# Impact of Matrix Metallopeptidase-9 Supplementation During In Vitro Maturation of Bovine Oocytes

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## DEDICATION

I would like to dedicate this thesis to my parents and my wonderful husband Alex. I could not have made it this far without their constant love and support.

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## ABSTRACT

Heat-induced decreases in bovine blastocyst development have been related to reductions in latent matrix metallopeptidase-9 (proMMP9) production from maturing cumulus-oocyte complexes. Elevated intrafollicular proMMP9 levels at the time of oocyte retrieval have been positively related to pregnancy following human IVF. Thus, we hypothesized that heat-induced reductions in proMMP9 levels during oocyte maturation may be responsible for decreased blastocyst development. As a first step towards testing this hypothesis, bovine cumulus-oocyte complexes were matured at 38.5°C for 24 h with 0 or 300 ng/mL recombinant human proMMP9 (rhMMP9) added at 0 h of in vitro maturation (hIVM). No differences were found in ability of oocytes to cleave or form blastocyst-stage embryos after IVF. In a second study, cumulus-oocyte complexes were matured at 38.5 or 41.0°C (first 12 h only, then transferred to 38.5°C). At 12 hIVM, 0 or 300 ng/mL of rhMMP9 was added. Heat stress exposure decreased 24 hIVM proMMP9 levels in 0 ($P = 0.006$) but not 300 ng/mL groups and elevated progesterone levels most when 300 ng/mL rhMMP9 was added ($P = 0.0002$). Heat stress exposure did not affect ability of oocytes to cleave but reduced blastocyst development ($P = 0.006$). Independent of maturation temperature, addition of rhMMP9 decreased cleavage ($P = 0.02$) and blastocyst development ($P = 0.08$). In a third study, 0, 30 or 300 ng/mL rhMMP9 was added at 18 hIVM to cumulus-oocyte complexes matured at 38.5 or 41.0°C (first 12 h only, then transferred to 38.5°C). Heat stress exposure decreased 24 hIVM proMMP9 levels in 0 ($P = 0.007$) and 30 ($P = 0.04$) but not 300 ng/mL groups and increased progesterone levels in 0 and 300 but not 30 ng/mL rhMMP9 groups ($P = 0.039$). Heat stress exposure decreased cleavage ($P < 0.0001$) and blastocyst development ($P < 0.0001$). Independent of maturation temperature, addition of rhMMP9 did not alter cleavage but decreased blastocyst development ($P = 0.02$). In summary,

addition of rhMMP9 at evaluated doses and times during IVM did not restore development of heat-stressed oocytes. Addition of 30 or 300 ng/mL rhMMP9 after 12 hIVM, regardless of maturation temperature, was detrimental to development.

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# CHAPTER 1
# INTRODUCTION

In approximately 60% of cattle operations worldwide, heat stress causes economic losses, many of which result from heat-induced decreases in fertility (Pegorer *et al.* 2007). Heat stress during oocyte maturation causes decreases in embryo development both in vivo (Putney *et al.* 1989) and in vitro (Edwards & Hansen 1996). Maturing oocytes are most susceptible to heat stress when connected through gap junctions to transcriptionally active cumulus cells (Thomas *et al.* 2004), whose premature removal compromises the ability of the oocyte to develop to the blastocyst stage (Zhang *et al.* 1995). Cumulus cells respond to heat stress exposure, as evidenced by increased Heat Shock Protein 70 transcription in cumulus derived from cumulus-oocyte complexes matured under physiologically relevant heat stress conditions (Payton *et al.* 2011). Therefore, cumulus cells may mediate effects of heat stress on the oocyte. In a subsequent microarray study, the abundance of several cumulus-derived transcripts was altered when heat stress was applied during maturation (Rispoli *et al.* 2013). Notably, heat stress decreased transcript abundance of matrix metallopeptidase-9 (*MMP9)* mRNA and increased mRNA abundance for two enzymes in the progesterone synthesis pathway (Rispoli *et al.* 2013). These changes in mRNA expression translated into reduced secretion of the latent form of MMP9 (proMMP9) and increased progesterone production, which may in part explain decreased developmental competence of heat-stressed oocytes (Rispoli *et al.* 2013).

Heat-induced reductions in MMP9 levels are noteworthy because MMP9 (a zinc-dependent gelatinase) has been associated with reproductive success and developmental competence of oocytes in multiple species. Mice with MMP9 knocked out were shown to have impaired fertility (Dubois *et al.* 2000). Bovine follicles that secreted MMP9 during in vitro culture were more likely to have healthy theca and granulosa cells (McCaffery *et al.* 2000).

Also, bovine follicular granulosa cells that expressed *MMP9* were more likely to be associated with developmentally competent oocytes (Robert *et al.* 2001). Furthermore, proMMP9 levels in conditioned maturation medium from bovine cumulus-oocyte complexes and fluid from human preovulatory follicles have been positively related to blastocyst development (Rispoli *et al.* 2013) after IVF and pregnancy rates (Lee *et al.* 2005, Horka *et al.* 2012), respectively.

Progesterone production in murine follicles may be regulated by MMP9 (Carbajal *et al.* 2011), although in most tissues including human endometrium (Marbaix *et al.* 1992, Cornet *et al.* 2002), trophoblast (Shimonovitz *et al.* 1998) and rabbit cervical fibroblasts (Imada *et al.* 1997), progesterone regulates MMP9 activity. Progesterone supplementation during in vitro maturation (IVM) of bovine oocytes in thermoneutral conditions decreased blastocyst development (Silva & Knight 2000, Schlüter *et al.* 2014). Heat stress has been found to increase progesterone production during IVM of bovine oocytes (Rispoli *et al.* 2013). Heat-induced reductions in development may be explained in part by increased progesterone and/or decreased MMP9 levels during IVM.

Because MMP9 has been positively associated with fertility and correlated with blastocyst development in the bovine, we hypothesized that heat-induced decreases in blastocyst development may be a consequence of reduced MMP9 levels during oocyte maturation. Initial efforts examined effects of adding recombinant human proMMP9 (rhMMP9) to maturing bovine cumulus-oocyte complexes under thermoneutral conditions. Subsequently, rhMMP9 was added to heat-stressed cumulus-oocyte complexes at 12 or 18 hours of IVM (hIVM) in an attempt to ameliorate effects of heat stress on developmental competence. Progesterone levels were evaluated in each effort because it has been inversely related to MMP9 production.

# CHAPTER 2
# REVIEW OF LITERATURE

## Introduction

The following is a literature review covering the impact of heat stress on bovine reproduction, the effects of heat stress on the cumulus-oocyte complex and MMP9 production, the importance of MMP9 in reproduction, and the connection between MMP9 and progesterone. This review will also discuss MMP9 as an indicator of developmental competence of oocytes and the possible roles of MMP9 during oocyte maturation. Finally, this review will cover the importance of in vitro oocyte maturation in human assisted reproduction and how the bovine oocyte is a suitable model for testing ways to improve in vitro maturation of human oocytes.

## Impact of Heat Stress on Bovine Female Reproduction

Over 60% of cattle operations worldwide experience economic loss as a result of environmental heat stress (reviewed by Pegorer *et al.* 2007). The United States Dairy Industry in particular loses approximately $900 million each year due to heat-induced decreases in milk yield and fertility (reviewed by Collier *et al.* 2006). Much of the decreases in fertility are a consequence of hyperthermia. Under normal environmental circumstances, rectal temperatures in the cow average 38.5˚C, but in cases of hyperthermia, rectal temperature may reach or exceed 41.0˚C (Seath & Miller 1946, Monty Jr & Wolff 1974, Ealy *et al.* 1993). Increasing rectal temperature (i.e., hyperthermia) has a detrimental effect on pregnancy rates. For instance, when rectal temperature was measured 12 hours after insemination, there was a 25% decrease in the number of cows pregnant for every 1.5°C increase in rectal temperature beyond 37.5°C (Ulberg & Burfening 1967).

Decreased pregnancy rates associated with environmental heat stress near the time of estrus (i.e., the period in which the cow is sexually receptive to mating) and insemination have

been well documented in cattle (Stott & Williams 1962, Ulberg & Burfening 1967, Gwazdauskas *et al.* 1975, Badinga *et al.* 1985). Putney *et al*. (1989) applied heat stress to superovulated heifers for only the first ten hours of estrus through the use of thermal chambers, elevating rectal temperature to 41.3°C. Heifers were then allowed to cool down before being artificially inseminated at both 15 and 20 hours after the onset of estrus. The embryos collected seven days after insemination from heat-stressed heifers were of poorer quality than embryos from non-heat-stressed heifers. This study illustrates that exposure to environmental heat stress, when limited to the first half of estrus (when the oocyte has resumed meiosis within the ovarian follicle), decreases the ability of oocytes to develop into good quality embryos after fertilization. Decreased embryo quality may be due to heat stress affecting the maternal environment (i.e., ovulatory follicle containing the maturing oocyte), which may then cause alterations in the oocyte. Alternatively, heat stress may directly affect the oocyte.

**The Impact of Heat Stress on the Cumulus-Oocyte Complex**

When heat stress was directly applied to oocytes during in vitro maturation (IVM), blastocyst development decreased, similar to the developmental decreases seen when environmental heat stress was applied to heifers during estrus (Putney *et al.* 1989). Edwards and Hansen (1996) applied physiologically relevant heat stress (41.0°C) to bovine cumulus-oocyte complexes for the first 12 hours of IVM (hIVM) and switched to 38.5°C thereafter. By 7 to 9 days after fertilization, the proportion of blastocysts that developed from heat-stressed cumulus-oocyte complexes was lower compared to the proportion of blastocysts from non-heat-stressed cumulus-oocyte complexes. Direct effects of heat stress on the oocyte to decrease developmental competence have been noted by several other studies as well (Edwards & Hansen

1996, Edwards & Hansen 1997, Lawrence *et al.* 2004, Roth & Hansen 2004, Edwards *et al.* 2005, Schrock *et al.* 2007, Payton *et al.* 2011, Rispoli *et al.* 2013).

The mechanisms through which heat stress affects the bovine cumulus-oocyte complex during maturation remain unclear although several factors have been implicated. For instance, when 41.0°C heat stress was applied during IVM, the time required for oocytes to reach metaphase I and II was decreased, yielding oocytes of aged quality by 24 hIVM (Edwards *et al.* 2005). Aged oocytes are less likely to develop to the blastocyst stage after fertilization (Ward *et al.* 2002), which may partially explain why heat-stressed oocytes are less developmentally competent when fertilized at 24 hIVM. In the lab, the impact of heat stress on development was partially alleviated but not eliminated by earlier in vitro fertilization (IVF) of heat-stressed oocytes (Schrock *et al.* 2007). In the cow, however, earlier fertilization is not yet an option because heat stress hastens oocyte maturation but not ovulation.

