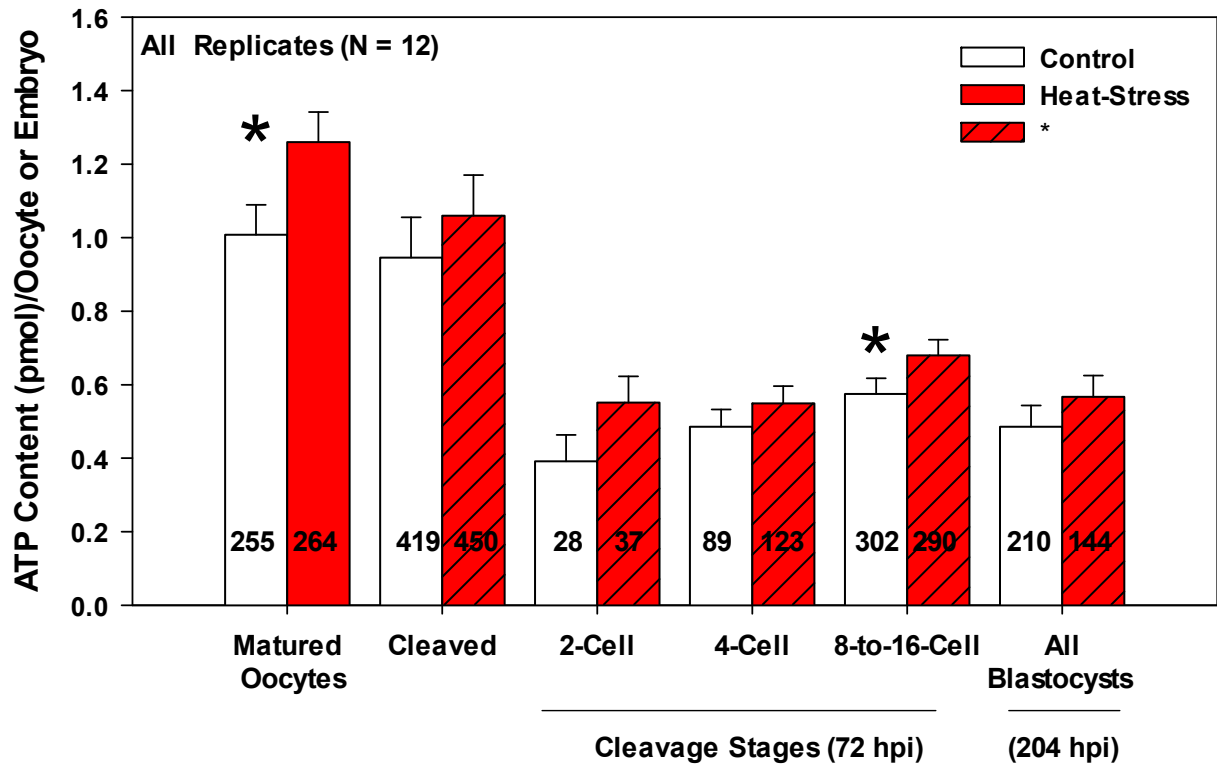


Figure 3.3. ATP content (pmols $\pm$ SED) in matured oocytes, cleaved embryos and blastocyst-stage embryos from control and heat-stressed oocytes (pairwise comparison).

* Means within stage differ.  * Embryos from heat-stressed oocytes

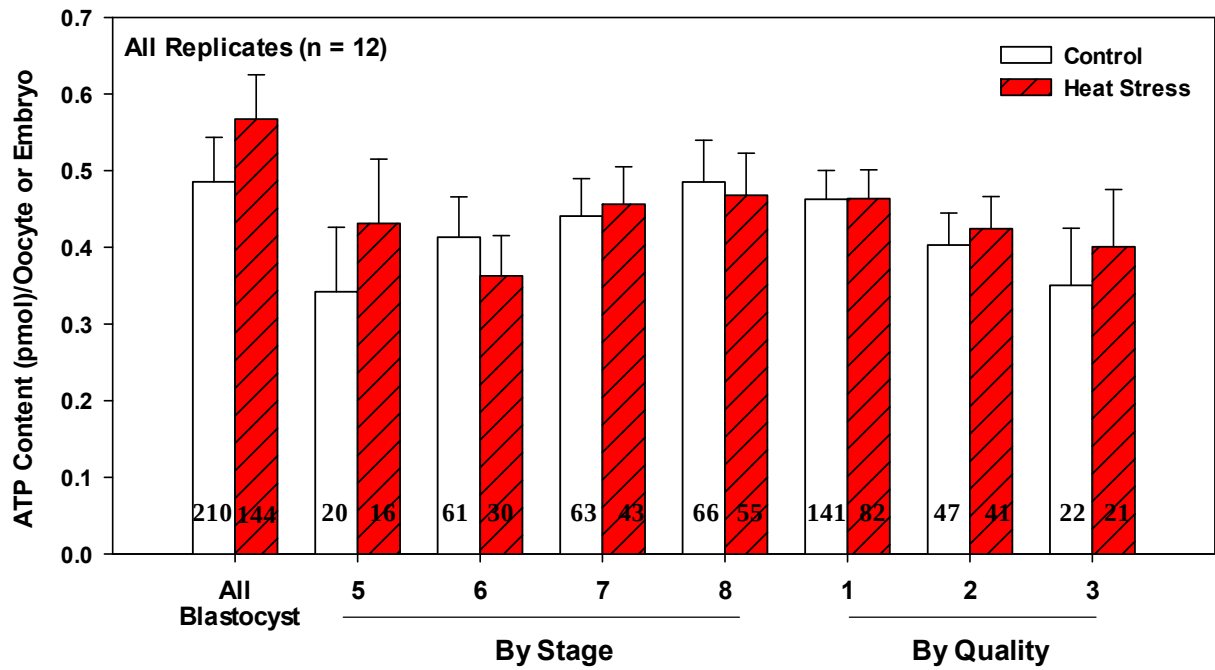


Figure 3.4. ATP content (pmols $\pm$ SED) in blastocysts of different stages and quality scores from control and heat-stressed oocytes.

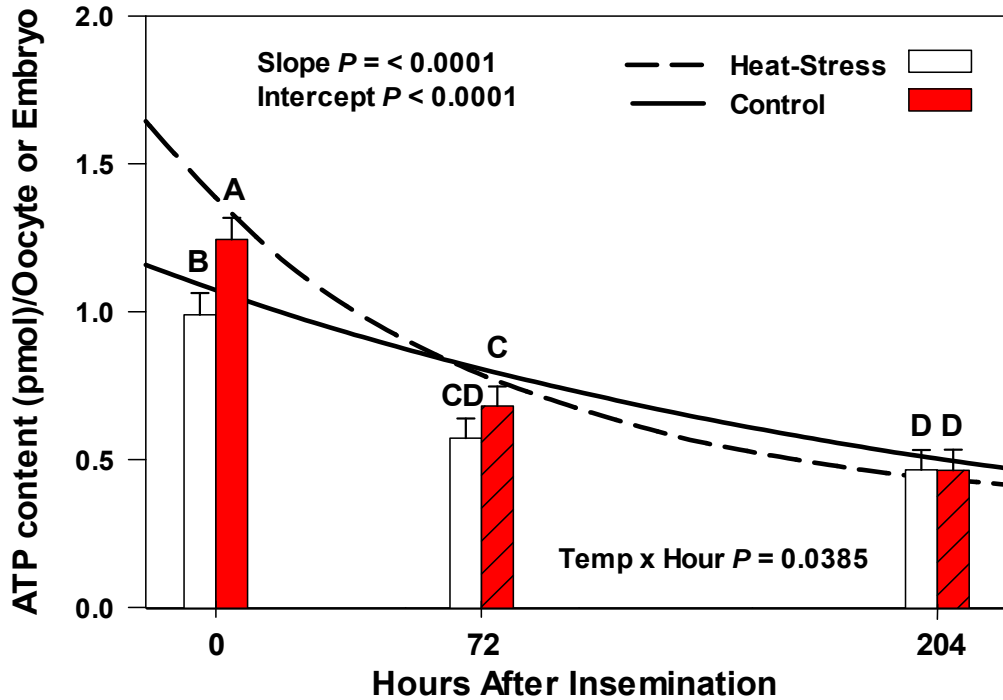


Figure 3.5. Exponential decay of ATP content in control and heat-stressed matured oocytes, and in resultant 8-to-16-cell and blastocyst-stage embryos.

