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Impact of Heat Stress on Germinal Vesicle Breakdown and Lipolytic Changes during In Vitro Maturation of Bovine Oocytes

Leah Marie Hooper
University of Tennessee - Knoxville, lhooper@vols.utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Leah Marie Hooper entitled "Impact of Heat Stress on Germinal Vesicle Breakdown and Lipolytic Changes during In Vitro Maturation of Bovine Oocytes." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

J. Lannett Edwards, Major Professor

We have read this thesis and recommend its acceptance:

Arnold M. Saxton, Travis J. Mulliniks

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Impact of Heat Stress on Germinal Vesicle Breakdown and Lipolytic Changes during In Vitro Maturation of Bovine Oocytes

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DEDICATION

I would like to dedicate this thesis to my family. Their support and encouragement throughout the pursuit of my goals have been invaluable.

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ABSTRACT

The main objective of this research was to examine the lipolytic changes in triglyceride and phospholipid as well as the incidence of germinal vesicle breakdown during IVM of heat-stressed oocytes compared to non-stressed oocytes. To this end, cumulus-oocyte complexes were matured for 0, 2, 4, 6 or 24 hIVM at 38.5 or 41.0°C (first 12 h only, then transferred to 38.5°C). Triglyceride and phospholipid levels decreased by 2 hIVM ($P = 0.0009$ and $P = 0.0005$, respectively) but remained fairly constant to 24 hIVM; lipid decline was not affected by maturation temperature. Elevated maturation temperature hastened meiotic progression by 4 hIVM ($P < 0.0001$). Incidence of germinal vesicle breakdown was associated, though not directly related, to lipolytic changes in oocyte triglyceride and phospholipid content (R^2 [R-squared] = 0.2123 and $P = 0.0030$; $R^2 = 0.2243$ and $P = 0.0026$, respectively). Oocyte ATP content was measured as an indirect indicator of lipolysis (i.e., mitochondrial fatty acid β-oxidation [beta-oxidation] of fatty acids freed during lipolysis of triglyceride/phospholipid for ATP production). The ATP content of oocytes increased during IVM and was greater in heat-stressed oocytes at 24 hIVM compared to controls ($P = 0.0082$). Levels of ATP were associated, though not directly related, to changes in oocyte triglyceride and phospholipid content ($R^2 = 0.1086$ and $P = 0.0184$; $R^2 = 0.1252$ and $P = 0.0096$, respectively). In summary, heat stress-induced hastening of oocyte germinal vesicle breakdown was not directly explained by lipolytic changes in triglycerides and phospholipids nor the oocyte ATP content.

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CHAPTER 1

INTRODUCTION

The impact of environmental heat stress on the dairy cattle industry is both a present and future concern. Economically, the US dairy cattle industry incurs losses of almost one billion dollars yearly due to production deficits resulting from heat stress including decreased rate of growth (West 2003), decreased milk production (Collier *et al.* 2008), and fertility (reviewed by Rensis & Scaramuzzi 2003; St-Pierre *et al.* 2003). Heat stress-induced decreases in fertility have been attributed to an unstable hyperthermic maternal environment, which may indirectly increase embryonic loss or directly impact the oocyte (reviewed by Rensis & Scaramuzzi 2003). Edwards and Hansen (1996) determined that some of the decreased fertility caused by heat-induced hyperthermia when occurring during estrus may stem from direct alterations to the oocyte as it undergoes meiotic maturation in preparation for fertilization.

Although the mechanism(s) underlying heat-induced perturbations occurring in the oocyte after resumption of meiosis remain largely unclear, multiple studies reported that exposure to a physiologically relevant heat stress hastens this process. Specifically, heat-stressed oocytes mature faster than non heat-stressed oocytes. Baumgartner and Chrisman (1981) reported that a greater proportion of murine oocytes were classified as bicellular (i.e., evidence of first polar body formation) after exposure to in vivo heat stress. In a different study, murine oocytes matured at an elevated temperature for a short time period in vitro showed evidence of accelerated germinal vesicle breakdown (GVBD; Kim *et al.* 2002). In the bovine, more heat-stressed oocytes reached metaphase I (MI) by 8 h of in vitro maturation (hIVM) and metaphase II (MII) by 18 hIVM than did non-stressed oocytes (Edwards *et al.* 2005). Meiotic hastening likely results in an aged oocyte at fertilization, which may not be without consequence, as

Rispoli et al. (2011) showed that fertilization of aged oocytes resulted in decreased blastocyst development similar to that seen in heat-stressed oocytes. In support of this notion, insemination of heat-stressed oocytes 4 to 6 h earlier improved blastocyst development (Edwards *et al.* 2005; Schrock *et al.* 2007).

Although it is unclear what heat-induced mechanism(s) serve to hasten the onset of meiotic maturation, in other cell types mild hyperthermia exposure increases mitogen activated protein kinase (MAPK) activity (reviewed by Park *et al.* 2005). Interestingly, when Fissore et al. (1996) microinjected bovine oocytes with M-mos sufficient to activate MAPK, the incidence of GVBD was higher. In addition to its role in promoting meiotic maturation, MAPK is also well-known for its role as a lipolytic driver in other cell types (Greenberg *et al.* 2001; Jaworski *et al.* 2007). Furthermore, lipolytic activity is increased in other cell types after exposure to elevated temperatures. Specifically, upon trigger of increased body temperatures in Malignant Hyperthermia-susceptible patients, fatty acid concentration in muscle cell homogenates was increased (Fletcher & Rosenberg 1986) most likely due to elevated triglyceride catabolism (Fletcher *et al.* 1989). Research has shown that triglyceride catabolism is actually an important occurrence during bovine oocyte IVM. Triglyceride content is significantly decreased (Ferguson & Leese 1999; Kim *et al.* 2001) concurrent with increased lipase activity (Cetica *et al.* 2002; Auclair *et al.* 2013) when comparing mature to immature bovine oocytes. Furthermore, when downstream lipid catabolism (fatty acid β-oxidation) is promoted, progression of nuclear maturation and embryonic development is increased (reviewed by Dunning & Robker 2012).