Heat stress alters cytoplasmic components within the bovine oocyte. The formation of meiotic spindles was impaired when an intense stress of 42.0°C was applied for only the last 4 out of 24 hIVM (Ju *et al.* 2005). Additionally, mitochondrial activity may be affected by heat stress as the abundance of ATP in oocytes at 24 hIVM was increased when oocytes were exposed to 41.0°C for the first 12 hIVM (Nagle 2011). Although heat stress during oocyte maturation did not affect fertilization rates, it may alter the intracellular calcium response that follows fertilization. When cumulus-oocyte complexes were heat-stressed at 41.0°C for 12 hIVM and chemically activated with ionomycin at 24 hIVM in media without calcium, less intracellular calcium was released compared to levels within non-heat-stressed oocytes (Rispoli *et al.* 2011).

**The Importance of Cumulus Cells**

Cumulus cells are differentiated granulosa cells that surround and project into the oocyte contributing to acquisition of developmental competence during oocyte maturation. Premature cumulus removal or inhibition of cumulus communication with the oocyte impairs oocyte maturation, fertilization, and embryo development in cattle (Sirard & First 1988, Zhang *et al.* 1995, Fatehi *et al.* 2002, Tanghe *et al.* 2003, Ali *et al.* 2005), pigs (Wongsrikeao *et al.* 2005), and humans (Goud *et al.* 1998). Additionally, when bovine oocytes were matured with additional cumulus isolated from other oocytes, subsequent blastocyst development increased as cumulus cell density increased (Hashimoto *et al.* 1998).

Cumulus cell signaling participates in regulating the onset of oocyte maturation. Initiation of maturation and resumption of meiosis are prevented in the oocyte by a high concentration of cAMP secreted from cumulus cells, sustaining arrest at prophase I (reviewed by Seli *et al.* 2014). Aside from regulating initiation of maturation, cumulus can also affect events occurring during oocyte maturation. In mice, cumulus removal altered meiotic spindle formation, mitochondrial relocation, and premature expulsion of cortical granules that hastened zona pellucida hardening, and likely decreased sperm penetration (Ge *et al.* 2008). Similarly, in the bovine, meiotic spindle formation was compromised when cumulus cell function during IVM was altered by the addition of 10 μM trilostane, a progesterone synthesis inhibitor (Daly *et al.* 2014). After oocyte maturation has begun, cumulus cells disconnect from the oocyte due to the influence of FSH, allowing for degradation of cAMP, as seen in mice (Eppig 1979, Eppig 1982, reviewed by Seli *et al.* 2014) and cattle (reviewed by Thomas *et al.* 2004).

**Responsiveness of Cumulus to Heat Stress**

Cumulus cells are intimately connected to the bovine oocyte during the first half of maturation (Thomas *et al.* 2004), when oocytes are most susceptible to heat stress. Like the oocyte, cumulus cells are susceptible to the effects of elevated temperature. When cumulus-oocyte complexes were exposed to 41.0°C heat stress for an extreme period of 24 hours, cumulus expansion was decreased (Lenz *et al.* 1983). Cumulus cells respond to heat stress by increasing transcription of *HSP70* mRNA (Payton *et al.* 2011). The realization that cumulus cells exhibit changes in transcription in response to heat stress prompted a microarray study, revealing 24 transcripts that differed more than two-fold in abundance between cumulus from heat-stressed and non-heat-stressed cumulus-oocyte complexes. A threefold heat-induced decrease in matrix metallopeptidase-9 (*MMP9*) translated into a reduction in MMP9 protein secreted into maturation medium. The differences in MMP9 levels in media between control and heat-stressed groups increased over time, with levels becoming significantly less in media from heat-stressed cumulus-oocyte complexes at 18 and 24 hIVM (Rispoli *et al.* 2013).

Matrix metallopeptidase-9 is a zinc-dependent gelatinase that cleaves gelatin and collagen types I, II, IV, V, and XVII as well as several functional proteins such as myelin, serine proteases, members of the transforming growth factor-β family, and epidermal growth factor (Van den Steen *et al.* 2002). In most cells, MMP9 is typically involved in cellular growth and maintenance of the extracellular matrix. The enzyme is usually expressed at low levels, but may be up regulated, especially during remodeling of the extracellular matrix. While other members of the matrix metallopeptidase family including MMPs 1, 2, 3, 7, 10, and 13 directly activate MMP9 by cleavage of the pro-domain, furin, plasmin, urokinases- and tissue-type plasminogen activators indirectly activate MMP9 through other factors downstream (Reviewed by Van den

Steen *et al.* 2002). Regulation of MMP9 activation is accomplished through tissue inhibitors of metallopeptidases (TIMPs), with TIMPs 1 and 3 having the highest affinity for binding MMP9 (Reviewed by Curry and Osteen, 2003).

**MMP9 Is Important for Fertility**

A heat-induced decrease in *MMP9* expression in cumulus cells is intriguing because MMP9 has been implicated in fertility in mammals. Breeding pairs of mice with the MMP9 gene knocked out were able to reproduce but averaged fewer litters and fewer pups per litter than mice with unimpaired MMP9 production (Dubois *et al.* 2000). In women, a single nucleotide polymorphism (SNP) on the MMP9 promoter caused downregulation of MMP9 translation, and increased risk for idiopathic recurrent spontaneous abortion (Pereza *et al.* 2012). Women with elevated levels of MMP9 in blood serum on the day of oocyte retrieval were also more likely to become pregnant after IVF (Horka *et al.* 2012).

**MMP9 Is Associated with Follicle Health**

Follicle health is essential to reproductive success, because the follicle houses the cumulus-oocyte complex as it proceeds through maturation. Follicle health has also been related to MMP9 expression, as bovine follicles that produced MMP9 during in vitro culture were more likely to have healthy theca and granulosa cells (McCaffery *et al.* 2000). In humans, IVF patients with higher levels of MMP9 in follicular fluid at the time of oocyte retrieval had a greater chance of becoming pregnant after embryo transfer (Lee *et al.* 2005). When follicular fluid was analyzed through gelatin zymography, samples that had less than 50,000 densitometric units of MMP9 were associated with a 0% chance of embryonic implantation whereas samples with more than 50,000 densitometric units were associated with a 32% chance of implantation (Lee *et al.* 2005). In a separate human IVF study, MMP9 levels in both follicular fluid and blood

serum on the day of oocyte retrieval were assessed via an enzyme-linked immunosorbent assay (Horka *et al.* 2012). Women who became pregnant following IVF had 2.5 ng/mL and 104.4 ng/mL more MMP9 in follicular fluid and blood serum, respectively, than women who did not become pregnant after IVF (Horka *et al.* 2012). Similarly, bovine follicles that contained MMP9 mRNA within granulosa cells were more likely to contain oocytes that could develop to blastocyst-stage embryos after in vitro fertilization (Robert *et al.* 2001).

**Role of MMP9 during Oocyte Maturation**

In bovine cumulus-oocyte complexes, production of MMP9 during IVM was positively correlated with subsequent blastocyst development as assessed by gelatin zymography of conditioned maturation medium at 24 hIVM (Rispoli *et al.* 2013), suggesting that MMP9 is beneficial during in vivo oocyte maturation. Once the oocyte has matured, MMP9 continues to contribute to development, as MMP9 inhibition with 20 μM 1, 10-phenanthroline immediately before IVF resulted in decreased fertilization of porcine cumulus-oocyte complexes (Beek *et al.* 2012).

Conversely, some have reported that MMP9 activity during oocyte maturation is related to reproductive dysfunction. At time of oocyte retrieval from IVF patients, MMP9 levels were higher in follicular fluid (~100 vs 150 ng/mL for control vs endometriosis, respectively; $P < 0.05$) and blood serum (~575 vs 775 ng/mL for control vs endometriosis, respectively; $P < 0.05$) from patients with endometriosis and measured via ELISA (Singh *et al.* 2013). Endometriosis patients also had lower TIMP1 levels in follicular fluid (~110 vs 85 ng/mL for control vs endometriosis, respectively; $P < 0.05$) and blood serum(~725 vs 650 ng/mL for control vs endometriosis, respectively; $P < 0.05$), as assessed by ELISA (Singh *et al.* 2013). This same study showed that elevated MMP9 and decreased TIMP1 follicular fluid from endometriosis

patients was associated with a greater percentage of immature oocytes at time of retrieval and decreased embryo quality following IVF (Singh *et al.* 2013). In this instance, however, inferences related to MMP9 may be confounded due to the added effects of endometriosis. Another study reported that the abundance of MMP9 mRNA was twenty times higher in cumulus surrounding in vitro matured bovine oocytes than in the cumulus surrounding in vivo matured oocytes (Salhab *et al.* 2013), which are more likely to develop to the blastocyst stage. On one hand, this data could be interpreted to support the idea that MMP9 mRNA abundance is increased in the less efficient maturation system and is therefore associated with lower developmental competence. However, this could also mean that MMP9 is so critical to the maturing oocyte that MMP9 mRNA abundance must be increased twenty-fold to compensate for the deficiencies in the in vitro maturation system.

**Progesterone Regulation of MMP9**

Progesterone may be most known for maintaining pregnancy as it is released from the corpus luteum (Siiteri *et al.* 1977), but progesterone has also been shown to regulate MMP9 activity (Marbaix *et al.* 1992), and is produced by the cumulus-oocyte complex during maturation (Rispoli *et al.* 2013). Transcripts for *CYP11A1* and *HSD3β1*, two of the enzymes in the progesterone synthesis pathway, were increased 1.6 and 1.8 fold, respectively, when 41.0°C was applied to bovine cumulus-oocyte complexes for the first 12 hIVM (Rispoli *et al.* 2013). Production of progesterone was also increased 1.2 fold by heat stress (Rispoli *et al.* 2013). In fact, the heat-induced increase in progesterone was significant at 12 hIVM, while the decrease in MMP9 was not significant until six hours later (Rispoli *et al.* 2013).

Progesterone has been known to down regulate MMP9 in other reproductive tissues. Progesterone decreases *MMP9* expression in murine uterine endometrium (Li *et al.* 2012), rabbit

cervical fibroblasts (Imada *et al.* 1997), and human trophoblasts (Shimonovitz *et al.* 1998). Progesterone may also regulate MMP9 secretion (Marbaix *et al.* 1992) and activation (Cornet *et al.* 2002) in human endometrium. Likewise, progesterone withdrawal may up regulate *MMP9* transcription, evidenced by increased *MMP9* expression from murine endometrial stromal cells during in vitro culture up to 16 h after administration of 1 μM RU486 (Li *et al.* 2012). Progesterone may decrease MMP9 transcription by preventing the binding of NF-κB to the MMP9 promoter region (Li *et al.* 2012, Halasz & Szekeres-Bartho 2013) or decrease activation through upregulation of TIMP1, an MMP9 inhibitor (Chaffin & Stouffer 1999, Lahav-Baratz *et al.* 2003). Conversely, a study in mice has shown that MMP2 and MMP9 play a role in increasing progesterone production in follicles post LH surge, (Carbajal *et al.* 2011) suggesting that MMP9 may be a regulator of LH-induced progesterone in murine follicles. When follicles were cultured in vitro with LH and doxycycline, a nonspecific MMP inhibitor, progesterone production decreased as doxycycline increased (Carbajal *et al.* 2011). Similarly, when Galardin, another MMP inhibitor, was supplemented with LH during in vitro culture of murine granulosa cells, production of progesterone was decreased compared to follicle cells cultured with LH alone (Jamnongjit *et al.* 2005). It seems, therefore, that the relationship between MMP9 and progesterone is reciprocal and not merely one-sided. It should be noted, however, that while a relationship may exist between progesterone and MMP9 in mammals, a recent study of chicken granulosa cells found no effect of progesterone supplementation on *MMP9* expression (Zhu *et al.* 2014). Thus, while there is substantial evidence for interactions between progesterone and MMP9 in certain scenarios, the two factors may be unrelated in some tissues.