ATP content over development = intercept + slope*log10(hour). Slope ($P < 0.0001$; -0.2245 and -0.3244) and intercept differed ($P < 0.0001$; 0.9873 and 1.2485) between control and heat stress, respectively. Solid line reflects culture throughout at 38.5°C while dashed line was cultured at 41.0°C for the first 12 hIVM but 38.5°C thereafter. Bar graph indicate data points from which regression analysis derived the equations (0 hpi (matured oocytes), 70 to 72 hpi (8-to-16-cell embryos), and 204 to 216 hpi (expanded (7) and hatched (8) blastocysts). An IVM temperature by time (hour) interaction was seen ($P < 0.0001$). As time after insemination increased, ATP content within the resultant embryos decreased.

ABCD Means within hour differ.  Embryos from heat-stressed oocytes

evident between IVM temperature, blastocyst development, and pmols of ATP content when converting a log-likelihood into an R^2 .

DISCUSSION

The present findings help to discern heat-induced alterations of mitochondrial activity within the oocyte and the potential for perturbations to carry over to resultant embryos. To this end, it was shown that heat stress exposure during the first 12 h of meiotic maturation increased ATP content in matured oocytes. This consequence persisted into cleavage stage embryos, most evident at the 8-to-16-cell stage. By the blastocyst stage however, differences in ATP content in embryos from control and heat-stressed oocytes were no longer detected. While ATP content in matured oocytes did not correlate with blastocyst development; poor quality blastocysts had less ATP content than those that would be deemed acceptable for embryo transfer (Hasler, 1995).

It is intuitive for heat-induced perturbations in the oocyte to carry over to early embryos since the oocyte contributes >99.9% of the cytoplasm containing mitochondria, to the resultant embryo. Heightened mitochondrial activity (indicated by ATP content; Van Blerkom et al., 1995) is suggestive of increased metabolic activity (Leese et al., 2008). This is concerning when observed in early embryos since a 'quiescent' embryo (i.e., less metabolic activity) tend to exhibit a more viable phenotype (Leese, 2002, Leese et al., 2008a).

Heat-induced increases in mitochondrial function in blastocyst stage embryos may not be evident since it is more complex than an oocyte and 8-to-16

cell embryos. For instance, it is transcriptionally active (Braude, 1979), mitochondria have matured and are replicating (Pikó and Taylor, 1987, Smith et al., 2004), and it has undergone the first differential stages (trophectoderm and inner cell mass). Embryos from oocytes exposed to elevated temperatures and make it to the blastocyst stage might be 'good' since some have the ability to establish pregnancies (Cartmill et al., 2001; Rutigliano et al., 2008); however, a subset is likely incompetent. Carry over effects of heat-induced alterations of mitochondrial function in the oocyte to resultant embryos make sense, even though increases in ATP content were not prominent. Resultant embryos seem morphologically equivalent but it is evident that resultant embryos of heat-stressed oocytes are not as developmentally competent compared to controls (Edwards et al., 2009, Cartmill et al., 2001).

The underlying mechanisms are unclear of heat-induced to alterations in ATP content, but may be a result of one or several possibilities. For example, exposure to hyperthermic conditions can induce conformational changes to intermembrane protein particles (reviewed by Streffer, 1985). Specifically, exposure of HeLa cells to heat stress causes an inactivation or loss of $\text{Na}^+\text{K}^+\text{ATPase}$ from cell membranes (Burdon et al., 1984). Disassociation of $\text{Na}^+\text{K}^+\text{ATPase}$ would result in ATP accumulation. Furthermore, data suggest that cells and tissues exposed to elevated temperatures need more energy and therefore metabolic turnover rates are enhanced (Streffer, 1982), such that more ATP production might be expected.

In addition, protein synthesis is one of the most energy consuming processes (Kelly, 1990) and an oocyte is translationally active. Edwards and Hansen (1996) reported that de novo protein synthesis decreases 30 to 50% in oocytes exposed to heat stress for the first 12 h of meiotic maturation. Thus, decreased protein synthesis suggests decreased utilization of ATP, which would result in an accumulation of ATP content in heat-stressed matured oocytes.

Altered protein function may not be the underlying cause for heat-induced increases of ATP in oocytes and resultant 8-to-16-cell embryos, but rather an increase in the abundance of proteins responsible for ATP production. For instance, a decrease in the relative abundance of transcripts involved with the ETC, ATP producing machinery, of heat-stressed oocytes at 24 hIVM was previously reported by Payton (2009). Decreased transcript abundance is typically correlated with a homodirectional change in protein expression (Fargnoli et al., 1990). However, in yeast, Preiss et al. (2003) reported a decrease in RNA levels corresponding with an increase in protein content following rapamycin treatment in 4.2% of cases. Moreover, Preiss et al. (2003) reported that heat shock (37°C) also demonstrated the same effect, where 36.7% of genes with decreased mRNA abundance showed a corresponding increase in being preferentially translated. This scenario may be true for an oocyte, since it is transcriptionally quiescent after the resumption of meiosis and relies on stored maternal mRNA for protein expression until maternal-embryonic genome transition (Bettegowda, 2007, Lodde et al., 2008). This is further authenticated by evidence of heat stress to alter translational machinery of HeLa S3 cells (Duncan

and Hershey, 1989). Theoretically, translation of stored RNA would increase functional protein levels available to hypothetically increase ATP production.

Increased ATP content by itself may not be detrimental, but the consequences of increased production of potentially harmful by-products to induce oxidative stress (reviewed by Dumollard et al., 2009) may be the underlying culprit. Loven (1988) noted that the production of free radicals increases in response to heat stress. Loven (1988) also stated that the damage caused by reactive oxygen species (**ROS**) includes lipid peroxidation, which causes a disruption of the cytoskeleton and calcium metabolism, both important aspects of the maturing ovum. Regarding impact of heat stress, Payton et al. (unpublished) showed increases in mitochondrial ROS following culture at 41°C for the first 6 hIVM compared to controls. Heat-induced elevations in ROS were not completely unforeseen since use of retinol (an antioxidant) while maturing oocytes at elevated temperature for the first 12 h improved blastocyst development (Lawrence et al., 2004). Furthermore, Nabenishi et al. (2011) reported a reduction in GSH in mature bovine oocytes following exposure to elevated temperatures during maturation which was in agreement with findings of Payton et al. (unpublished) and Wang et al. (2009). A reduction in GSH is indicative of increased utilization of this intracellular antioxidant to neutralize heat-induced increases of free radicals.