Depending upon the extent to which heat stress may be altering MAPK or other mechanisms to hasten GVBD, lipolysis may also be altered within the bovine oocyte. As a first step, we characterized the timing of GVBD within our IVM system to determine repeatability

and timeline agreement with previous research. In the second study, changes in triglyceride and phospholipid content were characterized during early IVM as an indirect measure of lipolysis to allow for direct comparison with incidence of GVBD. Heat stress was applied in the third study to assess the effects of elevated temperature on triglyceride and phospholipid changes in association with incidence of GVBD during early IVM. In a subset of oocytes, ATP content was evaluated to serve as an indirect measure of lipid catabolism in the oocyte (as mitochondria utilize fatty acid β-oxidation as a means to fuel ATP production). Furthermore, when measured at 24 hIVM ATP concentration is greater in heat-stressed oocytes (Nagle 2011).

CHAPTER 2

REVIEW OF LITERATURE

Introduction

The following is a literature review covering the impact of heat stress on bovine reproduction, focusing mostly on the effects of heat stress on the cumulus-oocyte complex during meiotic maturation. In an attempt to determine potential alterations which could induce a hastened nuclear maturation, this review also focuses on oocyte lipid content, lipid catabolism, and subsequent mitochondrial production of ATP.

Impacts of Environmental Heat Stress on the US Dairy Cattle Industry

The impact of environmental heat stress on the dairy cattle industry is both a present and future concern. Economically, the dairy industry incurs losses of approximately one billion dollars yearly due to production deficits which occur during the hot summer months experienced in the southern region of the country (Rensis & Scaramuzzi 2003; St-Pierre *et al.* 2003). Heat stress effects on cattle production is also of great concern, as by year 2050 the consumption of dairy products is expected to increase by 150 to 200% as the world's human population is predicted to expand by over three billion (McLeod 2011; U.S. Department of Agriculture 2012).

Cattle under the influence of heat stress experience hyperthermia, influencing multiple facets of production. In dairy cows, milk production is severely affected by increased environmental temperatures; the average decrease in milk production is about 1,100 kg/cow/year across the southern US (St-Pierre *et al.* 2003). Reproduction efficiency of dairy cows and heifers under the influence of heat stress suffers in that sexual receptivity and overall fertility coincidently decrease (reviewed by Hansen & Arechiga 1998; Drost *et al.* 1999). The pregnancy

rate of cattle is inversely related to ambient temperature, as rates are reduced by almost 20% when environmental temperatures increase by 10°C (reviewed by Hansen & Arechiga 1998).

Oocyte Maturation and Early Embryo Development are Affected by Elevated Temperature

Some of the reproductive losses caused by heat stress (i.e. induced hyperthermia) may stem from the alterations occurring during estrus, which is when the oocyte resumes meiotic maturation in preparation for fertilization (Putney *et al.* 1989). Though fertilization rate was unaffected, greater than 85% of embryos collected from superovulated heifers exposed to an elevated ambient temperature sufficient to induce hyperthermia for the first 10 hours of estrus (during the period of oocyte maturation) were retarded or abnormal (Putney *et al.* 1989). Heat stress seems to be able to impact the oocyte directly as both in vivo and in vitro matured oocytes show incidence of decreased embryonic development when heat-stressed during meiotic maturation. Specifically, Gendelman and Roth (2012) observed a 15% reduction in embryonic development (assessed at 42 h and 7–8 d post fertilization) from oocytes exposed to a hyperthermic maternal environment during the summer or to 41.2°C for the first 16 hours of in vitro maturation (hIVM). Decreased blastocyst development was also evident in bovine oocytes exposed to 41.0°C for the first 12 hIVM, then cultured at 38.5°C thereafter compared to non-stressed oocytes (Edwards & Hansen 1996, 1997; Lawrence *et al.* 2004) similar to the in vivo observations from Putney *et al.* (1989).

Organelle arrangement and morphology are altered in oocytes experiencing heat stress during this pertinent 24 h maturation period. When bovine oocytes are incubated at 41.0°C for the first 12 hIVM and 38.5°C for the second 12 h, there was a significant shift in cortical granule type (Edwards *et al.* 2005). Heat-stressed oocytes may also experience other organelle alterations like those found in rat fibroblast cells after experiencing heat shock for 3 hours at 42

to 43°C (Welch & Suhan 1985). Specifically, heat-shocked cells contained fragmented Golgi, swollen mitochondria, and alternatively arranged cytoskeletal elements (Welch & Suhan 1985).

Transcript abundance may also be altered in oocytes matured under heat stress conditions, both in vivo and in vitro (Payton 2009; Gendelman & Roth 2012). Payton (2009) heat stressed bovine oocytes for the first 12 hIVM at 41.0°C (transferred them to 38.5°C for the second 12 h), then completed a microarray analysis of the RNA present within those oocytes. A multitude of transcripts significantly differed in abundance between the two treatment groups (control versus heat stress). As at least 21 of these altered transcripts are important for proper mitochondrial function, it would seem that heat stress is capable of inducing mitochondrial dysfunction within the bovine oocyte. The developmental potential of oocytes is negatively impacted when mitochondrial function is disrupted (Thouas *et al.* 2004; Takeuchi *et al.* 2005). After a mere 5 seconds of photoirradiation to induce mitochondrial dysfunction, the percentage of murine oocytes progressed to MII after IVM was decreased by nearly 56% (Takeuchi *et al.* 2005). Blastocyst development was also decreased (by about 20%) when mitochondrial dysfunction was induced in murine oocytes by application of photoirradiation for 40 seconds before fertilization (Thouas *et al.* 2004).