**The Effects of Progesterone during Oocyte Maturation**

The presence of progesterone in the oocyte's environment has been associated with developmental competence. During IVM of rhesus monkey oocytes, for example, supplementation of 3 μg/mL progesterone resulted in a higher percentage of blastocyst development (Zheng *et al.* 2003). Blastocyst development was positively associated with increased progesterone concentration in follicular fluid when matured oocytes were retrieved from superstimulated heifers and then fertilized and cultured in vitro (Aardema *et al.* 2013). Similarly, in human IVF patients, a lower estrogen to progesterone ratio in follicular fluid when oocytes were retrieved 36 h after hCG treatment was related to good embryo quality (Thuesen *et al.* 2014).

Progesterone has been found to prompt the onset of and facilitate progression through meiosis in oocytes. When mifepristone, a progesterone receptor antagonist, was injected into bovine ovarian follicles immediately following treatment with a systemic GnRH agonist to initiate an LH surge, fewer oocytes underwent germinal vesicle breakdown (GVBD) within the next 15 h (Siqueira *et al.* 2012). Similarly, when canine oocytes were supplemented with 0.2 μg/mL progesterone and matured in vitro, a larger proportion underwent GVBD compared to those without exogenous progesterone (Vannucchi *et al.* 2009).

Increases in GVBD may be related to an increased percentage of oocytes completing meiotic maturation. Canine oocytes that were collected during the follicular phase of the bitch and matured in vitro were more likely to reach MII by 72 hIVM when they were matured in the presence of 1.0 or 2.0 μg/ml supplemented progesterone than were groups matured with 0 or 0.5 ng/mL added progesterone (Kim *et al.* 2005). The same trend is evident in the bovine; when oocytes were matured in vitro within follicular hemisections and supplemented with 0, 10, 100,

1000, or 10000 ng/mL progesterone, the group cultured with 100 ng/mL progesterone yielded the highest percentage of MII oocytes at 22 hIVM (Siqueira *et al.* 2012). When progesterone was added to bovine oocytes at a concentration of 1 or 5 μg/mL at the beginning of IVM in a serum-free environment, the percentage of oocytes reaching MII by 22 hIVM was more than doubled compared to controls (Sirotkin 1992). In this same study, when an antiserum to progesterone was incubated with maturing oocytes to inhibit progesterone activity, the percentage of oocytes at MII by 22 hours of maturation was only 25% of that seen in controls (Sirotkin 1992). In porcine cumulus-oocyte complexes, cumulus cells were found to produce progesterone during maturation, and higher concentrations of secreted progesterone corresponded with a hastened rate of GVBD (Yamashita *et al.* 2003).

The hastening effects of progesterone on oocyte maturation may be deleterious if there is an excess of progesterone during maturation, as several studies have noted reductions in embryo development when progesterone levels were increased during maturation. When progesterone was added to bovine cumulus-oocyte complexes during in vitro maturation in doses ranging from 50 to 450 ng/mL, no differences were seen in cleavage, but blastocyst development was reduced compared to that seen from cumulus-oocyte complexes matured without progesterone supplementation (Schlüter *et al.* 2014). Likewise, when 300 nmol/L (94.3 ng/mL) progesterone was added to maturing bovine cumulus-oocyte complexes, no changes in cleavage were noted, but blastocyst development was significantly lower than controls (Silva & Knight 2000). In the mare, when IVM medium was supplemented with 0, 50, 250 or 1250 ng/mL progesterone, followed by intracytoplasmic sperm injection and culture up to the 8-cell stage, embryonic cleavage was lower when supplementation occurred with 250 or 1250 ng/mL progesterone compared to 0 and 50 ng/mL progesterone (Schmid *et al.* 1999). When bovine cumulus-oocyte

complexes were heat-stressed, they produced on average 7.5 to 8.6 ng/mL more progesterone than non-heat-stressed counterparts (Rispoli *et al.* 2013) that may be related to subsequent reductions in blastocyst development from heat-stressed oocytes.

The effects of excessive progesterone during oocyte maturation are similar to the effects of heat stress on maturing cumulus-oocyte complexes. Heat stress during oocyte maturation, like excessive progesterone, reduces blastocyst development but does not usually alter cleavage rates (Payton *et al.* 2011, Rispoli *et al.* 2011, Rispoli *et al.* 2013). Heat stress has also been shown to hasten the onset of meiotic maturation. When murine oocytes were matured in vitro and exposed to a short-term heat stress of either 41-42°C for 30 minutes or 43°C for 15-30 minutes, the percentage of oocytes that went through GVBD increased (Kim *et al.* 2002, LaRosa & Downs 2007). Heat stress of maturing bovine oocytes accelerated progression to MI and MII, and therefore, oocytes were aged and less developmentally competent oocyte by 24 hIVM (Edwards *et al.* 2005). Heat stress also increases progesterone production from follicle walls, as demonstrated by heat stress of bovine ovarian follicle cultures (Bridges *et al.* 2005) and from cumulus cells surrounding the oocyte (Rispoli *et al.* 2013).

**Summary**

Matrix metallopeptidase-9 has been implicated in reproductive success in several instances (Dubois *et al.* 2000, McCaffery *et al.* 2000, Robert *et al.* 2001). Increased levels of MMP9 during oocyte maturation have been positively associated with blastocyst development (Rispoli *et al.* 2013) and pregnancy (Lee *et al.* 2005, Horka *et al.* 2012) following in vitro fertilization. Heat stress during bovine IVM decreased MMP9 production during maturation and subsequent blastocyst development but increased progesterone production during maturation (Rispoli *et al.* 2013). Elevated progesterone levels have been related to reductions in blastocyst

development (Silva & Knight 2000, Schlüter *et al.* 2014) that resemble reductions resulting from heat stress exposure (Edwards *et al.* 2005). Progesterone has been shown to down regulate MMP9 in other reproductive tissues and may contribute to the decreases in MMP9 seen in heat stress (Marbaix *et al.* 1992, Imada *et al.* 1997, Shimonovitz *et al.* 1998, Cornet *et al.* 2002, Li *et al.* 2012). It is possible that heat-induced reductions in blastocyst development may be explained in part by increases in progesterone secretion and decreases in MMP9 secretion from cumulus cells. It has thus been hypothesized that making levels of MMP9 equivalent between heat-stressed and non-stressed cumulus-oocyte complexes during oocyte maturation may improve blastocyst development from heat-stressed ova. In order to examine our hypothesis, recombinant human MMP9 will be added to maturing cumulus-oocyte complexes first at 0 hIVM in thermoneutral temperature. As MMP9 production changes over time and heat-induced decreases are not detectable until 12 hIVM, MMP9 will be added to heat-stressed cumulus-oocyte complexes at 12 or 18 hIVM. Because progesterone has been inversely related to MMP9 production, progesterone levels will also be assayed in each of these efforts.

**Importance of In Vitro Maturation in Human Assisted Reproduction**

In addition to livestock applications, the present study of MMP9 addition during IVM may aid in developing techniques to improve IVM efficiency in humans. In the United States, in vitro maturation of human oocytes is still considered experimental and is not widely practiced, although there are many individuals who would benefit from this procedure (Practice Committees of the American Society for Reproductive & the Society for Assisted Reproductive 2013). For instance, some cancer patients seeking fertility preservation may be unsuitable candidates for in vivo maturation because they are prepubescent, have estrogen-sensitive cancers that respond adversely to ovarian stimulation protocols, or have cancers that require immediate

treatment without time for hormonal stimulation (Loren *et al.* 2013). In these cases, efforts have been made to cryopreserve or vitrify ovarian cortex samples, but oocytes retrieved from cortex tissues will likely require in vitro maturation before being subjected to in vitro fertilization (Kagawa *et al.* 2009, Imesch *et al.* 2013). Additionally, in vivo maturation of oocytes may not be advisable for women with polycystic ovary syndrome (PCOS) who wish to undergo in vitro fertilization, because the hormonal stimulation required poses an increased risk for ovarian hyperstimulation syndrome in these patients (Jurema & Nogueira 2006).

Unfortunately, in vitro maturation of human oocytes has yielded little success thus far (Imesch *et al.* 2013). There have been recent advancements in in vitro maturation of human oocytes, especially in PCOS patients, due to optimizing methods for FSH priming of ovaries and selecting optimal follicle size for oocyte retrieval, but there remains much room for improvement (Lindenberg 2013). The associations between MMP9 levels in human follicular fluid and pregnancies resulting from in vitro fertilization suggest that MMP9 could be used to improve the success of in vitro maturation of human oocytes (Lee *et al.* 2005, Horka *et al.* 2012).

**The Bovine as a Model for Human Oocyte Maturation**

Because of the precious nature of human oocytes, bovine oocytes, which are more readily available and highly similar in terms of maturation, are often used as a model in place of human gametes. To begin with, bovine oocytes are comparable in diameter to human oocytes (Ménézo & Hérubel 2002), and must reach a size threshold of 110 μm in diameter in order to resume meiosis and reach metaphase II (Fair *et al.* 1995). Human oocytes must likewise reach a diameter of 90 μm in order to proceed with meiosis and develop to metaphase II (Trounson *et al.* 2001). Resumption of meiosis, however, does not depend solely on size of the oocyte; hormonal stimuli also play a role in regulating nuclear and cytoplasmic maturation. When a combination

of LH and FSH was added to in vitro maturation media, human and bovine, but not murine, oocytes displayed increased percentage of oocytes achieving metaphase II status and developing into blastocyst-stage embryos by 8 days post fertilization (Anderiesz *et al.* 2000).

Once maturation has begun, the human and the bovine oocytes follow very similar timelines. From about 0-6 hours, the germinal vesicle is present. From around 6-12 hours, the germinal vesicle breaks down. From 12-18 hours, the oocyte progresses through metaphase I, anaphase I, and telophase I, and by 24 hours the oocyte arrests at metaphase II in the cow (Sirard *et al.* 1989, Combelles *et al.* 2002) and in the human (Combelles *et al.* 2002). As these events take place within the nucleus, cytoplasmic maturation occurs simultaneously, including events such as polyadenylation of mRNA to be stored and later translated into protein (reviewed by Hennet & Combelles 2012). The bovine and the human have a similar degree of mRNA polyadenylation that is less than that seen in the mouse (reviewed by Ménézo & Hérubel 2002). Inhibition of protein synthesis in the cumulus-oocyte complex, especially at or before 18 hours of maturation, has been shown to impede meiosis, illustrating the importance of synchrony between cytoplasmic and nuclear events (Sirard *et al.* 1989). As maturation progresses, the Golgi bodies within the oocyte decrease in abundance as they produce cortical granules that disperse and migrate toward the periphery of the oocyte for humans (Sathananthan 1994) and cows alike (Sathananthan 1994, Hennet & Combelles 2012). For both species, maturation is complete when the oocyte rearrested at metaphase II with cortical granules prepared to empty their contents into the perivitelline space.