In aging cells oxidative stress may introduce mutations in mitochondrial DNA (**mtDNA**) causing defective mitochondrial respiratory function (reviewed by Wei and Lee, 2002). Mitochondria and mtDNA of an embryo are inherited solely

from the mature oocyte; therefore, perturbations occurring within the oocyte are potentially carried over to the resultant embryo. This is concerning since mtDNA copy number of a matured oocyte or embryo does not increase after fertilization until the blastocyst stage (Pikó and Taylor, 1987, Smith et al., 2004), when mitochondrial replication begins (Dvorak and Tesarik, 1985). Therefore, perturbations in mitochondrial activity (i.e., increased ATP production) or mutations in mtDNA of an oocyte are inherited by consequential embryos and possibly contribute to the decrease in proportion of 8-to-16-cell embryos of heat-stressed oocytes compared to controls. Those 8-to-16-cell embryos that did develop from heat-stressed oocytes subsequently also had elevated ATP levels compared to nonheat-stressed controls. This persistent elevation in mitochondrial activity may indicate damaged mitochondria or altered function, thus decreasing developmental competence of resultant blastocyst even further. Extent to which increased mitochondrial function in heat-stressed oocytes accounts for some of the decrease development to blastocyst stage is unclear, but any molecular defect leading to alterations of the mtDNA-encoded genes or impairment in biogenesis of mitochondria could result in a deficiency of energy metabolism of affected cells, thus affecting developmental competence of resultant embryos.

Increased ATP content is suggestive of heightened mitochondrial and metabolic activity (Leese et al., 2008). If heat-induced increases in metabolic activity persists in oocytes and resultant cleavage stage embryos the energy substrates and synthetic precursors provided in culture media may not be substantial to support heightened metabolic demands. A depletion of nutrients

within the media during embryo culture could negatively impact blastocyst development (Krisher et al., 1999).

In addition, heat-induced increased ATP content in matured oocytes may have an effect on sperm and oocyte reprogramming following fertilization. For instance, chromatin decondensation and chromatin remodeling are early chromosomal events in male pronuclear formation during fertilization (Green and Poccia, 1985) and requires ATP hydrolysis for reorganization (Banerjee et al., 1995). Altered levels of ATP content could potentially have a negative effect on the processing of spermatozoa chromatin reducing developmental competence. It has been shown by Edwards et al. (unpublished) that without spermatozoa, chemical activation of heat-stressed oocytes increases blastocyst development closer to control oocytes; therefore it may be a sperm alteration reducing developmental potential.

Independent of heat stress, ATP content has been correlated with development by others. Mitochondrial activity and the production of ATP have been utilized to identify the most developmentally competent (highest potential of establishing a pregnancy) oocytes and early embryos (Stojkovic et al., 2001, Van Blerkom, 2004a, 2004b). ATP content between 1.8 and 2.2 pmol in human MII oocytes and pronuclear zygotes has been correlated with increased embryo development and quality (Stojkovic et al., 2001, Van Blerkom, 2004a, 2004b) as well as an increase in pregnancy rates (Van Blerkom et al., 1995). This is consistent with Van Blerkom et al. (1995) and Zeng et al. (2007) who indicated MII oocytes should have a cytoplasmic ATP concentration ranging from 1.7 to 2.0

pmol. ATP content reported herein in bovine oocytes is consistent with previous reports by Payton et al. (unpublished), Nagano et al. (2006), and Iwata et al. (2011) but subsequent efforts herein to relate ATP content in matured oocytes and developmental competence revealed no relationship. This may be the case since we had a 20% developmental competence cut off, up to 37%, which may be too narrow to have shown any positive correlations.

The study herein provides evidence that ATP content in blastocyst stage embryos is dependent upon blastocyst quality. For instance, blastocysts with poor quality had less ATP than those deemed good in quality. A poorer quality blastocyst likely has more defragmented cells where the embryo mass is <50% of all cellular material (IETS; Robertson and Nelson, 1998), are usually slower developing, and have fewer overall cell numbers (Gardner et al., 2000). In addition, Hasler et al. (1995) reported that poorer quality blastocysts result in reduced pregnancy rates after transfer.

Further investigation is required to assess the extent to which heat-induced alterations of mitochondrial function persist in resultant embryos to hinder developmental competence. A potential next step may be to 'quiet' the oocyte using inhibitors or uncouplers of the ETC and OXPHOS systems to dissipate the proton gradient in efforts to assess whether reducing ATP production in heat-stressed oocytes would improve subsequential developmental competence of resultant embryos.

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CHAPTER 4

Relative Abundance of NDUFC2 and COQ3 in Bovine Oocytes, and IHH Transcript in Surrounding Cumulus after Heat Stress Exposure

ABSTRACT

Perturbations in the transcriptome profiles of oocytes and their associated cumulus heat-stressed during first 12 h of meiotic maturation were reported by Payton (2009) using bovine microarray analysis. The aim of this study was to validate the relative abundance of NDUFC2, COQ3, and IHH transcripts identified by microarray (Payton, 2009) to be perturbed by heat stress in oocytes and surrounding cumulus cells using qPCR. RNA originated from oocytes and associated cumulus cells that were matured at 38.5°C or 41°C for the first 12 h followed by 38.5°C for the remainder of maturation from previous experimental efforts (Payton, 2009, Payton et al., 2011). Results were in agreement with previous microarray data (Payton, 2009) reporting heat-induced decreases of NDUFC2 and COQ3 in oocytes and heat-induced increases in relative abundance of IHH in cumulus cells. While functional significance of these findings remains unclear, heat-induced perturbations at the molecular level in the oocyte are evident and may provide explanation for some of the reductions in developmental competence of heat-stressed oocytes.

INTRODUCTION

During the first half of meiotic maturation, exposure to elevated physiological-relevant temperatures decreases developmental competence of the cumulus-oocyte complex by reducing the number of resultant embryos following fertilization (Putney et

al., 1989, Edwards and Hansen, 1996, Payton et al., 2011). Heat induced reductions in developmental competence are coincident with alterations in numerous developmentally-important processes (Roth and Hansen, 2005, Edwards et al., 2005, Wang et al., 2009b, Tseng et al., 2009). Oocytes exposed to elevated temperatures have the ability to be fertilized and go through subsequent cleavage divisions, but fewer undergo compaction likely providing some explanation for heat-induced reductions in blastocyst rates (Edwards and Hansen, 1996, Edwards et al., 1997, Lawrence et al., 2004, Schrock et al., 2007).