Oocytes that experience elevated temperatures during meiotic maturation show evidence of an accelerated progression to MII. Approximately 30% of murine oocytes from superovulated mice exposed to heat stress conditions for 15.5 h after hCG injection were classified as bicellular (i.e. evidence of first polar body formation) compared to only 15% from the control mice (Baumgartner & Chrisman 1981). In vitro, when Kim et al. (2002) heat shocked murine oocytes for 15 or 30 min at 43°C, greater than 20% had undergone GVBD (assessed 1 h after application of heat shock) compared to 10% of control oocytes. Edwards et al. (2005) observed a greater

proportion of heat-stressed bovine oocytes, matured at 41°C for the first 12 hIVM (then at 38.5°C for the second 12 h), reach the MI stage as early as 8 hIVM compared to non-stressed oocytes. Also, a greater proportion of heat-stressed oocytes had undergone GVBD compared to controls when assessed at 8 hIVM (Edwards *et al.* 2005). Furthermore, a greater proportion of heat-stressed oocytes reached the MII stage by 18 hIVM than did non-stressed oocytes (Edwards *et al.* 2005). This hastening of maturation likely results in aged oocytes at the time of fertilization, which is not without consequence. Fertilization of non-stressed bovine oocytes (matured at 38.5°C) after 30 hIVM, effectively aging them by approximately 6 h, reduced blastocyst development similar to the reduction observed when heat-stressed oocytes (cultured at 41.0°C for the first 12 hIVM, then at 38.5°C thereafter) are fertilized at the physiologically relevant 24 hIVM (Rispoli *et al.* 2011). In support of this notion, Edwards *et al.* (2005) reported that performing IVF 5 h earlier, at 19 hIVM instead of at 24 hIVM, improved blastocyst development of heat-stressed oocytes (matured at 41.0°C for the first 12 h, then at 38.5°C thereafter) from about 17% (IVF at 24 hIVM) to 22% (IVF at 19 hIVM).

Lipid Content in Oocytes

The immature bovine oocyte contains approximately 57 ng of lipid (Ferguson & Leese 1999). When bovine oocytes are viewed under bright field microscopy, the cytoplasm appears dark due to the abundance of this lipid (Jeong *et al.* 2009; Barcelo-Fimbres & Seidel Jr 2011). The amount of lipid in an oocyte is species specific; both porcine and ovine oocytes contain more lipid than those of the bovine, averaging 160 ng, 89 ng, and 63 ng, respectively (McEvoy *et al.* 2000). Though, the three most abundant fatty acids which make up this lipid were the same in oocytes of all three species – palmitic, oleic, and stearic acids (McEvoy *et al.* 2000). In contrast, murine oocytes contain smaller amounts of lipid such that quantification is difficult.

Lowenstein and Cohen (1964) reported the lipid content of murine oocytes at approximately 4 ng.

Though the zona pellucida of an oocyte contains some lipid, the majority of lipid is housed within the oocyte's cytoplasm in the form of lipid droplets (Kruip *et al.* 1983; Ferguson & Leese 1999; Genicot *et al.* 2005). These droplets are distributed throughout the cytoplasm, and placement can vary between oocytes. An oocyte contained within a primordial follicle has only a few droplets, but numerous more develop as the follicle continues growth to the tertiary stage (Fair *et al.* 2007). Each of the droplets is chiefly neutral in charge due to a largely triglyceride-based composition (McEvoy *et al.* 2000; Genicot *et al.* 2005). Kim et al. (2001) reported triglyceride at 57 pmol, cholesterol at 16 pmol, phospholipid at 15 pmol, and non-esterified fatty acids at 11 pmol as the major fractions contained within lipid droplets in the immature bovine oocyte.

Composition of Oocyte Lipid

Lipids, such as triglyceride and phospholipid, are metabolically synthesized by cells using various fatty acids with other molecules made available to the cell such as glycerol or phosphate groups. The majority of the fatty acids present in the bovine oocyte are saturated, and there are four fatty acids which make up greater than 80% of the total fatty acid fraction (Khandoker *et al.* 1997; McEvoy *et al.* 2000). The four most abundant fatty acids, as determined by gas chromatography of immature bovine oocyte lipid fractions, are palmitic, stearic, oleic, and linoleic acids (Khandoker *et al.* 1997; McEvoy *et al.* 2000; Lapa *et al.* 2011).

These four fatty acids are also abundant in follicular fluid of follicles present on bovine ovaries (Leroy *et al.* 2005; Aardema *et al.* 2013). Concentrations of both palmitic and linoleic acid change during follicular growth (Homa & Brown 1992; Bender *et al.* 2010). Bender et al.

(2010) found palmitic acid concentrations to be significantly greater in fluid from subordinate follicles than dominant bovine follicles. Homa and Brown (1992) reported linoleic acid concentrations to be significantly greater in small bovine ovarian follicles compared to large follicles.

Supplementing in vitro maturation media with singular, or a mixture of, fatty acids affects bovine oocyte developmental competence. Researchers found linoleic acid to negatively impact bovine oocytes when supplemented in the maturation media. Marei et al. (2010) observed a significant decrease in cumulus cell expansion along with impaired oocyte maturation (specifically oocyte nuclear maturation to MII) when bovine oocytes were matured in maturation media supplemented with 50 µM, 100 µM and 200 µM linoleic acid. When Homa and Brown (1992) supplemented maturation media with 50 µM linoleic acid, they observed a significantly decreased percentage of COCs to undergo germinal vesicle breakdown (a very important step of the oocyte nuclear maturation process). Homa and Brown (1992) suggested that linoleic acid may be important for the maintenance of bovine oocyte meiotic arrest until the surrounding follicle is selected for dominance since follicular concentrations of linoleic acid decrease as the follicle becomes dominant (Marei *et al.* 2010; Renaville *et al.* 2010).