# CHAPTER 3
# MATERIALS AND METHODS

## *Materials*

Reagents were purchased from Sigma Chemical Co. unless otherwise noted. Oocyte collection medium was M-199 with Hank's salts (Mediatech, Manassas, VA, USA), containing 1-2% standard FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine, and 50 U/mL penicillin with 50 µg/mL streptomycin (EMD Millipore Corp., Billerica, MA, USA). Oocyte maturation medium was M-199 with Earle's salts (Gibco Life Technologies, Grand Island, NY), containing 10% premium FBS (BioWhittaker, Walkersville, MD, USA), 50 µg/mL gentamicin (BioWhittaker), 5 µg/mL FSH (Bioniche, Belleville, ON, Canada), 0.2 mM sodium pyruvate, and 2 mM L-glutamine. Media for sperm and putative zygote (PZ) handling consisted of sperm Tyrode's albumin lactate pyruvate (Sperm-TALP), IVF-TALP, and HEPES-TALP, prepared according to Parrish *et al*. (1988). Potassium simplex optimized medium was prepared according to Biggers *et al*. (2000) and modified to contain 1 mM glutamine, 10 mM glycine, 1X non-essential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin (mKSOM). Ovaries were obtained from a mixture of beef and dairy Bos taurus breeds at a local abattoir (Southeastern Provision, Bean Station, TN, USA). All gelatin zymography gels and buffers were prepared according to Toth and Fridman (2001). Coomassie Brilliant Blue and glycine were purchased from MD Biomedicals (Santa Ana, California, USA).

## *In Vitro Production of Embryos*

In vitro maturation (IVM), fertilization, and embryo culture were performed as previously described (Lawrence *et al.* 2004, Edwards *et al.* 2005, Schrock *et al.* 2007). Unless otherwise specified, groups of ~36 ± 5.6 (mode = 30) cumulus-oocyte complexes per well of 500 µL maturation medium were matured at 38.5°C in 5.5% $CO_2$ and 21% $O_2$ in a humidified

environment. After 24 h of IVM (hIVM), groups of cumulus-oocyte complexes were photographed with a Nikon Digital Sight camera attached to a SMZ 800 bright field microscope so an individual uninformed of treatment could evaluate cumulus expansion. Also, conditioned maturation medium was harvested for assessment of matrix metallopeptidase-9 and progesterone levels. Immediately thereafter, IVF-TALP and sperm were added to cumulus-oocyte complexes at a concentration of 500,000 motile sperm/mL (final volume 500 µL). Putative zygotes (PZs) were denuded of cumulus by vortexing in hyaluronidase at 16 to 18 hours after IVF and then cultured in mKSOM at 38.5°C, 5.5% $CO_2$, and 7% $O_2$ in a humidified environment. The ability of PZs to cleave beyond the one cell stage was assessed at approximately 72 hours post IVF (hpi). Essential amino acids (1X) were added to mKSOM before returning PZs to the incubator. Blastocyst development was assessed at approximately 210 hpi; stage and quality scores were assigned as per Schrock *et al.* (2007). Blastocyst stage embryos were fixed using 3% paraformaldehyde and then stained with 0.5 µg/mL Hoechst 33342 to enumerate nuclei.

***Assessment of MMP9 by Gelatin Zymography***

Gelatin zymography was used to assess the levels of MMP9 in conditioned maturation medium as per Rispoli *et al.* (2013) with a few modifications. Protein concentration of maturation medium was determined using Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Rockford, IL, USA); then samples were mixed with Tris-glycine SDS sample buffer to achieve a 1 µg/µL concentration. Gelatin-impregnated 7.5% polyacrylamide gels were loaded with 20 µg of protein per sample and run at 125 V for 3 h prior to development at 37.0°C for 18 h. Gels were stained in Coomassie for 2 h at room temperature and then destained for 4 h. Dried gels were scanned and analyzed according to Leber and Balkwill (1997) using ImageJ software (ver. 1.45s; Rasband 1997-2013).

***Progesterone Assay***

Progesterone concentrations in conditioned maturation medium were determined using a commercially-available solid phase radioimmunoassay kit after diluting samples at a 1:8 ratio with unconditioned media (Coat-A-Count; Siemens Medical Solutions Diagnostic, Los Angeles, CA). Assay sensitivity was 0.02 ng/mL. Average intra- and inter-assay CVs were 5.35% and 5.39%, respectively.

***Study One: Effects of Adding MMP9 during 24 hIVM at 38.5°C***

An initial effort testing the effects of 0, 30 or 300 ng/mL rhMMP9 added at 0 hIVM was suggestive that 300 ng/mL may be beneficial to embryo development. Soon after collection, cumulus-oocyte complexes were evenly and randomly distributed to maturation medium containing 0, diluent, or 300 ng/mL recombinant human latent MMP9 (rhMMP9). Recombinant human proMMP9 was purchased from Calbiochem (San Diego, CA, USA) and diluted in 50 mM Tris-buffered saline with 0.05% Brij-35 and 10 mM calcium (four replicates) or from Sino Biological (Beijing, China) and diluted in phosphate buffered saline (PBS; six replicates). This experiment was replicated on ten different occasions.

***Study Two: Effects of Adding MMP9 during last 12 hIVM on Control and Heat-Stressed Cumulus-Oocyte Complexes***

Bovine cumulus-oocyte complexes were cultured for 24 h at either 38.5 (control) or 41.0°C (heat stress; first 12 h only and then transferred to 38.5°C) as previously described by Edwards *et al.* (2005) and Schrock *et al.* (2007). At 12 hIVM, maturation medium was supplemented with diluent or 300 ng/mL rhMMP9, resulting in a 2 x 2 factorial treatment arrangement (Figure 1). This experiment was replicated on ten different occasions using rhMMP9 sourced from Sino Biological (PBS diluent).

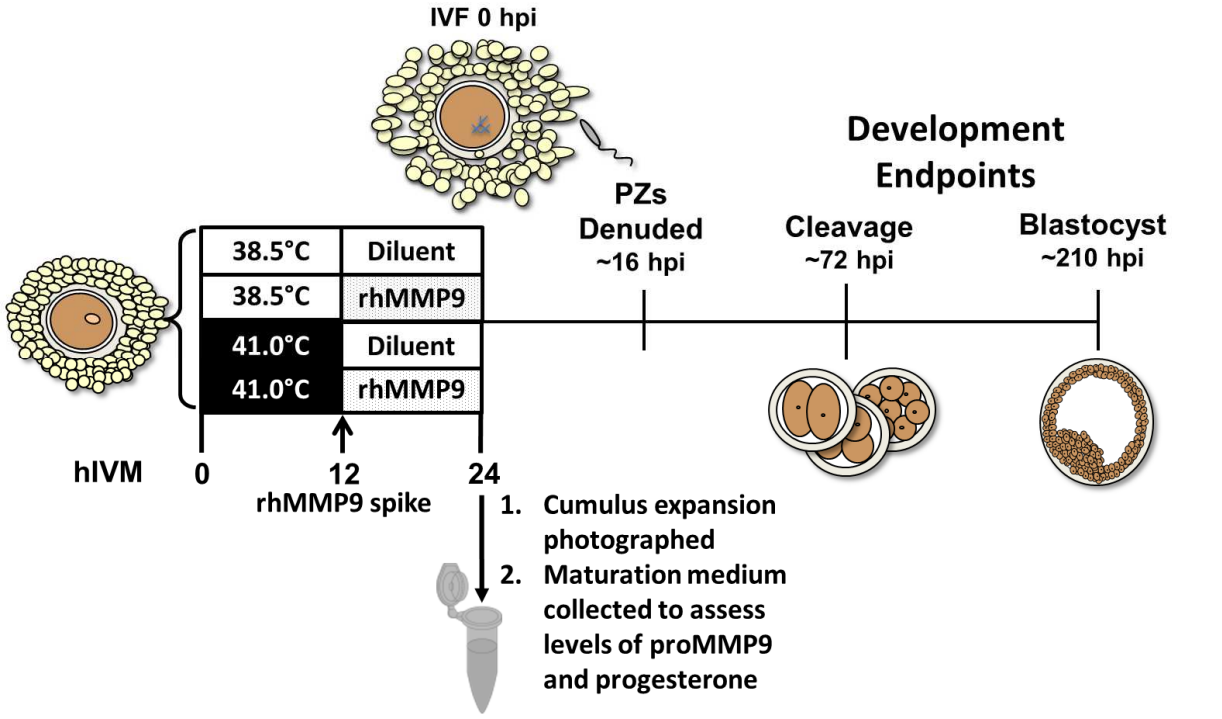



**Figure 1**. Experimental schematic for Study Two. Bovine cumulus-oocyte complexes were cultured at either 38.5 or 41.0°C (first 12 h only and then transferred to 38.5°C). At 12 hours of in vitro maturation (hIVM), all treatment groups were removed from the incubator and maturation medium was supplemented with diluent or 300 ng/mL recombinant human latent MMP9 (rhMMP9). At 24 hIVM, cumulus expansion was photographed and conditioned maturation medium collected to assess levels of proMMP9 and progesterone. Putative zygotes (PZs) were denuded of cumulus at 16 h post IVF (hpi). Cleavage and blastocyst development were assessed at 72 and 210 hpi, respectively.

***Study Three: Effects of Adding MMP9 during last 6 hIVM on Control and Heat-Stressed Cumulus-Oocyte Complexes***

Because a previous study did not detect differences in proMMP9 levels between heat stress and control cumulus-oocyte complexes until 18 hIVM (Rispoli *et al.* 2013), maturation medium was supplemented with MMP9 at 18 hIVM. Bovine cumulus-oocyte complexes were cultured for 24 h at either 38.5 (control) or 41.0°C (heat stress; first 12 h only then transferred back to 38.5°C). At 18 hIVM, maturation medium was supplemented with diluent, 30, or 300 ng/mL rhMMP9, resulting in a 2 x 3 factorial treatment arrangement. This experiment was replicated on twelve different occasions. Recombinant human proMMP9 was purchased from Sino Biological and diluted in PBS (four replicates) or from Abcam (Cambridge, MA, USA) and diluted in 50% glycerol and 250 mM NaCl in PBS (eight replicates).

***Statistical Analyses***

Data were analyzed as a randomized block design, blocking on replicate, using generalized linear models (PROC GLIMMIX) in SAS (9.3, SAS Inst., Inc., Cary, NC, USA). For the first study, model included rhMMP9 as a fixed effect. For Studies Two and Three, fixed effects included maturation temperature, rhMMP9 dose, and the interaction of maturation temperature x rhMMP9 dose. Experimental unit was defined as a plate containing well(s) of oocytes. Treatment differences were determined using protected least significant differences and reported as least squares means ± SEM using the inverse link option.