As a first step towards identifying molecular mechanisms underlying heat-induced reductions in development, Payton (2009) examined the transcriptome profiles of oocytes and their associated cumulus after exposure to heat stress during the first 12 h of meiotic maturation using bovine genome microarray analysis. Total RNA was derived from oocytes and associated cumulus after 24 h of in vitro maturation (**hIVM**). Gene ontology analysis showed differential abundance of transcripts important in mitochondrial function due to exposure to a physiologically-relevant heat stress for the first 12 h of meiotic maturation. Specifically, transcripts involved with the electron transport chain (**ETC**), the ATP producing machinery (Figure 4.1.), exhibited a decrease in mRNA abundance. While functional significance of these findings remain unclear, subsequent efforts of Payton et al. (unpublished) and Nagle (see Chapter 3) reported higher ATP content in heat-stressed oocytes when examined at 24 hIVM compared to control counterparts. It was hypothesized that alterations in ATP content could be a result of heat-induced alterations in the relative abundance of individual transcripts in

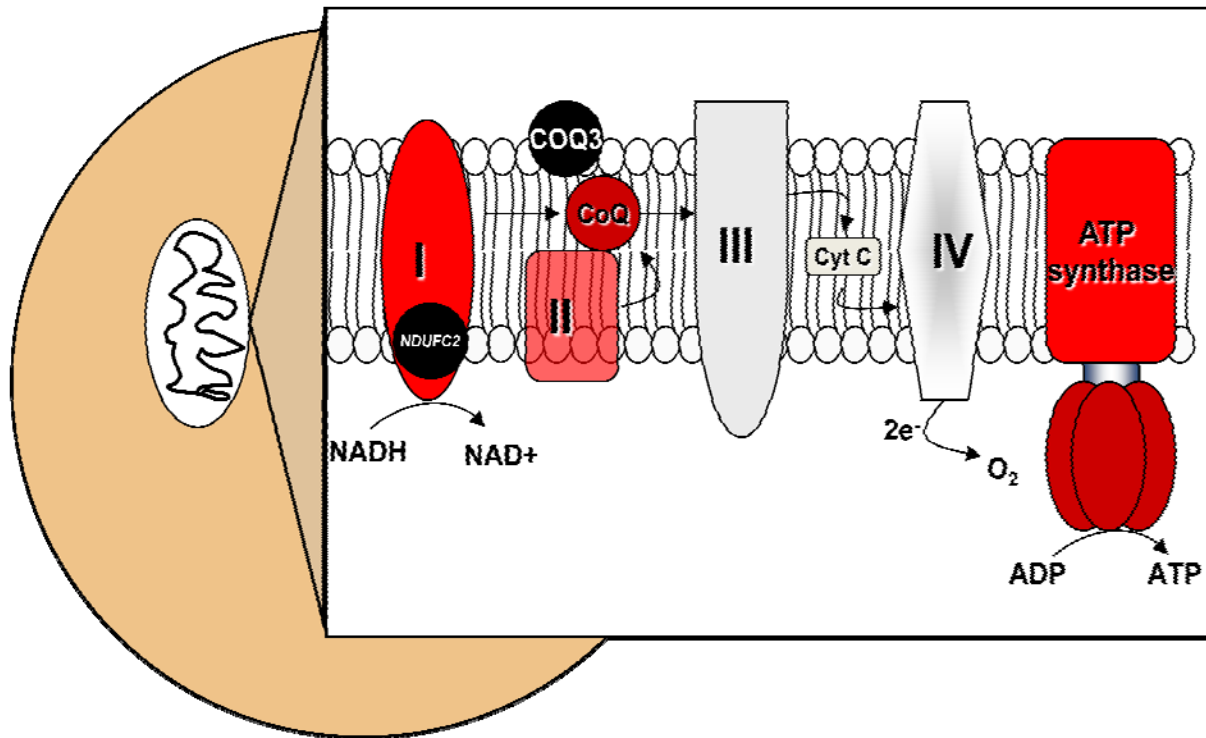


Figure 4.1. Model of the electron transport chain and oxidative phosphorylation for ATP production, highlighting the multi-protein subcomplexes which contain NDUFC2 and COQ3 (Adapted from Payton, 2009).

the ETC. For example, microarray results of coenzyme Q3 homolog (**COQ3**), a methyltransferase located in subcomplex III of the ETC, showed a 1.5 fold decrease comparing heat-stressed oocytes at 24 hIVM to control counterparts. NADH dehydrogenase (**NDUFC2**), a ubiquinone in subcomplex I, was decreased by 2.0 fold in heat-stressed versus control oocytes. Each transcript belongs to a different multi-protein complex within the inner mitochondrial matrix involved in the production of ATP (Figure 4.1). A common validation technique for validating alterations in gene expression results of microarray studies is quantitative real-time polymerase chain reaction (**qPCR**). To this end, COQ3 and NDUFC2 identified by microarray analysis of polyA amplified oocyte RNA will be further investigated using qPCR.

The oocyte is intimately associated with companion cumulus cells through gap junctions (Thomas et al., 2004) for the first part of meiotic maturation (Sutovsky et al., 1993). Oocytes matured without cumulus cells exhibit a significant reduction in blastocyst development (Zhang et al., 1995, Atef et al., 2005). Unlike the oocyte, cumulus cells are transcriptionally active (Regassa et al., 2011) and can respond to heat stress with changes to gene expression. Theoretically, heat-induced changes to cumulus transcriptome may explain some of the reductions in embryo development following fertilization.

Payton (2009) reported heat-induced alterations in relative abundance of transcriptome profiles within associated cumulus cells. KEGG analysis identified from a list of transcripts determined to be differentially expressed in heat-stressed derived cumulus polyA amplified total RNA, the Hedgehog signaling pathway was over-represented. In particular, Payton (2009) noted that the relative abundance of Indian

hedgehog homolog (**IHH**) was 2.62 fold higher following heat stress exposure during the first 12 h of meiotic maturation compared to control counterparts. To further investigate heat-induced perturbations in RNA populations of cumulus cells, expression of IHH will be examined using qPCR.

MATERIALS AND METHODS

Source of Oocyte Samples

Oocyte RNA samples were derived from a study previously published by Payton (2009), a dissertation titled “Direct effects of heat stress during meiotic maturation on bovine oocyte and cumulus RNA” (Chapter 5. Department of Animal Science, University of Tennessee, Knoxville). Payton (2009) isolated genomic DNA-free total RNA at 24 hIVM from oocytes matured at 38.5°C (control) or matured at 41°C for first 12 hIVM followed by 38.5°C for the remainder of maturation (heat stress) from replicates where heat-induced reductions in blastocyst development were >25% (Payton, 2009; Figure 4.2). After RNA isolation, samples were spiked with 12 pg of green fluorescent protein (GFP) cRNA per oocyte and underwent reverse transcription (**RT**) with 500 ng oligo-d(T) primers. Samples were processed and stored at -80°C by Payton in accordance with Minimum Information for publication of Quantitative real-time PCR Experiments (**MIQE**; Bustin et al., 2009) guidelines.

Source of Cumulus Samples

RNA samples utilized for examining the relative abundance of IHH in cumulus cells were derived from a different experimental effort described in Payton et al. (2011)

and published in the Journal of Reproduction and Development (doi:10.1262/jrd.10-