Studies researching the effects of other fatty acids on oocyte maturation have been performed. When bovine oocytes were matured in media supplemented with palmitic or stearic acid, post-fertilization development was significantly decreased compared with oocytes matured in control media (Leroy *et al.* 2005; Aardema *et al.* 2011). Though palmitic and stearic acid supplementation at 100, 250 or 500 µM did not seem to decrease the ability of bovine oocytes to mature to MII after 23 hIVM, it did decrease the amount of cumulus expansion during maturation (Aardema *et al.* 2011). Supplementing maturation media with 100, 250 or 500 µM

oleic acid did not affect bovine oocyte maturation to MII or cleavage of embryos 5 days post fertilization; however, the 500 µM concentration did increase the proportion of blastocysts developed from fertilized MII-stage oocytes (Aardema *et al.* 2011). When maturation media is supplemented with 50 µM linolenic acid, bovine oocytes contained greater concentrations of cAMPi after 3 hIVM and phosphorylated MAPK1/3 after 3 and 6 hIVM (Marei *et al.* 2009). Each of the aforementioned fatty acids and compounds are considered to be promoting or inhibiting factors for bovine oocyte maturation.

Changes in Lipid Content of Oocytes during In Vitro Maturation

The profile of intracellular lipid droplets changes over bovine oocyte maturation, specifically these droplets are degraded during maturation (Kruip *et al.* 1983). As triglyceride is the major constituent of these lipid droplets, Ferguson and Leese (1999) utilized a dual-reagent metabolic assay to measure triglyceride content in bovine oocytes (before IVM and after 24 hIVM) and even a variety of embryonic stages. Immature bovine oocytes contained 59 ng of triglyceride, which decreased significantly after 24 hIVM to 46 ng (Ferguson & Leese 1999). Triglyceride content significantly decreased once more when fertilized oocytes cleaved to 2-cells (34 ng) before plateauing throughout the rest of embryonic development to the hatched blastocyst stage (Ferguson & Leese 1999). Upon utilization of a colorimetric triglyceride assay, Kim et al. (2001) also reported immature bovine oocytes to contain more triglyceride than oocytes matured for 24 hIVM (measuring 57.6 pmol and 36.6 pmol, respectively). Though triglyceride levels decreased in bovine oocytes over IVM, this lipid fraction was still the most abundant in in vitro matured oocytes (Kim *et al.* 2001). Furthermore, Auclair *et al.* (2013) observed a decrease in bovine oocyte lipid content from 0 to 24 hIVM when using a lipid-specific fluorescent stain (Nile Red).

As the triglyceride levels within bovine oocytes and early embryos decrease during energetically taxing developmental stages, many researchers theorize that it is being metabolized to produce ATP (energy). Cetica et al. (2002) provided evidence for triglyceride metabolism in immature and in vitro matured bovine oocytes and their surrounding cumulus cells. Measurement of the rate-limiting enzymes of glycolysis, the pentose phosphate pathway, and lipolysis (phosphofructokinase, G6PDH and lipase, respectively) showed that while cumulus cells are most capable of utilizing glucose as an energy source, the oocytes themselves are most capable of utilizing lipid as an energy source (Cetica *et al.* 2002). Not only was lipase activity greater in oocytes than in cumulus cells, but specific activity was significantly increased in oocytes matured for 22–24 h compared to immature oocytes (Cetica *et al.* 2002). Though Cetica's research group did not try to determine the identity of the lipase(s) present in bovine oocytes, Auclair et al. (2013) detected inactive hormone sensitive lipase in oocytes before and after IVM for 22 h. The active (phosphorylated) form of hormone sensitive lipase was only detected after IVM (Auclair *et al.* 2013). At this time, it is unknown whether other types of lipase are present within the bovine oocyte.

Fatty Acid β-oxidation in Oocytes during In Vitro Maturation

In order to utilize stored lipid for energy production, the lipid must be hydrolyzed and processed through lipolysis followed by β-oxidation of the resultant fatty acids. Oocytes likely utilize this process as other cell types do, as evidenced by the decrease in triglyceride levels and concomitant increase in lipase levels through oocyte maturation. In fact, inhibition of fatty acid oxidation during IVM of oocytes negatively impacted their developmental competence (Ferguson & Leese 2006; Dunning *et al.* 2010; Paczkowski *et al.* 2013). Paczkowski et al. (2013) inhibited fatty acid β-oxidation in murine, bovine, and porcine oocytes by supplementing

the maturation medium with 10, 25, 100 and 250 µM etomoxir (a fatty acid oxidation-specific inhibitor). Meiotic maturation to MII was inhibited in murine oocytes at the 250 µM concentration, bovine oocytes at the 100 and 250 µM concentrations, and porcine oocytes at the 10, 100 and 250 µM concentrations of etomoxir (Paczkowski *et al.* 2013). Cleavage to the 4 to 8-cell stage by Day 3 and blastocyst formation by Day 5 after fertilization of murine oocytes matured in the presence of 100 µM etomoxir was also decreased (Dunning *et al.* 2010). When bovine oocytes were matured for 24 h in maturation media supplemented with 1.0 or 5.0 mM methyl palmoxirate (an inhibitor of mitochondrial beta-oxidation), development to the blastocyst stage after fertilization was reduced from 21.6% in controls to 14.0 and 6.75%, respectively (Ferguson & Leese 2006). Furthermore, promotion of oocyte fatty acid β-oxidation by L-carnitine supplementation of the maturation media improved embryonic development after fertilization (reviewed by Dunning & Robker 2012).

Mitochondrial Translocation and ATP Production during Oocyte Maturation

During oocyte maturation mitochondria translocate within the cytoplasm. Between 0 and 8 h of maturation within the follicle, mitochondrial organization within the cytoplasm of oocytes changed from a seemingly random distribution to aggregation around lipid droplets with close association to portions of smooth endoplasmic reticulum (Kruip *et al.* 1983). Mitochondrial aggregation occurs similarly in in vitro matured bovine oocytes (Kątska-Książkiewicz *et al.* 2011).

Mitochondrial respiration is indicative of ATP production; thus as respiratory activity increases, so should the production of ATP. The respiratory activity of mitochondria was greater (Kątska-Książkiewicz *et al.* 2011), as was the concentration of ATP in bovine oocytes matured for 24 h (Machatkova *et al.* 2012) compared to immature oocytes before IVM. These results are

similar to those of other studies measuring the ATP content of bovine oocytes. When Stojkovic et al. (2001) measured ATP content in bovine oocytes before maturation for any length of time as well as after IVM, concentration increased from 1.8 pmol/oocyte to 2.4 pmol/oocyte. Iwata et al. (2011) also observed greater ATP concentrations in bovine oocytes after IVM compared to immature oocytes (1.2 and 2.0 pmol/oocyte, respectively).