For the second study, an equivalence test was used to further examine the proportion of putative zygotes that developed to the blastocyst stage for groups treated with 0 or 300 ng/mL rhMMP9. Equivalence between 0 and 300 ng/mL rhMMP9 blastocyst development means was

set at 6% (two one-sided t tests). In order for two means to be considered equivalent ($P \leq 0.05$), means and their confidence intervals must be within 6% of each other.

To examine proportional relationship between the heat-induced changes in proMMP9 and progesterone levels, percent differences between thermoneutral and heat stress levels per cumulus-oocyte complex were calculated for each replicate of Studies Two and Three for only samples without rhMMP9 addition. To increase the power of this analysis, seven replicates from the microarray study conducted by Rispoli *et al.* (2013) were included in the correlation. A Pearson correlation of MMP9 percent differences and progesterone percent differences was conducted without blocking on replicate (PROC CORR).

# CHAPTER 4
# RESULTS

***Study One: Effects of Adding rhMMP9 during 24 hIVM at 38.5°C***

Adding rhMMP9 at 0 hIVM increased proMMP9 levels at 24 hIVM compared to control counterparts (P = 0.0005; Figure 2A). Levels of progesterone in the conditioned maturation medium supplemented with 0, diluent, or 300 ng/mL rhMMP9 did not differ at 24 hIVM (P = 0.5049; Figure 2B). After 24 hIVM, no visual differences were detected in cumulus expansion among any of the treatment groups examined (data not shown). The proportion of putative zygotes that cleaved and developed to the 8 to 16-cell stage was similar across all three treatments (Table 1). Likewise, blastocyst development, stage, quality score, and total number of nuclei were comparable (Table 1). There were no differences between diluent and control groups.

***Study Two: Effects of Adding rhMMP9 during last 12 hIVM on Control and Heat-Stressed Cumulus-Oocyte Complexes***

A priori comparison of 38.5 vs 41.0°C diluent groups (0 ng/mL rhMMP9) revealed a heat-induced decrease in proMMP9 production from cumulus-oocyte complexes (75.04 vs 42.05 ± 14.44 arbitrary units for 38.5°C and 41.0°C, respectively; P = 0.0097). When 300 ng/mL rhMMP9 was added at 12 hIVM, proMMP9 levels were equivalent at 24 hIVM (270.54 vs 279.05 ± 77.04 for 38.5°C and 41.0°C, respectively; P = 0.8732), thus allowing for a more precise test of the hypothesis. Progesterone production per cumulus-oocyte complex was increased by heat stress exposure (P = 0.0002; Figure 3). Heat-induced increases in progesterone at 24 hIVM were highest when 300 ng/mL rhMMP9 was present in maturation medium (Figure 3). After 24 hIVM, no visual differences in cumulus expansion were detected in any examined treatment groups (Figure 4).

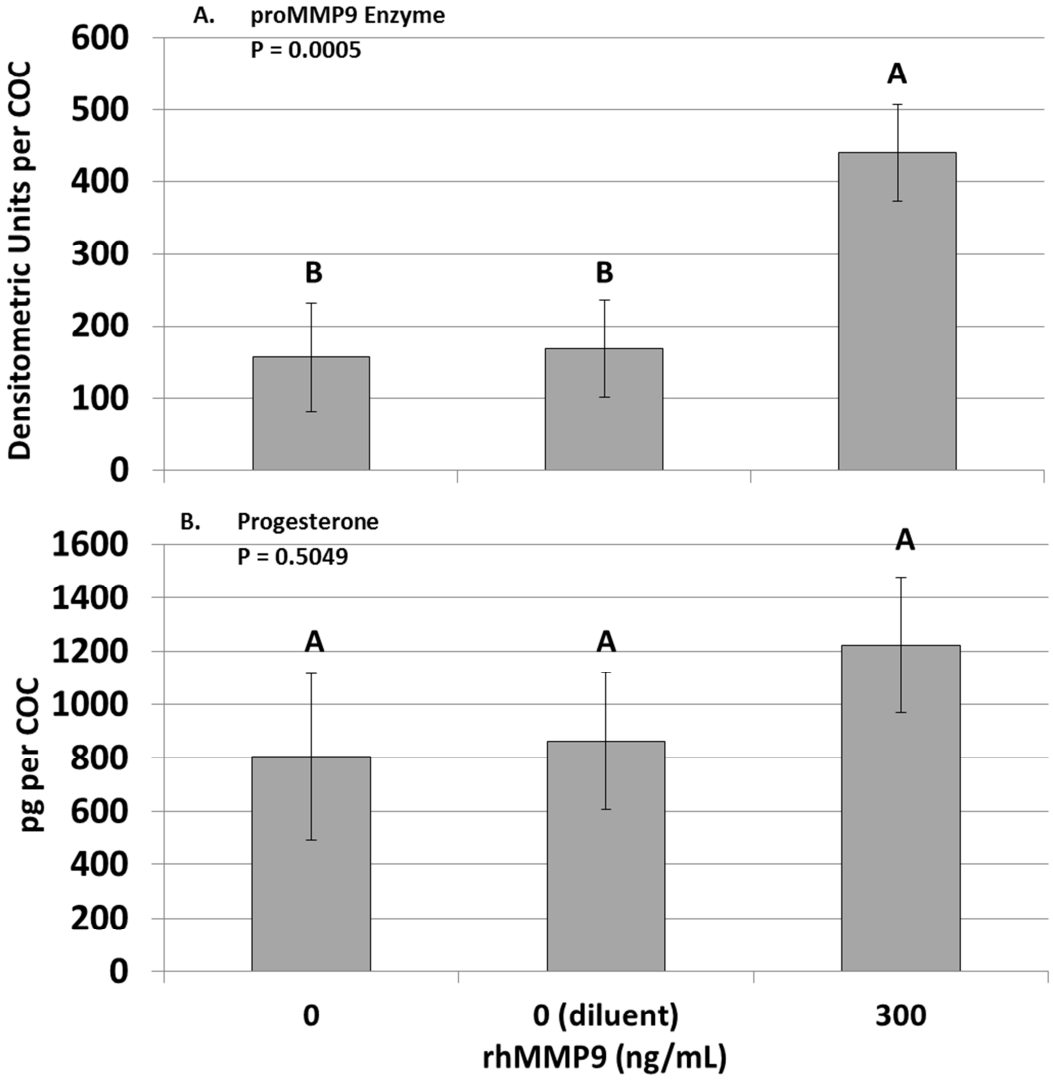



**Figure 2.** Levels of latent matrix metallopeptidase-9 (proMMP9) and progesterone per cumulus-oocyte complex (COC) after 24 h of in vitro maturation (hIVM). Media were supplemented with 0 or 300 ng/mL recombinant human proMMP9 (rhMMP9) at 0 hIVM. Effects of the diluent were evaluated by inclusion of a group without diluent (0 vs diluent control.) [AB]Different letters denote statistical difference within a comparison.

**Table 1. Cleavage and blastocyst development after supplementing rhMMP9 at 0 h of oocyte maturation**

| | | Cleavage (72.3 ± 1.5 hpi) | | Blastocyst Development (207.5 ± 0.5 hpi) | | | | |
|---|---|---|---|---|---|---|---|---|
| rhMMP9 (ng/mL) | No. PZs | % Cleaved of PZs | % 8 to 16-Cell of Cleaved | % Blastocysts of PZs | % Blastocysts of Cleaved | Stage | Quality | Nuclei |
| 0 | 460 | 70.94 | 69.72 | 29.06 | 41.49 | 7.15 | 2.00 | 147.35 |
| 0 (Diluent) | 681 | 67.47 | 64.11 | 27.21 | 40.65 | 7.02 | 2.06 | 130.43 |
| 300 | 685 | 68.07 | 61.38 | 26.78 | 39.85 | 7.12 | 1.90 | 141.20 |
| | SEM | 2.83 | 4.71 | 2.14 | 3.13 | 0.1383 | 0.0803 | 8.8792 |
| | *P-value* | 0.4735 | 0.1258 | 0.7020 | 0.9060 | 0.5433 | 0.1576 | 0.2294 |

Abbreviations: hpi = hours post in vitro fertilization; PZs = Putative Zygotes

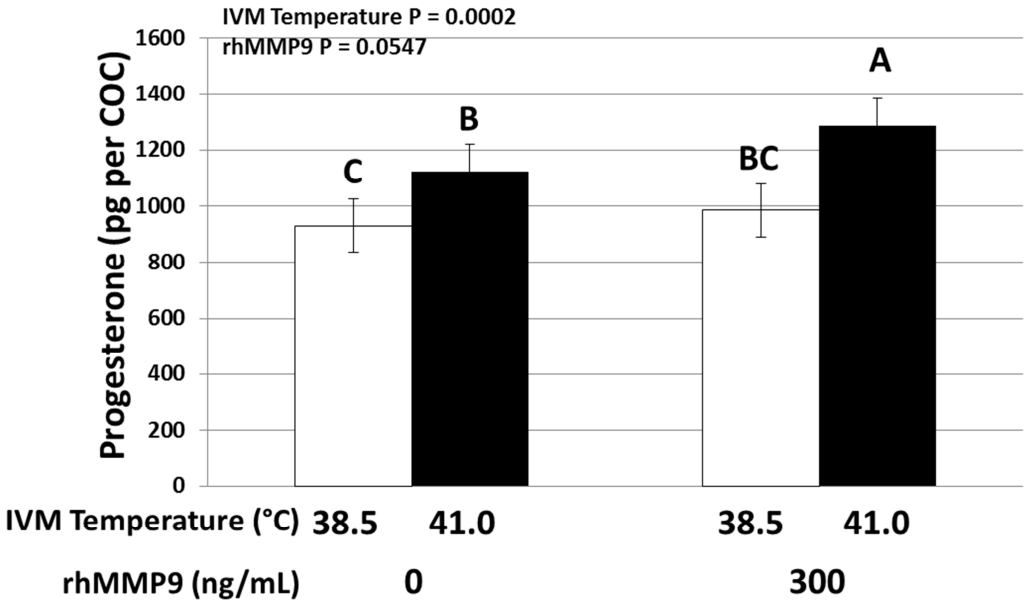



**Figure 3.** Levels of progesterone per cumulus-oocyte complex (COC) at 24 h of in vitro maturation (hIVM) when 0 (diluent) vs 300 ng/mL recombinant human proMMP9 (rhMMP9) was added at 12 h of in vitro maturation (hIVM) to cumulus-oocyte complexes matured at 38.5 or 41.0°C (first 12 h only and then transferred to 38.5°C). [AB]Different letters denote statistical difference within a comparison.