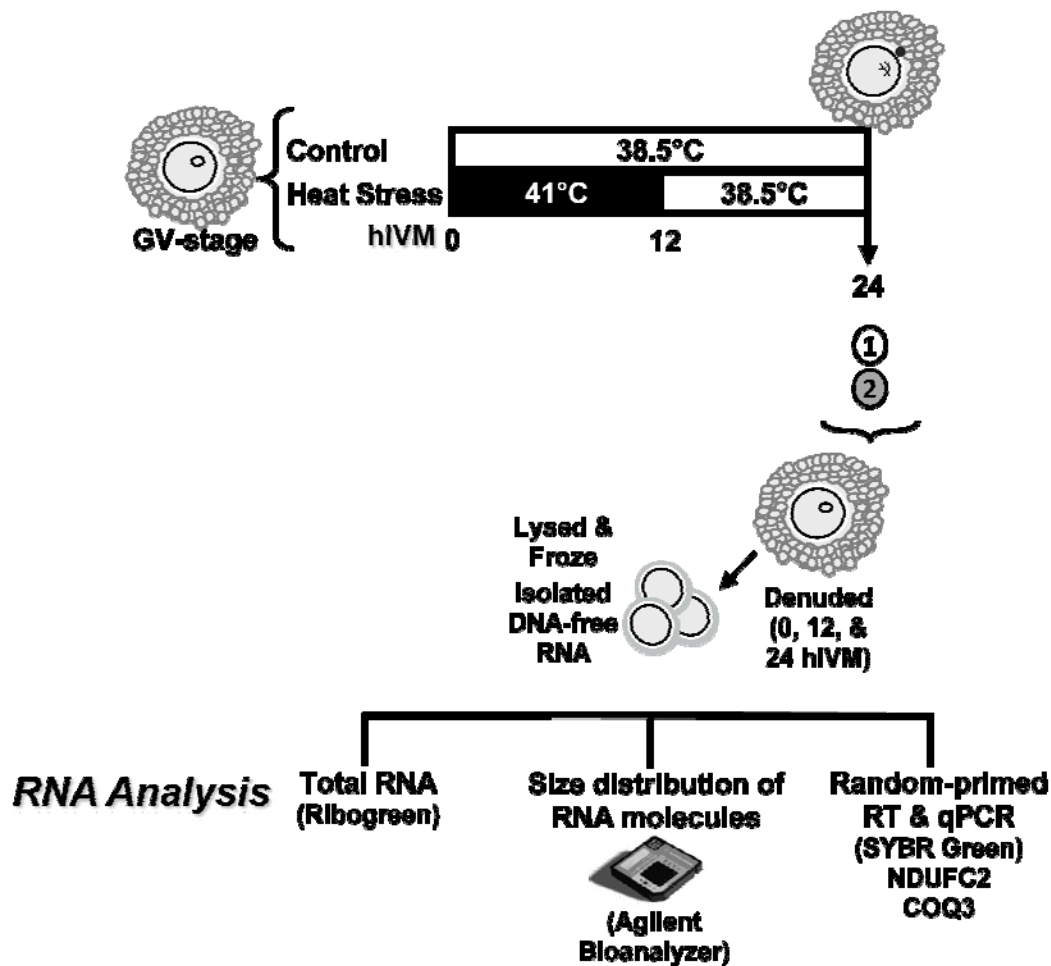


Figure 4.2: Schematic of experimental design for oocytes samples adapted from Payton (2009). Cumulus-oocyte complexes were randomly assigned to culture at 38.5°C for 24 h of in vitro maturation (**hIVM**; white circle) or at 41.0°C for the first 12 h of meiotic maturation followed by 38.5°C for the remaining 12 hIVM (gray circle). Payton (2009) denuded oocytes of their associated cumulus cells and lysed in extraction buffer before storage at -80°C until RNA isolation. Subsequently, total RNA was spiked with green fluorescent protein cRNA and underwent reverse transcription. Relevant for this study, samples were thawed and analysis of coenzyme Q3 homolog (**COQ3**) and NADH dehydrogenase (**NDUFC2**) was performed with qPCR.

163M). Cumulus samples were derived from experimental replicates where their associated oocytes had heat-induced reductions in blastocyst development >10% after undergoing in vitro fertilization. In brief, total RNA was isolated directly after removal from antral follicles of abattoir-derived ovaries (0 hIVM; i.e., GV-stage) or after in vitro maturation at 38.5°C or 41°C for the first half of meiotic maturation, at 12 or 24 h (Payton et al., 2011; Figure 4.3). Payton et al. (2011) isolated genomic DNA-free total RNA from cumulus, spiked with GFP cRNA (500 pg per 100 ng total RNA) and reverse transcribed with 500 ng random hexamers before storage at -80°C until qPCR analysis.

Quantitative Polymerase Chain Reaction

Relevant for this study, primers for NDUFC2, COQ3 and IHH were designed using FastPCR 6.0 software for both the 5'- and 3'-ends of mRNA from bovine sequences in GenBank in effort to ensure evaluation of intact mRNA (Table 4.1). To assess specificity of target gene, PCR products were sequenced. Optimization of primer concentration and annealing temperature was performed to target reaction efficiencies between 90 to 110% (efficiency = $10^{(-1/\text{slope})-1}$). Relative quantification using qPCR was performed as previously described by Payton et al. (2011). In brief, the equivalent of 0.1 oocytes or 100 pg cumulus RNA was analyzed in either duplicate or triplicate for each transcript of interest using a 7300 ABI Real-Time PCR System (Applied Biosystems) with conditions of 50°C for 2 min, 95°C for 10 min, and 40 cycles of denaturing at 95°C for 15 sec, annealing (temperature stated in Table 4.1) for 30 sec, and extension at 72°C for 30 sec, followed by a dissociation curve. Data was normalized using GFP and analyzed using $\Delta\Delta C_t$ method (calculated with RQ study,

7300 System SDS software as per manufacturer's specifications; RQ Study Software,

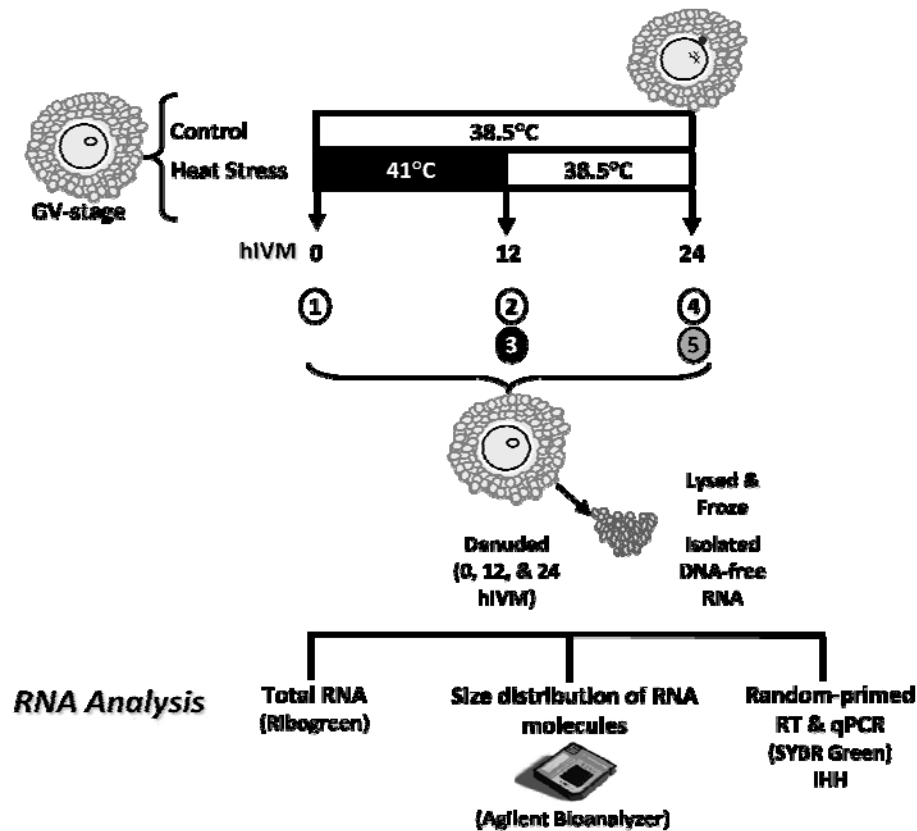


Figure 4.3: Schematic of experimental design for cumulus samples adapted from Payton et al. (2011). Payton et al. (2011) randomly assigned cumulus-oocyte complexes (**COCs**) to culture at 38.5°C for 0 (soon after removal from antral follicles), 12 or 24 h in vitro maturation (**hIVM**; white circles). Remaining COCs were matured at 41.0°C for 12 h and removed for processing (black circle) or cultured for another 12 h at 38.5°C (gray circles). At 0, 12, and 24 hIVM, cumulus were removed from associated oocyte and lysed in extraction buffer and stored at -80°C until RNA isolation and analysis of concentration and integrity (Payton et al., 2011). Subsequently, total RNA was spiked with green fluorescent protein cRNA and underwent reverse transcription. Relevant for this study, stored samples were thawed and analyzed for the relative abundance of Indian hedgehog (**IHH**) using qPCR.