Impact of Elevated Temperature on Oocyte Energy Production

The concentration of ATP in bovine oocytes is increased even more so if oocytes are matured at an elevated temperature (Nagle 2011). After maturation at 41.0°C during the first 12 hIVM and 38.5°C for the remaining 12 h, heat-stressed oocytes contained 2.01 pmol ATP/oocyte as compared to the 1.65 pmol/oocyte in oocytes matured at 38.5°C for the entire 24 h maturation period (Nagle 2011).

Though no previous research has been conducted on the effects of elevated temperature on fatty acid β-oxidation or lipolytic activity in oocytes, heat stress has been observed to alter lipolysis in other cell types. Lipolytic activity is increased in skeletal muscle cell homogenates of patients with Malignant Hyperthermia, a genetic disease causing a rapid increase in body temperature (fever) when administered any sort of general anesthetic (Fletcher & Rosenberg 1986). The increased release of fatty acids in the muscle cells of these patients is likely resultant of enhanced triglyceride turnover (Fletcher *et al.* 1989). In adipocytes, lipolysis is increased through procedures such as Laser Body Sculpting; a technique that claims to "melt away fat" where a laser is applied to a fatty area of the body in order to increase adipocyte temperature to approximately 41.0°C (Weiss 1996). Thus, it is possible that heat stress may also increase the rate of lipolysis in oocytes.

CHAPTER 3

MATERIALS AND METHODS

Unless otherwise stipulated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Collection and In Vitro Maturation of Oocytes

In general, the methods used to collect and mature bovine oocytes in vitro were as described previously by Edwards et al. (2005). Oocyte collection medium was prepared using M199 with Hank's salts (Mediatech; Manassas, VA, USA) containing 12.5 mM HEPES, and 4.2 mM sodium bicarbonate, 1 – 2% standard fetal bovine serum (Atlanta Biologicals; Lawrence, GA, USA), 1% L-glutamine, and 0.5% Penicillin/Streptomycin. Depending on study, approximately 35 to 50 cumulus-oocyte complexes (COCs) were randomly grouped for maturation at 38.5°C and/or 41.0°C (heat-stressed COCs were transferred to 38.5°C after the first 12 hIVM) for up to 24 h. Oocyte maturation medium was prepared using M199 with Earle's salts (Gibco, Life Technologies; Grand Island, NY, USA) with 26.2 mM sodium bicarbonate, and supplemented to contain 10% premium fetal bovine serum (Biowhittaker; Walkersville, MD, USA), 50 µg/mL gentamicin (Biowhittaker), 5 µg/mL FSH (Bioniche; Belleville, Ontario, Canada), 0.2 mM sodium pyruvate, and 2 mM L-glutamine (Schrock *et al.* 2007).

Either immediately before placement into maturation media (0 hours), or some time thereafter, a subset of COCs was removed from culture and denuded completely of cumulus. For the 0 to 12 h groups, oocytes were denuded by vortexing in HEPES-TALP (Sirard *et al.* 1988); whereas COCs from the 24 h groups were vortexed in HEPES-TALP containing 0.3 mg/mL of hyaluronidase. Successfully denuded oocytes were fixed in 3% paraformaldehyde-Dulbecco's

Phosphate Buffered Saline (DPBS, without $CaCl_2$ or $MgCl_2$) for 1 h at room temperature, protected from light.

Study One: Timing of Germinal Vesicle Breakdown in Bovine Oocytes Undergoing In Vitro Maturation at 38.5°C

The timing of GVBD was assessed by evaluating oocytes every 2 h during the first 12 hIVM. Oocytes were determined to have undergone GVBD if the germinal vesicle (GV) was no longer detectable and the nuclear material was in a condensed chromatin (CC) configuration or at MI. Fixed oocytes were stained with Hoechst 33342 before mounting to a slide under a coverslip. Nuclear stage of individual oocytes was determined using fluorescence (excitation 330 – 380/emission ≥ 420) on a Nikon TE300 Inverted Fluorescent microscope. Study One was conducted over seven days of oocyte collection using 1,325 oocytes in total (131 to 389 oocytes per each time period examined).

Study Two: Timing of Lipolytic Changes and GVBD in Bovine Oocytes Undergoing In Vitro Maturation at 38.5°C

Lipolysis was evaluated by examining triglyceride and phospholipid content in COCs cultured for 0, 2, 4, 6 or 24 hIVM as modified from Genicot et al. (2005) and Auclair et al. (2013). Fixed oocytes were washed in HEPES-TALP before incubation in 0.2 µg/mL Nile Red fluorescent lipophilic stain in 1% PVP-DPBS for 2 h at room temperature, protected from light. Stained oocytes were washed in 1% PVP-DPBS and then transferred in groups of ten per 100 µL DPBS-PVP into separate wells of a 96-well black microplate with a transparent bottom (Thermo Scientific Nunc – Thermo Fisher Scientific; Rochester, NY, USA). Fluorescent readings were obtained using a Synergy H1 microplate reader (BioTek Instruments, Inc., VT, USA) at two fluorescent settings: excitation 485/emission 588 (triglyceride) and excitation 549/emission 628

(phospholipid; Greenspan *et al.* 1985; Kimura *et al.* 2004). Once measurements were obtained, background fluorescence was subtracted and the corrected value from each well was divided by the number of oocytes in said well to determine the arbitrary fluorescent units (A.F.U.) per oocyte. After fluorescence was recorded, oocytes were removed from the 96-well microplate and Hoechst stained to determine nuclear stage as previously described. Study Two was replicated over six oocyte collection days using 1,237 oocytes in total (the total number of pools per each of the five treatment groups ranged from 17 to 25).