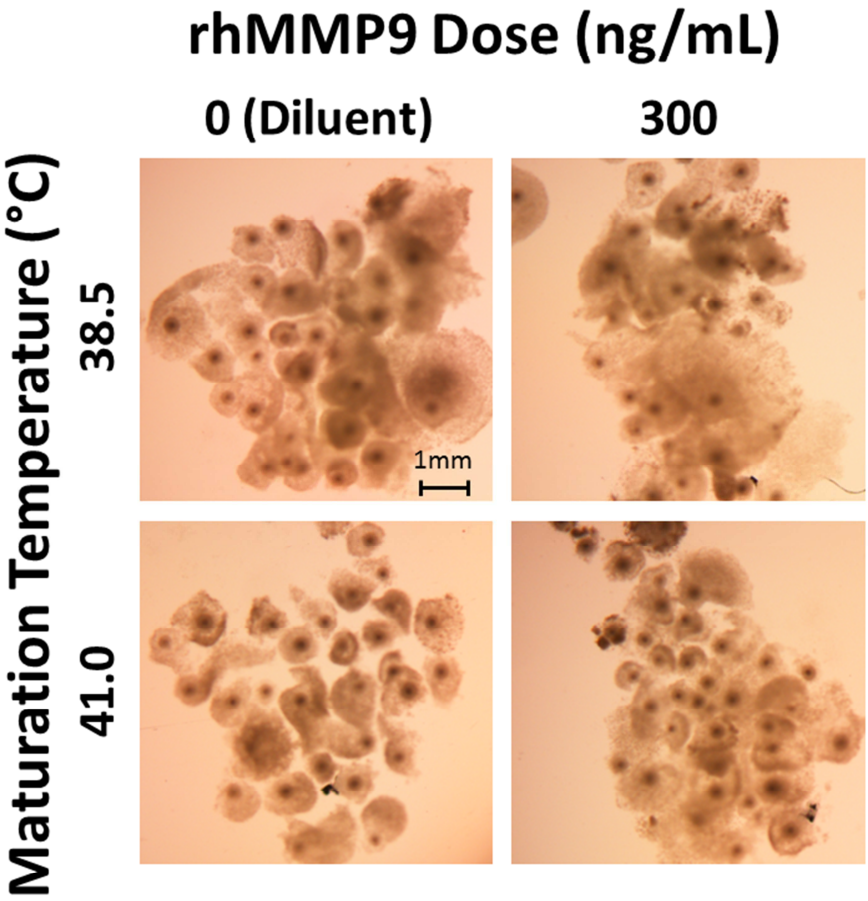



**Figure 4.** Representative images of cumulus expansion at 24 h of in vitro maturation (hIVM) of bovine cumulus-oocyte complexes cultured at either 38.5 or 41.0°C (first 12 h only and then transferred to 38.5°C) and supplemented at 12 hIVM with diluent (phosphate buffered saline) or 300 ng/mL recombinant human latent matrix metallopeptidase-9 (rhMMP9). Images were taken at 10X total magnification. There were no detectable differences in cumulus expansion among cumulus-oocyte complexes matured at 38.5°C with diluent (A) or 300 ng/mL rhMMP9 (B) or matured at 41.0°C (for first 12 h and then restored to 38.5°C) with diluent (C) or 300 ng/mL rhMMP9 (D).

Heat stress exposure during the first 12 hIVM had no effect on the ability of putative zygotes to cleave after IVF, but reduced the ability to cleave to the 8 to 16-cell stage ($P = 0.0006$) and develop to the blastocyst stage ($P = 0.0062$; Table 2). Heat stress exposure did not affect stage score or number of nuclei per blastocyst (Table 2).

The presence of rhMMP9 during the last 12 hIVM decreased the ability of putative zygotes to cleave ($P = 0.0157$) without affecting ability of cleaved embryos to progress to the 8 to 16-cell stage. The proportion of putative zygotes that developed to blastocyst stage tended to be less when rhMMP9 was present in maturation medium during last 12 hIVM ($P = 0.0823$; Table 2). An equivalence test confirmed blastocyst development from putative zygotes in 300 ng/mL rhMMP9 groups was not equivalent to diluent groups ($P = 0.0922$; means are considered equal when $P \leq 0.05$). Addition of rhMMP9 at 12 h of oocyte maturation did not affect blastocyst stage or number of nuclei per blastocyst. While blastocyst quality was influenced by an interaction between maturation temperature and addition of rhMMP9 that made the difference between means different ($P = 0.04$), the means themselves were not different (Table 2).

***Study Three: Effects of Adding rhMMP9 during Last 6 hIVM on Control and Heat-Stressed Cumulus-Oocyte Complexes***

Heat stress exposure during the first 12 h of oocyte maturation reduced proMMP9 levels in 0 and 30 ng/mL rhMMP9 groups, but addition of 300 ng/mL rhMMP9 resulted in equivalent levels between heat stress and control groups ($P = 0.0004$; Figure 5A). Heat stress during IVM increased progesterone production in 0 and 300 ng/mL rhMMP9 groups, but was equivalent to control in the presence of 30 ng/mL rhMMP9 groups (rhMMP9 x temperature interaction; $P = 0.039$; Figure 5B). After 24 hIVM, although not measured, no visual differences in cumulus expansion were detected across any examined treatment groups (data not shown).

**Table 2. Cleavage and blastocyst development after supplementing rhMMP9 at 12 h of oocyte maturation**

| | | Cleavage (72.32 ± 0.62 hpi ) | | Blastocyst Development (210.68 ± 0.81 hpi) | | | | |
|---|---|---|---|---|---|---|---|---|
| Effect | No. PZs | % Cleaved of PZs | % 8 to 16-Cell of Cleaved | % Blastocysts of PZs | % Blastocysts of Cleaved | Stage | Quality | Nuclei |
| Maturation Temperature (°C) | | | | | | | | |
| 38.5 | 1677 | 66.77 | 59.56$^{A}$ | 25.31$^{A}$ | 37.83$^{A}$ | 7.16 | 1.86 | 137.65 |
| 41.0 | 1633 | 66.55 | 51.01$^{B}$ | 19.74$^{B}$ | 29.41$^{B}$ | 7.19 | 1.86 | 133.99 |
| | SEM | 1.57 | 3.21 | 1.72 | 2.23 | 0.06 | 0.05 | 6.31 |
| | *P-value* | 0.8940 | **0.0006** | **0.0062** | **0.0003** | 0.5886 | 0.9855 | 0.4238 |
| rhMMP9 (ng/mL) | | | | | | | | |
| 0 (Diluent) | 1655 | 68.76$^{A}$ | 56.90 | 24.12 | 35.01 | 7.15 | 1.86 | 139.37 |
| 300 | 1655 | 64.50$^{B}$ | 53.74 | 20.77 | 32.00 | 7.20 | 1.85 | 132.27 |
| | SEM | 1.57 | 3.22 | 1.72 | 2.24 | 0.06 | 0.05 | 6.30 |
| | *P-value* | **0.0157** | 0.1561 | 0.0823 | 0.1489 | 0.4332 | 0.8982 | 0.1200 |
| Maturation Temperature x rhMMP9 | | | | | | | | |
| 38.5°C x 0 ng/mL (Diluent) | 855 | 69.40 | 60.20 | 27.85 | 40.19 | 7.17 | 1.78$^{A}$ | 143.72 |
| 41.0°C x 0 ng/mL (Diluent) | 800 | 68.11 | 53.53 | 20.75 | 30.17 | 7.13 | 1.94$^{A}$ | 135.02 |
| 38.5°C x 300 ng/mL | 822 | 64.04 | 58.92 | 22.93 | 35.52 | 7.14 | 1.93$^{A}$ | 131.58 |
| 41.0°C x 300 ng/mL | 833 | 64.95 | 48.48 | 18.77 | 28.67 | 7.26 | 1.77$^{A}$ | 132.96 |
| | SEM | 1.95 | 3.54 | 2.15 | 2.64 | 0.08 | 0.07 | 7.04 |
| | *P-value* | 0.5054 | 0.4018 | 0.5318 | 0.4926 | 0.2142 | **0.0400** | 0.2638 |

Abbreviations: hpi = hours post in vitro fertilization; PZs = Putative Zygotes

$^{AB}$Least squares means within a column differ.

Significant P-values are shown in bold.

Heat stress decreased the ability of putative zygotes to cleave ($P < 0.0001$) and develop further to the 8 to 16-cell stage ($P < 0.0001$; Table 3). Heat stress also decreased the proportion of putative zygotes that developed to blastocyst stage ($P < 0.0001$), yet did not affect stage score or number of nuclei per each blastocyst.

Supplementation of rhMMP9 during the last 6 hIVM did not affect the ability of putative zygotes to cleave or reach the 8 to 16-cell stage, but did reduce their ability to progress to the blastocyst stage ($P = 0.0220$; Table 3). Addition of rhMMP9 did not affect stage score or number of nuclei per blastocyst.

***Relationship between Heat-Induced Changes in Levels of MMP9 and Progesterone***

To examine the relationship between MMP9 and progesterone levels in the context of heat stress, data were combined from studies two and three (0 ng/mL observations only) and observations from a previously published study (Rispoli *et al.* 2013, n=7 replicates). Collectively, heat stress decreased proMMP9 production (224.06 vs 125.79 ± 28.52 units per cumulus-oocyte complex for control and heat stress, respectively; $P < 0.0001$) and increased progesterone production from cumulus-oocyte complexes during IVM (883.22 vs 1055.92 ± 68.97 pg per cumulus-oocyte complex for control and heat stress, respectively; $P < 0.0001$). A Pearson correlation analysis of MMP9 percent differences and progesterone percent differences revealed an inverse relationship between changes in MMP9 and progesterone ($r = -0.37$, $P = 0.069$; Figure 6).