Table 4.1. Sequences of primers and annealing conditions used for quantitative PCR analyses							
Gene Symbol	GenBank Accession Number		Amplicon Location	Primer Sets	Primer Conc.	Annealing Temp.	Amplicon Size
NDUFC2	NM_176642.3	5'	148 - 171 310 - 332	F - CGTCACAATGATGACTGGACGGCA R - GATGCAAACCGGCCAACAGCACC	200 nM	57°C	185
		3'	400 - 419 502 - 521	F - GTATGCTGTGAGGGACCACG R - TACTTCAACGCACTGGATGG	200 nM	60°C	122
COQ3	NM_0010776210.1	5'	114 -134 225 - 244	F - AACTACGTCGGCGGTTTATCC R - TCATCCAATTCAGGCAGGCA	200 nM	56°C	131
		3'	664 - 683 742 - 761	F- ACGTTTGATGCTGTCGTAGC R - GAACCATCAGGCTTTAACAC	400 nM	57°C	98
IHH	NM_00107687.2	5'	57 - 82 138 - 157	F - CCAACTACAATCCAGACATCATCTTC R - GATGGCCAGCGAGTTCAGGC	200 nM	60°C	101
		3'	1432 -1454 1532 - 1552	F - CCACACTCCTCGGGACACATACG R - AGAGGCTGGGCAGGACATCC	400 nM	60°C	121
GFP	--		615 -634 696 - 716	F - CAACTTCAAGACCCGCCACA R - TCTGGTAAAAGGACAGGGCCA	800 nM	56 - 60°C	102

version 1.3; Applied Biosystems) using control 24 hIVM oocytes or GV-stage cumulus within each replicate as calibrators to generate relative quantities.

Statistical Analyses

Relative abundance of NDUFC2 and COQ3 was analyzed as a randomized block design (**RBD**) with main effect of treatment (control and heat stress) and random effect of replicate (i.e., oocyte collection date). The relative abundance of IHH was analyzed also as a RBD with main effects of treatment (control and heat stress) and hours of in vitro maturation (0, 12, 24 hIVM). Data were analyzed using PROC GLIMMIX (SAS 9.2, SAS Institute, Inc., Cary, NC, USA) and results for all transcripts are reported as least squares means (**LSM**) $\pm$ standard error of the mean (**SEM**).

RESULTS

Relative Abundance of NDUFC2 and COQ3 Transcripts in Heat-Stressed Oocytes

Exposure to 41°C during first 12 h of meiotic maturation decreased the relative abundance of NDUFC2 in oocytes examined at 24 hIVM using both 5' and 3' primer sets ($P = 0.0001$ and $P = 0.0029$, respectively; Figure 4.4). Heat-induced differences in the relative abundance of COQ3 transcript were observed when 3'-end primers were used ($P = 0.6526$ and $P = 0.0201$ for 5'- and 3'-end, respectively; Figure 4.5).

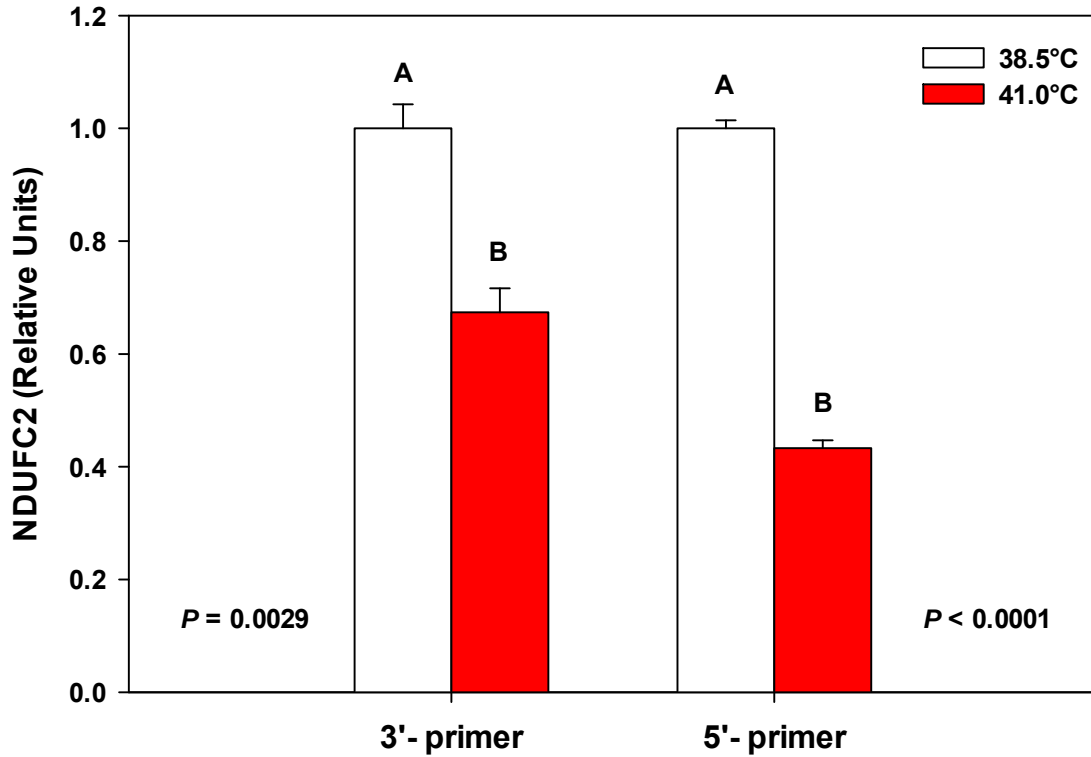


Figure 4.4. Relative abundance of NADH dehydrogenase (**NDUFC2**) transcript using 3' and 5' primers. Transcripts evaluated in oocytes that were cultured at 41.0°C during the first 12 h of maturation (**hIVM**) to reduce blastocyst development >25% following fertilization. Data are least squares means $\pm$ standard error of the mean with treatment differences indicated by letter designation at significance level indicated for each primer set. White bars indicate culture at 38.5°C for 24 hIVM. Red bars indicate culture at 41.0°C for the first 12 hIVM and 35.5°C thereafter (replicates: $n = 13$).