Study Three: Lipolytic Changes, GVBD and ATP Content during In Vitro Maturation of Bovine Oocytes at 38.5 and 41.0°C

Triglyceride and phospholipid content of control and heat-stressed oocytes was assessed at 0, 2, 4, 6 and 24 hIVM as previously described. Each plate was read ten times instead of once, and these values were averaged separately for each well to better control for variability. After the average fluorescence was recorded, oocytes were transferred from the 96-well microplate and prepared for nuclear stage assessment using Hoechst 33342.

Concurrent with efforts described above, ATP content was measured in a small subset of oocytes taken before fixation from each treatment group at five different time points (0, 2, 4, 6 and 24 and cultured at 38.5°C and 41.0°C). First, oocytes were denuded of surrounding cumulus cells and the zona pellucida was removed using 0.5% pronase. Oocytes were then transferred individually to microcentrifuge tubes, lysed in sterile water, and stored at -80°C. Oocyte lysates were assessed for ATP content using the ATP determination kit from Invitrogen (Division of Life Technologies; Carlsbad, CA, USA) and a tube-based luminometer (Berthold, Huntsville, AL, USA) set to read the sample for ten seconds after a three second hold-time. The total amount of ATP in each oocyte lysate was determined using a standard curve ranging from 0 to

10 pmol. Study Three was replicated over six oocyte collection days using 2,680 oocytes for lipid analysis (14 to 16 pools per each of nine treatment groups) and 270 for ATP analysis (21 to 39 oocytes per treatment group).

Statistical Analyses

Nuclear maturation data from Study One is presented as raw values to show variability among different batches of bovine oocytes collected on different days. Data from Studies Two and Three were analyzed as a randomized block design using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA), blocking on date of oocyte collection. The Study Two dependent variables were lipid data (triglyceride, phospholipid, or the triglyceride to phospholipid ratio) and nuclear maturation data (GVBD). Maturation time (0, 2, 4, 6 and 24 hIVM) was the fixed effect for Study Two. Study Three dependent variables included lipid data (triglyceride, phospholipid, or the triglyceride to phospholipid ratio), nuclear maturation data (GV, CC, MI, Anaphase I (AI), Telophase I (TI), MII or GVBD), and ATP data. Maturation time (0, 2, 4, 6 and 24 hIVM) and maturation temperature (38.5°C and 41.0°C) were the fixed effects for Study Three. The experimental unit for all data analyses was the 4-well Nunc plate in which the oocytes were housed during IVM, as treatments were applied to a "plate" rather than individual oocytes. Treatment differences from all analyses were determined using F-protected least significant differences and reported as least squares means ± standard error of the mean (SEM).

When analyzing the association of nuclear maturation to lipolytic changes during IVM, GVBD served as the dependent variable. The fixed effect for Study Two was lipid fluorescence, and the fixed effects for Study Three were lipid fluorescence and maturation temperature. When

analyzing the association of ATP content in oocytes to lipolytic changes during IVM, ATP was the dependent variable. The fixed effects were lipid fluorescence and maturation temperature.

CHAPTER 4

RESULTS

Study One: Timing of Germinal Vesicle Breakdown in Bovine Oocytes Undergoing In Vitro Maturation at 38.5°C

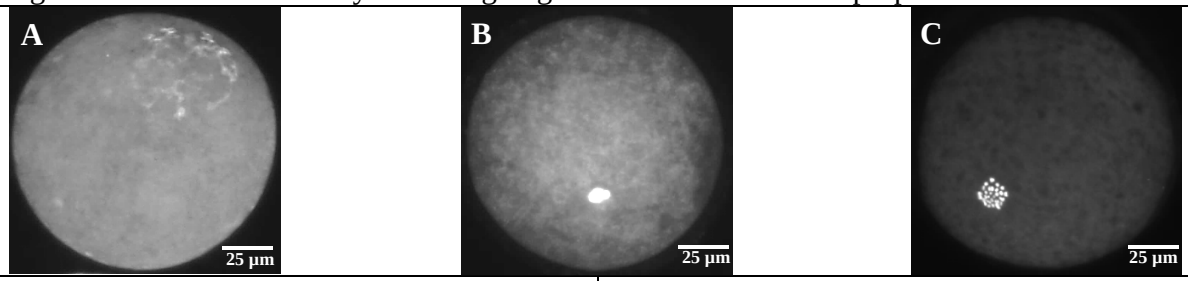
Either immediately before placement into maturation media or after 2 hIVM, the GV was present in 100% of oocytes (Table 4.1). The proportion of oocytes with an intact GV decreased progressively thereafter. Condensed chromatin was noticeable after 4 hIVM. Progression to MI was observed by 6 hIVM in a small subset of oocytes, and by 12 hIVM the majority of oocytes were at this nuclear stage.

Study Two: Timing of Lipolytic Changes and GVBD in Bovine Oocytes Undergoing In Vitro Maturation at 38.5°C

Triglyceride content per oocyte was greatest at 0 hIVM ($P < 0.0001$; Figure 4.1, panel A). By 2 hIVM, triglyceride levels decreased and levels were even lower at 24 hIVM ($P < 0.0001$; Figure 4.1, panel A). Similar to the triglyceride content, phospholipid content per oocyte was greatest at 0 hIVM, decreased by approximately half after 2 hIVM, and then decreased further by 6 hIVM ($P < 0.0001$; Figure 4.1, panel B). Independent of IVM time, triglyceride levels were almost double that of phospholipid (283.14 vs 156.56; SEM = 21.35) resulting in a ratio of 1.81 to 1 ($P = 0.4201$).