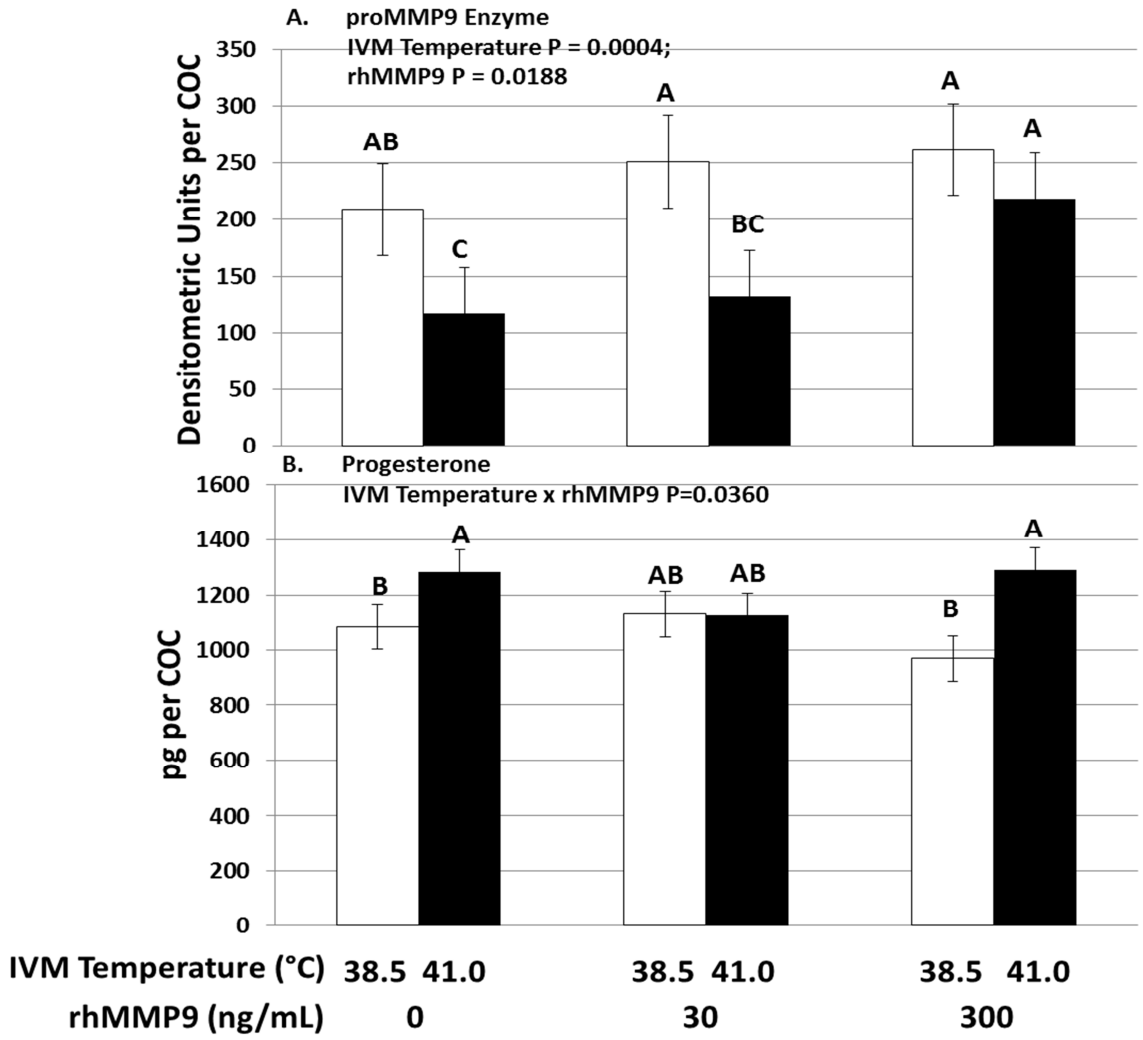



**Figure 5.** Levels of latent matrix metallopeptidase-9 (proMMP9) and progesterone per cumulus-oocyte complex (COC) at 24 h of in vitro maturation (hIVM) when 0, 30 or 300 ng/mL of recombinant human proMMP9 (rhMMP9) was added to oocyte maturation medium at 18 hIVM. Cumulus-oocyte complexes matured at 38.5 or 41.0 °C (first 12 h only and then transferred to 38.5°C). $^{AB}$Denotes means differ within a comparison.

**Table 3. Cleavage and blastocyst development after supplementing rhMMP9 at 18 h of oocyte maturation**

| | | Cleavage (69.94 ± 6.77 hpi) | | Blastocyst Development (211.37 ± 1.25 hpi) | | | | |
|---|---|---|---|---|---|---|---|---|
| Effect | No. PZs | % Cleaved of PZs | % 8 to 16-Cell of Cleaved | % Blastocysts of PZs | % Blastocysts of Cleaved | Stage | Quality | Nuclei |
| Maturation Temperature (°C) | | | | | | | | |
| 38.5 | 1642 | 69.76$^{A}$ | 72.72$^{A}$ | 26.83$^{A}$ | 38.85$^{A}$ | 7.22 | 1.78 | 140.71 |
| 41.0 | 1723 | 60.76$^{B}$ | 62.27$^{B}$ | 16.35$^{B}$ | 26.89$^{B}$ | 7.14 | 1.73 | 136.23 |
| | SEM | 1.72 | 3.05 | 1.96 | 2.58 | 0.047 | 0.055 | 5.39 |
| | *P-value* | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | 0.0926 | 0.5422 | 0.3798 |
| rhMMP9 (ng/mL) | | | | | | | | |
| 0 (Diluent) | 1166 | 67.23 | 69.00 | 24.07$^{A}$ | 36.04 | 7.15 | 1.83$^{A}$ | 131.24 |
| 30 | 1026 | 64.45 | 67.57 | 19.39$^{B}$ | 30.35 | 7.20 | 1.62$^{B}$ | 143.07 |
| 300 | 1173 | 64.48 | 66.56 | 20.10$^{B}$ | 31.51 | 7.18 | 1.82$^{A}$ | 141.09 |
| | SEM | 1.93 | 3.25 | 2.09 | 2.81 | 0.052 | 0.066 | 5.96 |
| | *P-value* | 0.2916 | 0.6079 | **0.0220** | 0.0639 | 0.6884 | **0.0396** | 0.1334 |
| Maturation Temperature x rhMMP9 | | | | | | | | |
| 38.5°C x 0 ng/mL (Diluent) | 580 | 70.33 | 74.83 | 30.17 | 43.10 | 7.17 | 1.79 | 135.74 |
| 41.0°C x 0 ng/mL (Diluent) | 586 | 63.97 | 62.49 | 18.86 | 29.55 | 7.14 | 1.86 | 126.75 |
| 38.5°C x 30 ng/mL | 485 | 71.64 | 72.30 | 26.41 | 37.04 | 7.27 | 1.72 | 148.21 |
| 41.0°C x 30 ng/mL | 541 | 56.55 | 62.45 | 13.88 | 24.39 | 7.13 | 1.52 | 137.93 |
| 38.5°C x 300 ng/mL | 557 | 67.20 | 70.95 | 24.11 | 36.54 | 7.21 | 1.82 | 138.17 |
| 41.0°C x 300 ng/mL | 596 | 61.65 | 61.85 | 16.62 | 26.87 | 7.15 | 1.82 | 144.00 |
| | SEM | 2.38 | 3.67 | 2.41 | 3.27 | 0.065 | 0.090 | 7.39 |
| | *P-value* | 0.0513 | 0.7364 | 0.3189 | 0.7603 | 0.5486 | 0.2980 | 0.3544 |

Abbreviations: hpi = hours post in vitro fertilization; PZs = Putative Zygotes

$^{AB}$Least squares means within a column differ.

Significant P-values are shown in bold.

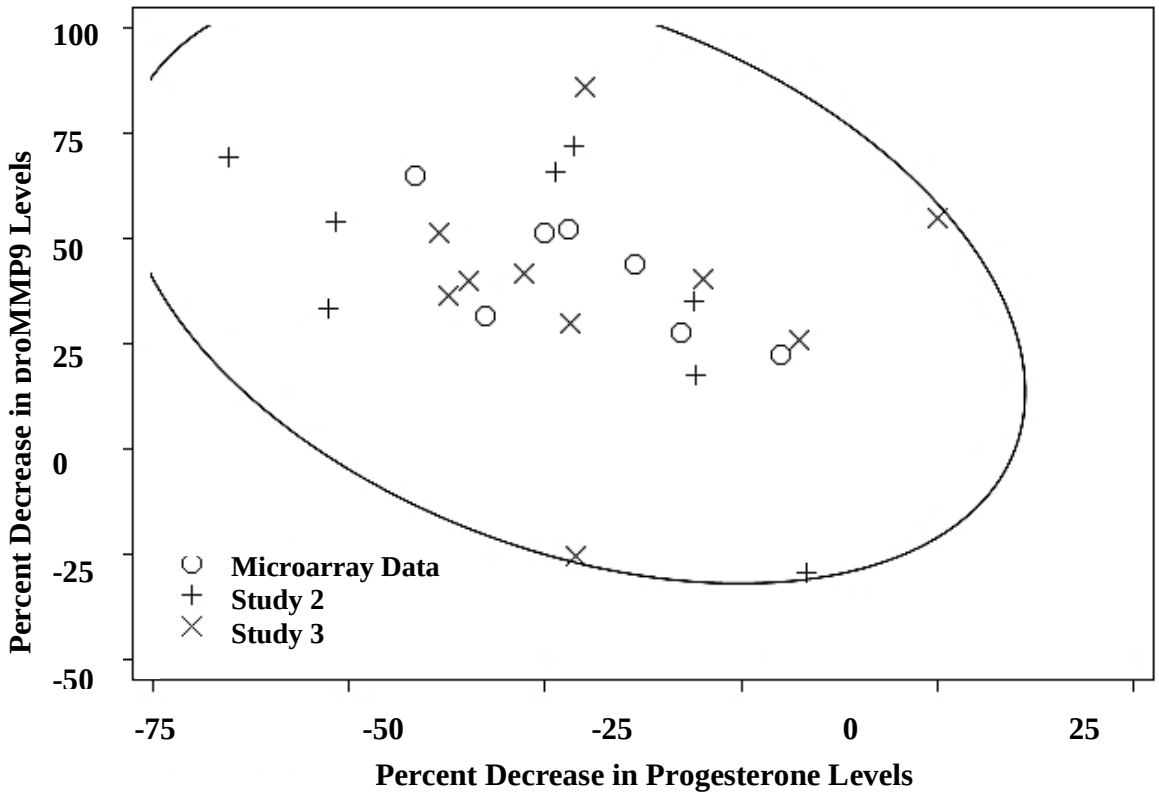



**Figure 6.** Correlation of heat-induced percent differences in latent matrix metallopeptidase-9 (proMMP9) and progesterone. Cumulus-oocyte complexes were cultured at 38.5°C for 24 h of in vitro maturation (hIVM) whereas heat-stressed cumulus-oocyte complexes were cultured at 41.0°C (first 12 hIVM only and then transferred to 38.5°C). Percent differences were calculated by expressing the difference in heat-stressed levels of proMMP9 and progesterone per cumulus-oocyte complex as a percentage of control levels for each replicate. Positive values indicate heat-induced reductions; negative values indicate heat-induced increases. ($r = -0.37$; $P = 0.069$).

# CHAPTER 5
# DISCUSSION

Heat-induced reductions in blastocyst development were previously related to decreases in MMP9 production from maturing bovine cumulus-oocyte complexes (Rispoli *et al.* 2013). Thus, it was hypothesized that effects of heat stress to decrease blastocyst development may be mediated in part by reductions in MMP9 production during oocyte maturation. The goal of this study was to make MMP9 levels equivalent between heat-stressed and non-stressed oocytes during IVM in an attempt to improve development from heat-stressed oocytes. Addition of 300 ng/mL rhMMP9 during IVM made MMP9 levels equal between heat-stressed and non-stressed oocytes but was not sufficient to restore development. In fact, rhMMP9 addition after 12 hIVM was detrimental to cleavage and blastocyst development.