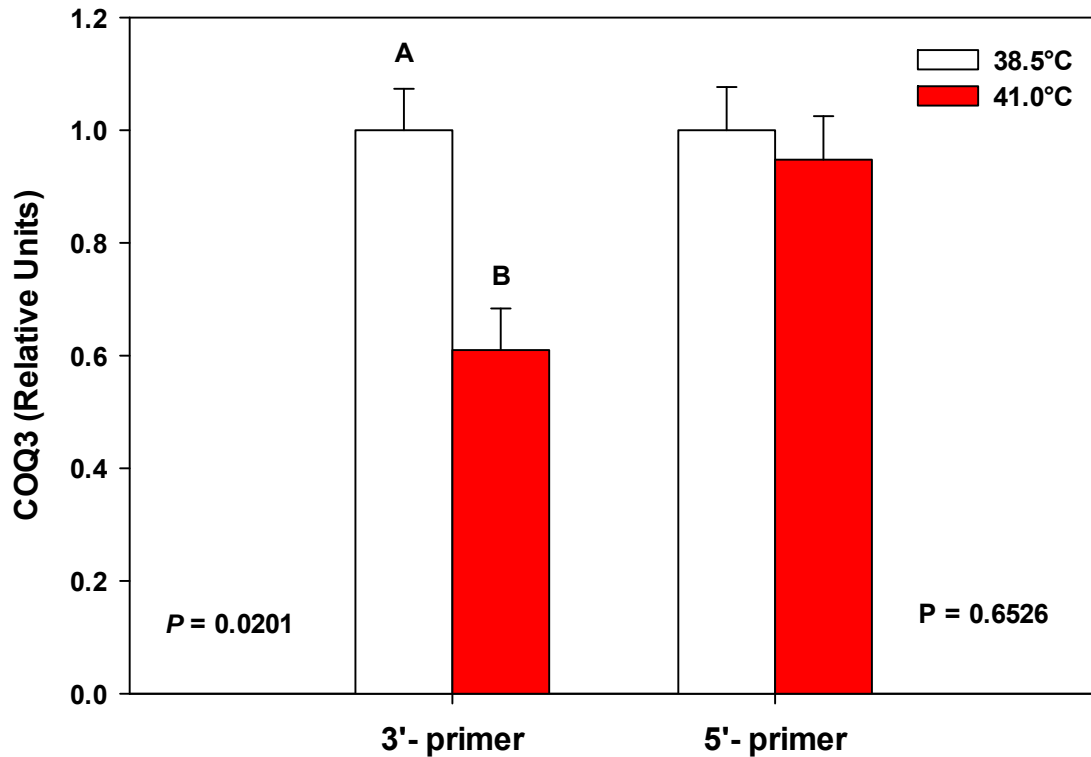


Figure 4.5. Relative abundance of Coenzyme Q3 homolog (**COQ3**) transcript using 3' and 5' primers. Transcripts evaluated in oocytes that were cultured at 41.0°C during the first 12 h of maturation (**hIVM**) to reduce blastocyst development >25% following fertilization. Data are least squares means $\pm$ standard error of the mean with treatment differences indicated by letter designation at significance level indicated for each primer set. White bars indicate culture at 38.5°C for 24 hIVM. Red bars indicate culture at 41.0°C for the first 12 hIVM and 35.5°C thereafter (replicates: n = 13).

Relative Abundance of IHH Transcript in Cumulus from Heat-Stressed Oocytes

A decrease in the relative quantity of IHH transcript was observed during meiotic maturation in the surrounding cumulus from control and heat-stressed oocytes (Figure 4.6). The relative abundance of IHH was significantly increased in heat-stressed cumulus cells compared to their control counterparts at 12 hIVM; differences were no longer evident at 24 hIVM for 3'-end primers and 5'-end primers (Temperature x hIVM interaction; $P = 0.007$ and $P = 0.008$, respectively; Figure 4.6)

DISCUSSION

Results generated via qPCR are consistent with microarray results of Payton (2009) for COQ3 and NDUFC2, transcripts of the electron transport chain in oocytes. For instance, microarray analysis showed a 1.5-fold decrease in the relative abundance of COQ3 in heat-stressed oocytes; whereas, qPCR results showed a 1.7 fold decrease (3'- primer set). For NDUFC2 transcript, microarray analysis showed a 2.00 fold decrease; whereas, qPCR validation showed a 2.3 and 1.5 fold decrease (5'- and 3'- primer set, respectively). In addition, microarray data reported by Payton (2009) showed a 2.6 fold increase in IHH at 24 hIVM in heat-stressed cumulus cells compared to control; whereas, current qPCR data showed a significant increase in the relative abundance of IHH at 12 hIVM of 4.55 and 4.79 fold for 5'- and 3'-ends, respectively. Obtaining similar

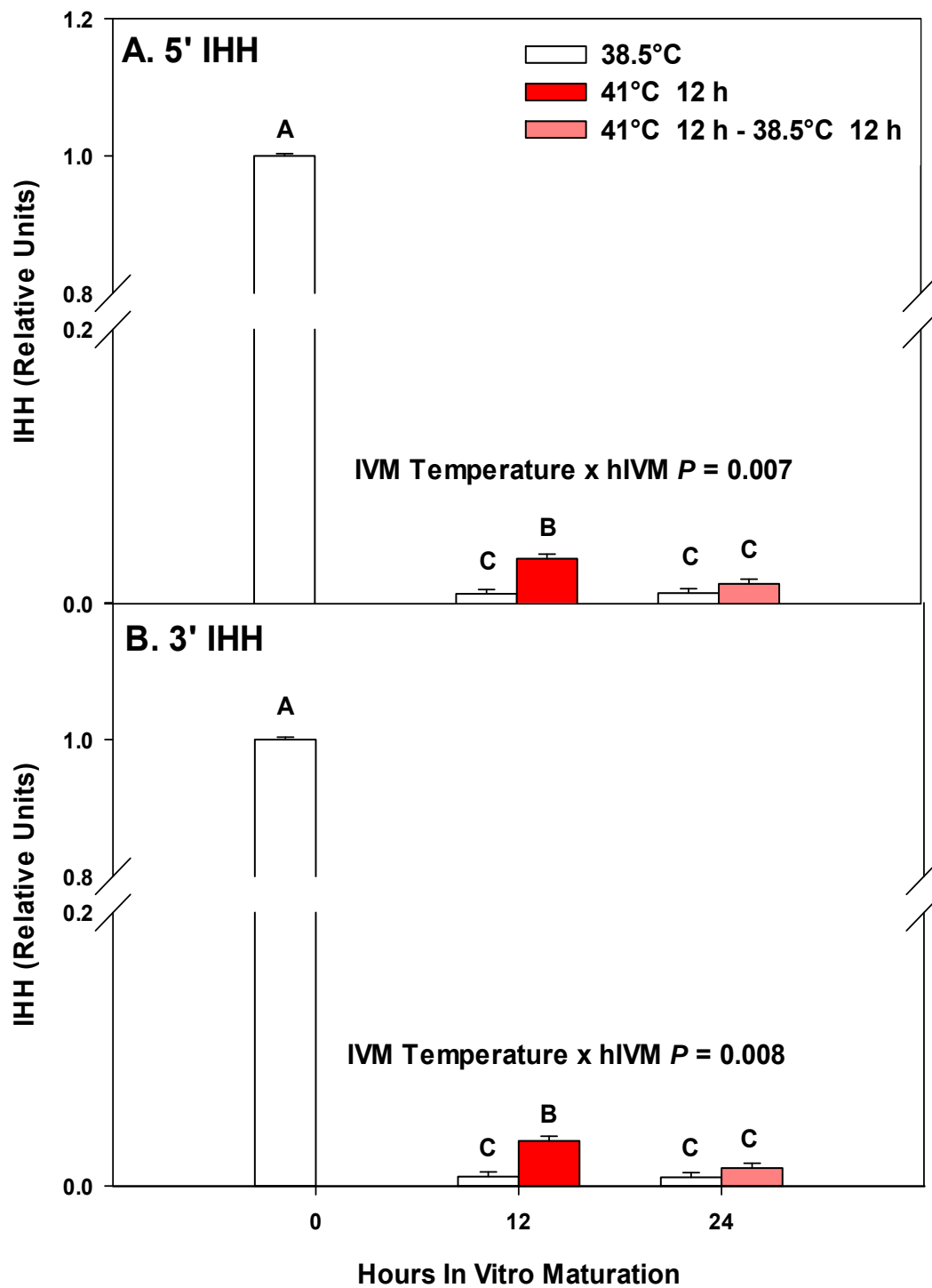


Figure 4.6: Relative abundance of Indian hedgehog (**IHH**) using the 5'-primer (Panel A) and 3'-primer (Panel B) where culture at 41.0°C during the first half of maturation reduced blastocyst development >10% following fertilization. Data is expressed as least squares means $\pm$ standard error of the mean shown with treatment differences. White bars indicate culture at 38.5°C for 0, 12, or 24 h of in vitro maturation (**hIVM**). Red bars indicate culture at 41.0°C for the first 12 h of maturation and the pink bars represent culture at 41°C for the first 12 hIVM followed by 38.5°C for 12 hIVM (replicates: n = 9).