All oocytes at 0 hIVM had an intact GV, and the percentage of oocytes having an intact GV decreased by 4 hIVM ($P = 0.0500$; Figure 4.1, panel C). Chromatin condensation was evident in almost all of the oocytes without an intact GV at 4 hIVM. Prevalence of GVBD in oocytes increased again after 6 hVIM ($P = 0.0500$; Figure 4.1, panel C); less than 1% had reached the MI stage and the remainder had condensing chromatin. Oocytes after 24 hIVM were

Table 4.1. Timing of GVBD in bovine oocytes undergoing IVM at 38.5°C and the proportion at MI.



hIVM	Germinal Vesicle Breakdown (%)							Metaphase I (%)						
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	-	-	0	0	0	0	0	0	0
4	8.0	3.2	0	0	0	-	-	0	0	0	0	0	-	-
6	21.2	6.3	16.7	11.9	13.8	-	-	3.0	3.1	4.8	1.7	0	-	-
8	-	63.3	64.5	71.4	74.1	75.0	93.2	-	20.0	19.4	30.1	37.0	42.9	43.2
10	-	-	-	-	93.8	100	92.9	-	-	-	-	65.5	93.0	64.3
12	-	-	-	-	93.1	100	100	-	-	-	-	81.3	100	95.6
Rep. No.	1	2	3	4	5	6	7	1	2	3	4	5	6	7

GVBD = Germinal Vesicle Breakdown
MI = Metaphase I
hIVM = hours of in vitro maturation
Images: **A.** Oocyte with intact germinal vesicle (GV), **B.** oocyte with condensing chromatin (CC), and **C.** oocyte at MI; scale bar = 25 µm
– = did not record for these time periods for a given replicate
Rep. No. = each number represents a different date of oocyte collection

Figure 4.1. Triglyceride (**Panel A**) and Phospholipid content (**Panel B**) (average fluorescence units (A.F.U.) ± SEM) in bovine oocytes matured in vitro to 0, 2, 4, 6 or 24 hIVM. **Panel C**: Percentage of bovine oocytes at 0, 2, 4, 6 or 24 hIVM in which the germinal vesicle was no longer intact. $^{A-D}$Letters within a panel are indicative of differing means at $P < 0.05$.

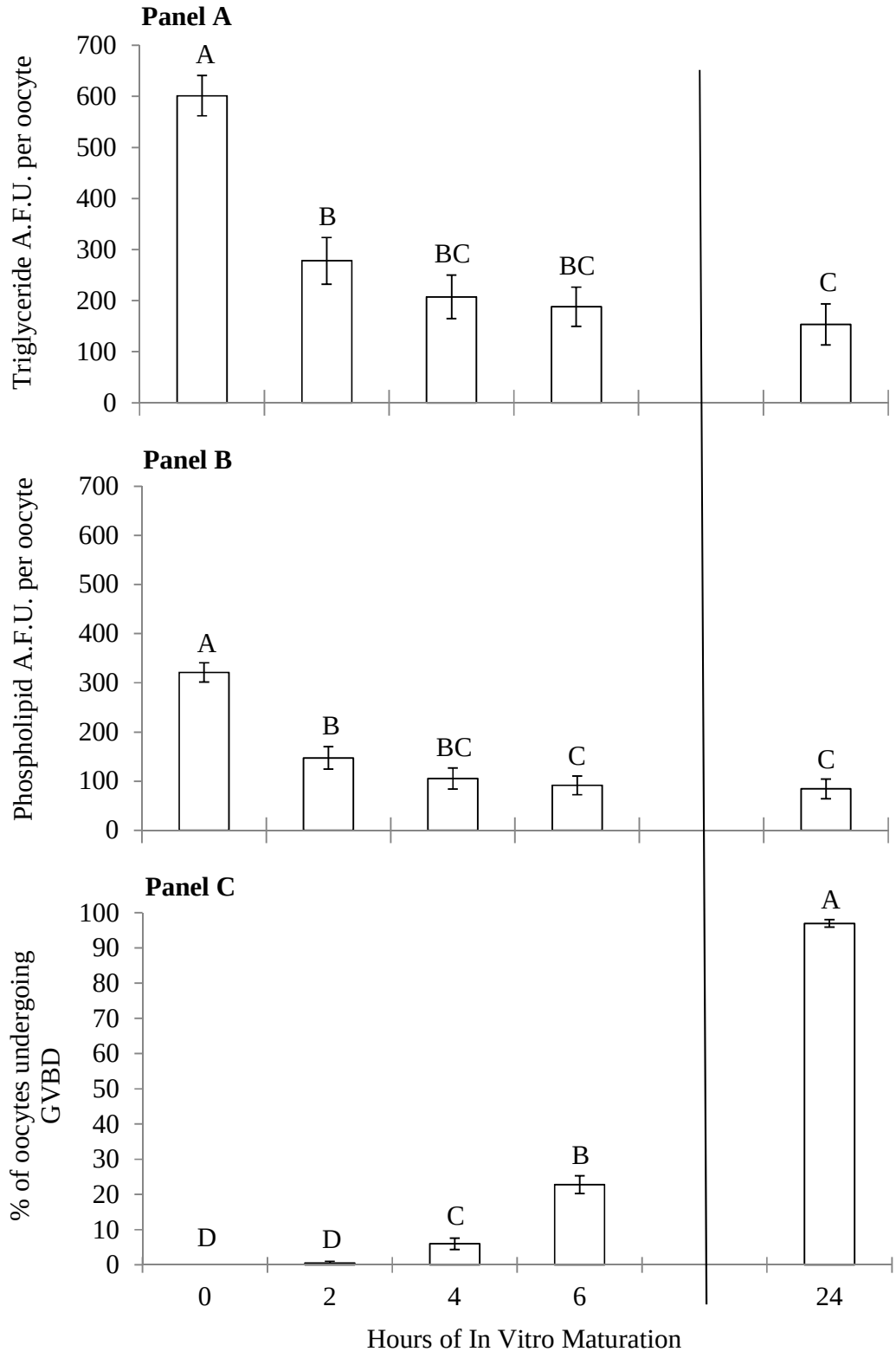


Figure 4.1. Cont.

predominantly at the MII stage (> 85%).

Lipolytic changes in triglyceride (R^2 = 0.2477; P = 0.0095) and phospholipid (R^2 = 0.2335; P = 0.0121) were associated with GVBD in bovine oocytes undergoing meiotic maturation (Figure 4.2). For example, when lipid content was typically high at 0 hIVM there was no evidence of GVBD; however, when lipid content was low at 24 hIVM incidence of GVBD was high (Figure 4.2).