It was surprising that addition of rhMMP9 had a negative effect because MMP9 has been positively related to fertility. When Dubois *et al.* (2000) knocked out MMP9 in mice, those mice had fewer litters and fewer pups per litter in a breeding season compared to mice that produced MMP9. McCaffery *et al*. (2000) found via gelatin zymography that bovine follicle sections that produced MMP9 during in vitro culture were more likely to have healthy granulosa and theca cells. Robert *et al.* (2001) later discovered that bovine follicles that expressed *MMP9* mRNA were also more likely to house oocytes capable of developing to the blastocyst stage after IVF. Similarly, Lee *et al*. (2005) noted that women with higher levels of MMP9 in follicular fluid and blood serum as assessed by zymography at the time of oocyte retrieval were more likely to become pregnant after IVF. A separate study by Horka *et al.* (2012) using an enzyme-linked immunosorbent assay (ELISA) confirmed that higher levels of MMP9 in follicles housing mature oocytes were positively associated with pregnancy rates after human IVF. Furthermore, in the bovine, MMP9 levels at 24 hIVM were positively related to blastocyst development

following IVF (Rispoli *et al.* 2013). In fact, when heat stress was sufficient to reduce blastocyst development in observations pooled from a study by Rispoli *et al.* (2013) and diluent observations (0 ng/mL rhMMP9) in this study, MMP9 production at 24 hIVM was decreased by heat stress 92% of the time (23/25 replicates). Although the present study is the first to add MMP9 to maturing oocytes, it was highly unexpected that doing so would have negative consequences, given the plethora of data positively implicating MMP9 in development.

While difficult to explain the negative impact of rhMMP9 on blastocyst development, consequences were likely not due to rhMMP9 sources used herein. Study One specifically examined the effect of Calbiochem and Sino Biological diluents compared to controls and found no difference in subsequent embryo development. Sino Biological rhMMP9 was then used for all replicates of Study Two and four replicates of Study Three, thus providing one common source across all three studies. When replicates with Abcam rhMMP9 were compared to Sino Biological replicates in Study Three, no apparent differences were noted. Furthermore, despite being derived from a different species, supplemented rhMMP9 was likely able to be used in the same way as bovine proMMP9. Human proMMP9 is 90% homologous to the bovine form (HomoloGene, NCBI) and has been shown to interact with other bovine tissues. For instance, when bovine bone slices were primed with rat osteoclasts and incubated with rhMMP9, collagen I degradation was increased (Parikka *et al.* 2001). Bovine-derived collagen type III can also be cleaved by rhMMP9 (Bigg *et al.* 2007). Bovine cumulus-oocyte complexes produce known activators of proMMP9 such as the inducible and endothelial isoforms of nitric oxide synthase (Tesfaye *et al.* 2006) and tissue- and urokinases-type plasminogen activators (Park *et al.* 1999), likely enabling activation of rhMMP9. As differences in cumulus expansion were not visually

detected across treatments in all three studies, regardless of rhMMP9 source, it is unlikely that effects of rhMMP9 on cumulus expansion contributed to differences in development.

Interestingly, a negative impact of rhMMP9 addition was only noted when present for the last 12 or 6 hIVM. When rhMMP9 was present for the last 12 hIVM, a four percentage point decrease in cleavage was observed, accompanied by a four percentage point decrease in blastocyst development. When rhMMP9 was present only for the last 6 hIVM, no effects were seen on cleavage, but blastocyst development decreased by close to four percentage points as well. When rhMMP9 was present during the entire period of maturation at the same dose, however, no effects on development were seen. It is possible that the deleterious effects of rhMMP9 supplementation may be due to alterations in sensitivity of the cumulus-oocyte complex as it progresses through maturation. When rhMMP9 was added in the last 12 or 6 hIVM, considerably more events were occurring and cumulus connections had been lost, perhaps making the oocyte more sensitive to changes in enzymatic activity. At the beginning of maturation, oocytes are arrested at the germinal vesicle stage; cumulus cells are tightly compacted and will not lose connection to the oocyte until about 9 hIVM (Thomas *et al.* 2004). The oocyte may be less susceptible to changes in MMP9 concentration at this time when cumulus cells are closely associated and meiosis has yet to resume. Germinal vesicle breakdown does not begin until 6 h after the initiation of oocyte maturation. From 10.3 to 15.4 hIVM, nuclear maturation progresses to metaphase I, anaphase I is reached by 16.6 hIVM, telophase I is achieved by 18 hIVM (Sirard *et al.* 1989) with the first polar body extruded around 12-18 hIVM (Hyttel *et al.* 1986), and most oocytes rearrest in metaphase II by 21 hIVM (Edwards *et al.* 2005). As maturation progresses in the cytoplasm, Golgi bodies decrease in abundance, lipid droplets associate with mitochondria to be metabolized for energy, and ribosomes and protein

synthesis increases (Kruip *et al.* 1983). Addition of protein synthesis inhibitors prior to 18 hIVM has been shown to prevent the majority of oocytes from reaching metaphase II, illustrating the important connection between cytoplasmic and nuclear elements during maturation (Sirard *et al.* 1989).

The rhMMP9 doses used in this study may have been above a beneficial range. A decrease in blastocyst development was noted when 300 ng/mL rhMMP9 was added during the last 12 or 6 hIVM, during which time MMP9 production naturally increases within the cumulus-oocyte complex. When a smaller dose of 30 ng/mL rhMMP9 was included during the last 6 hIVM, it was insufficient to make MMP9 levels equivalent between heat stress and control, but still decreased blastocyst development similar to 300 ng/mL rhMMP9. In these second and third studies, MMP9 levels may already have been near optimal, and rhMMP9 addition could have increased MMP9 concentration beyond the range of benefit. While MMP9 levels have been related to fertility, excessive MMP9 has also been related to reproductive dysfunction. In some bovine studies, when antral follicles were collected at various stages of the estrous cycle, the highest amount of MMP9 was detected through gelatin zymography in fluid from cystic or atretic follicles (Khandoker *et al.* 2001, Imai *et al.* 2003). Similarly, women with polycystic ovary syndrome were found to have higher levels of MMP9 in follicular fluid at time of mature oocyte recovery compared to a normal group (Shalev *et al.* 2001). Elevated intrafollicular levels of MMP9 detected via ELISA have also been found in human IVF patients with endometriosis and were associated with reduced in vivo oocyte maturation and decreased embryo quality (Singh *et al.* 2013). Most studies to date, including the present study, have used gelatin zymography, which quantifies MMP9 in relative but not absolute terms, so further effort using ELISA may be needed to more precisely determine an optimal range for MMP9 levels.

Consistent with previous efforts, heat stress decreased MMP9 production and increased progesterone production (Rispoli *et al.* 2013). These heat-induced changes were coincident with decreased blastocyst development noted by others both in vitro (Edwards & Hansen 1996, Lawrence *et al.* 2004, Roth & Hansen 2004) and in vivo (Putney *et al.* 1989). After combining observations from this study and a previously published one (Rispoli *et al.* 2013), when heat stress exposure during IVM was sufficient to reduce blastocyst development, progesterone production from cumulus-oocyte complexes measured at 24 hIVM was increased 72% of the time (18/25 replicates). Elevated progesterone production in response to elevated temperature may have superseded any positive effects rhMMP9 could have provided and may in part explain the lack of observed benefits from rhMMP9 supplementation. Furthermore, when examining these observations, it was noted that greater percent reductions in blastocyst development from control (38.5°C) to heat-stressed (41.0°C) cumulus-oocyte complexes correlated with greater increases in progesterone production from control to heat stress ($r = 0.37$; $P = 0.0669$; data not shown). A correlation of percent differences from control to heat stress showed that increases in progesterone were inversely related to decreases in MMP9, adding support to the theory that the two factors are related.

Independent of MMP9, increased progesterone levels during maturation reduces development to the blastocyst stage in the bovine (Silva & Knight 2000, Schlüter *et al.* 2014). The negative impact of elevated progesterone may be due to its hastening effect on oocyte maturation. Progesterone supplementation during IVM resulted in a higher percentage of bovine oocytes reaching MII by 22 hIVM whether cultured in a serum-free environment (Sirotkin 1992) or in follicular hemisections (Siqueira *et al.* 2012). In pigs, increased progesterone production

from cumulus-oocyte complexes corresponded with earlier onset of germinal vesicle breakdown (Yamashita *et al.* 2003).

Heat stress also hastens oocyte maturation, as heat stress application during the first 12 hIVM resulted in the majority of oocytes reaching MII by 18 hIVM (Edwards *et al.* 2005), yielding aged and less developmentally competent oocytes by 24 hIVM (Ward *et al.* 2002). The aging effect of heat stress may be mediated by progesterone as elevated temperature increased progesterone production both from cumulus cells (Rispoli *et al.* 2013) and from dominant follicle sections primed with LH during in vitro culture (Bridges *et al.* 2005). Because increased progesterone production has been observed in both cumulus and mural granulosa cells, the effect of heat stress to increase progesterone may be more pronounced in vivo. Chaffin *et al*. (2012) showed that cumulus revert to a mural granulosa-like state after exposure to gonadotropins. Cumulus observations may therefore provide insight into the actions of heat stress on mural granulosa after the LH surge, indicating a possible elevation in intrafollicular progesterone levels during in vivo oocyte maturation. An exhaustive search of the literature did not reveal any study examining follicular fluid from heat-stressed cows collected after the LH surge.

If progesterone is indeed contributing to decreased developmental competence, this would account for the lack of improvement seen when rhMMP9 was added during IVM, especially given that progesterone levels were unaffected or slightly elevated in the presence of rhMMP9. Interestingly, when 30 ng/mL rhMMP9 was added at 18 hIVM, progesterone levels at 24 hIVM were equivalent between control and heat stress groups, but blastocyst development was still lower than 0 ng/mL rhMMP9 groups. Neither progesterone nor MMP9 alone can account for changes in development, but a relationship between the two may come closer to providing an explanation.

Progesterone has been shown to regulate MMP9 production in other tissues, and its supplementation has decreased MMP9 activity in rat brain cortex (Ishrat *et al.* 2010) and human endometrium (Salamonsen *et al.* 1997). Inhibition of progesterone allowed for an increase in MMP9 production in murine (Li *et al.* 2012) and human (Marbaix *et al.* 1992) endometrium. Progesterone may decrease MMP9 activity through upregulation of TIMP1, an MMP9 inhibitor (Chaffin & Stouffer 1999, Lahav-Baratz *et al.* 2003) or inhibit transcription of MMP9 by preventing the binding of NF-κB to the MMP9 promoter region (Li *et al.* 2012, Halasz & Szekeres-Bartho 2013). While there are comparatively fewer studies documenting MMP9 effects on progesterone production (Jamnongjit *et al.* 2005, Carbajal *et al.* 2011), the present study revealed increased progesterone secretion from cumulus-oocyte complexes at 24 hIVM in response to heat stress exposure and rhMMP9 addition at 12 hIVM. This is suggestive of either a feedback relationship between MMP9 and progesterone or may simply indicate that the two factors are regulated through different pathways depending on the tissue examined.

Nevertheless, addition of rhMMP9 at evaluated doses and times did not restore development of heat-stressed oocytes and was actually detrimental. Bovine oocytes are comparable to human oocytes, having similar requirements and timelines for maturation (Sirard *et al*. 1989; Andriesz *et al.* 2000; Combelles *et al*. 2002). Supplementation of rhMMP9 should therefore not be considered for use in human embryo production at this time. While MMP9 may be useful as an indicator of oocyte developmental competence (Kim *et al.* 2006), the present study indicates that addition of rhMMP9 at doses tested is not sufficient to alleviate heat-induced decreases in blastocyst development. Other factors including progesterone may need to be examined as possible mediators of heat stress effects.

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