results using qPCR, provides increased confidence in microarray data generated by Payton (2009). However, whether perturbations are sufficient to explain heat-induced reductions in embryo development occurring after in vitro fertilization still needs further assessment.

Both *NDUFC2* and *COQ3* are transcripts for mitochondrial proteins important for ATP production and exhibited alterations in their relative abundance after heat stress exposure. It is possible that heat-induced increases in ATP content (Payton, unpublished; Nagle, see Chapter 3) may be related to changes in abundance of these transcripts and corresponding proteins. Despite most cells having a correlation between an increase in RNA and a subsequent increase in corresponding protein levels (Fagnoli et al., 1990), there have been instances where a decrease in transcripts coincided with an increase in protein. For instance, a microarray transcriptome study of yeast demonstrated a decrease in RNA levels with an increase in corresponding polysome association (indicator of protein expression) in 4.2% of cases after rapamycin treatment (Preiss et al., 2003). Furthermore, Preiss et al. (2003) reported that heat shock (37°C) also demonstrated the same effect, where 36.7% of genes with decreased mRNA abundance showed a corresponding increase in being preferentially translated. This scenario may be relevant for an oocyte, since it is transcriptionally quiescent after the resumption of meiosis and relies on stored maternal mRNA for protein synthesis until the maternal-embryonic genome transition (Bettegowda, 2007, Lodde et al., 2008). This seems possible since heat stress is known to affect translational machinery to increase preferential synthesis of certain proteins

(Duncan and Hershey, 1989). It could be that increases in ATP content are a result of heat-induced conformational changes to intermembrane protein particles (reviewed by Streffer, 1985) to enhance overall enzymatic activity rather than an increase in protein expression.

Regardless, data are suggestive of heat-induced increases in mitochondrial activity within the oocyte. Leese et al. (2002, 2008) provided evidence that a 'quiet' metabolism is important for obtaining a more developmentally viable phenotype of embryos in mice and humans. Therefore, any increase in metabolic related activity (i.e., increased mitochondrial activity) may be detrimental for developmental competence. Proper mitochondrial function (i.e., distribution and activity level) within a certain range is necessary for normal oocyte maturation (Takeuchi et al., 2005). Induced oocyte mitochondrial dysfunction by sublethal photosensitization has been shown to impair not only early embryonic development (Thouas et al., 2004) but also pregnancy (Thouas et al., 2006).

Heat stress not only impacted oocyte transcriptomes but also those within associated cumulus cells. Unlike the oocyte, the surrounding cumulus cells are transcriptionally active (Regassa et al., 2011) with the ability to respond to stressors like hyperthermia (Payton et al., 2011). Specifically, HSP70 transcripts were significantly increased in cumulus from heat-stressed oocytes at 12 hIVM compared to control counterparts (Payton et al., 2011).

Consistent with qPCR results from present study, others have observed that IHH decreases during maturation (Wijgerde et al., 2005, Hernandez-

Gonzalez et al., 2006, Ren et al., 2009). The relative abundance of IHH in cumulus cells in qPCR and microarray studies exhibited a decrease during maturation while heat-stress decelerated the decline. Payton's (2009) study reported increased IHH at 24 hIVM of heat-stressed cumulus but did not evaluate 12 hIVM. However, the current study showed increased IHH at 12 hIVM that was no longer evident at 24 hIVM. The discrepancy may be explained by variations in methodologies of derived cumulus RNA between the amplified microarray and qPCR studies. The microarray replicates had heat-induced reductions in blastocyst development by >25% where the qPCR study used replicates demonstrating a heat-induced reduction of blastocysts by >10%. Furthermore, individual abattoir derived, pools of oocytes may have inherent differences in regards to genotype (Rocha et al., 1998) and physiological status of donors including disease (Fray et al., 2000) and nutrition (Armstrong et al., 2003) which may add additional variation and probably explain disparity seen between two studies.

The Hedgehog signaling pathway is a complex signaling network which regulates the WNT pathway. Payton's (2009) transcriptome study revealed heat-induced changes in WNT2B, thus an increase in IHH expression in heat-stressed cumulus cells may explain an increase in WNT2B expression. This would be functionally important given that the WNT- β -catenin pathway is active in cumulus cells of maturing oocytes (Wang et al., 2009a) and potentially play a role in gap junctions and to anchor companion cumulus cells to the oocyte (Gershon et al., 2008). Transgenic mice, where a continuous activation of Hedgehog signaling

was induced, an elevation in Hedgehog signaling (revealed by microarray) in murine cumulus-cell complexes coincided with reductions in cumulus expansion (detected by fluorescent microscopy for expression of YFP); furthermore, resulting in the hindrance of ovulation (Ren et al., 2009). Others associate heat stress with a decrease in cumulus expansion (GAG expression; Lenz et al., 1983) and a decrease in expansion to have consequences on ovulation (Hernandez-Gonzalez et al., 2006), but the extent to which this is happening in our model is unclear, since no to slight reduction in cumulus expansion has been visually detected. In the presence of IHH, the WNT pathway is a target for up-regulation in gene translation, therefore alterations within the Hedgehog pathway may alter bi-directional communication impacting the oocyte, ovulation, and subsequent development.

Results generated in present study revealed heat-induced effects on the relative abundance of transcripts using both 5'- and 3'-end primers that were designed in effort to ensure evaluation of intact mRNA. However, oocyte samples provide an example in which results may differ depending on whether 5' or 3' primers are utilized. Heat stress may alter the expression of COQ3 but was not observed utilizing the 5'-end primer due to possible technical artifacts of the reverse transcription process. Reverse transcription used oligo-d(T) primers which anneal and extend from the 3'-end of the transcript, creating a 3'-bias (Dixon et al., 1998). If reverse transcription was incomplete, this may result in an un-transcribed 5'-end. However, the 5'-end of transcript may be absent if degraded in total RNA due to 5' to 3' exonucleolytic decay or endonucleolytic

cleavage (Beelman and Parker, 1995), making relative abundances difficult to determine.

In summary, qPCR data support previous microarray analyses showing heat-induced alterations in the relative abundance of transcripts important in ETC of oocytes and IHH in surrounding cumulus cells. However, further investigation is required to determine whether the differential expression of transcripts are a cause or merely 'associated' to heat-induced decrease in developmental competence. An important next step would be to discern whether alterations in the relative abundance of individual genes are associated with changes in protein levels to clarify functional consequences.

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