Study Three: Lipolytic Changes, GVBD and ATP Content during In Vitro Maturation of Bovine Oocytes at 38.5 and 41.0°C

Triglyceride content decreased by 2 hIVM in control and heat-stressed oocytes (P = 0.0009; SEM = 40.23). Independent of IVM temperature, triglyceride levels did not change from 2 to 6 hIVM; however, content was decreased by 24 hIVM (P = 0.0003; Figure 4.3, panel A). Application of heat stress did not alter triglyceride content during IVM (P = 0.9198; Figure 4.3, panel A).

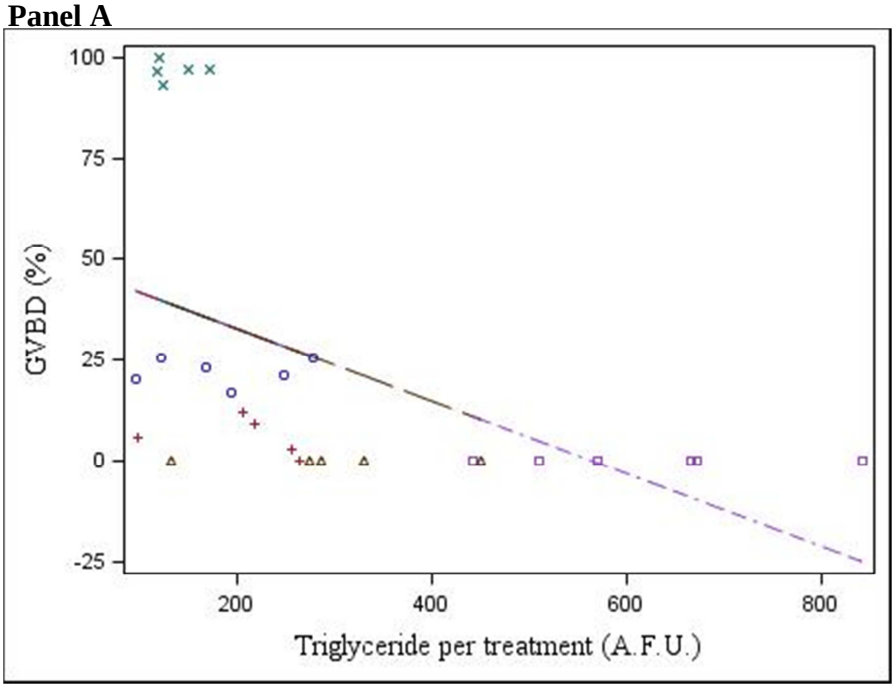
Phospholipid content also decreased by 2 hIVM in control and heat-stressed oocytes (P = 0.0005; SEM = 21.54). Independent of IVM temperature, phospholipid levels did not change from 2 to 6 hIVM; however, content was decreased by 24 hIVM (P < 0.0001; Figure 4.3, panel B). Heat stress exposure did not alter phospholipid content during IVM (P = 0.7861; Figure 4.3, panel B).

Triglyceride levels were almost double that of phospholipid (184.82 vs 93.33; SEM = 17.42) resulting in a ratio of 1.98 to 1 (P = 0.1841). Neither IVM time nor temperature affected this ratio (P = 0.3927).

There was a significant interaction of IVM temperature X time when evaluating the ability of oocytes to undergo GVBD (P < 0.0001; Table 4.2). The proportion of oocytes without

Figure 4.2. Relationship of triglyceride (**Panel A**) and phospholipid content (**Panel B**) (average fluorescence units (A.F.U.) ± SEM) to GVBD (%) in bovine oocytes matured in vitro at 38.5°C (R^2 = 0.2477 and P = 0.0095 for triglyceride; R^2 = 0.2335 and P = 0.0121 for phospholipid). Symbols correspond to different time points of IVM: □ = 0, Δ = 2, + = 4, ○ = 6 and x = 24.

Panel A



Panel B

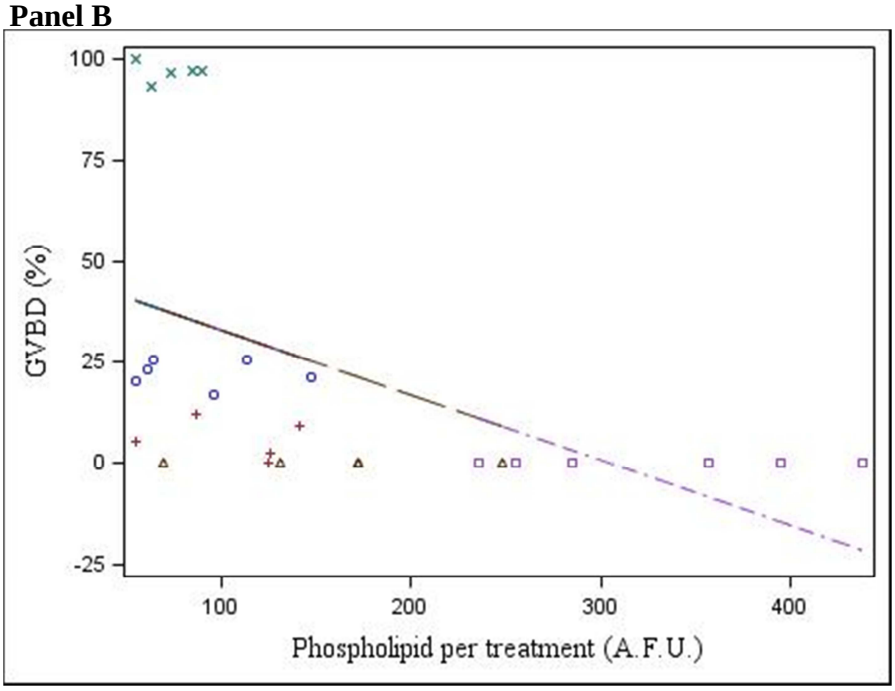


Figure 4.2. cont.

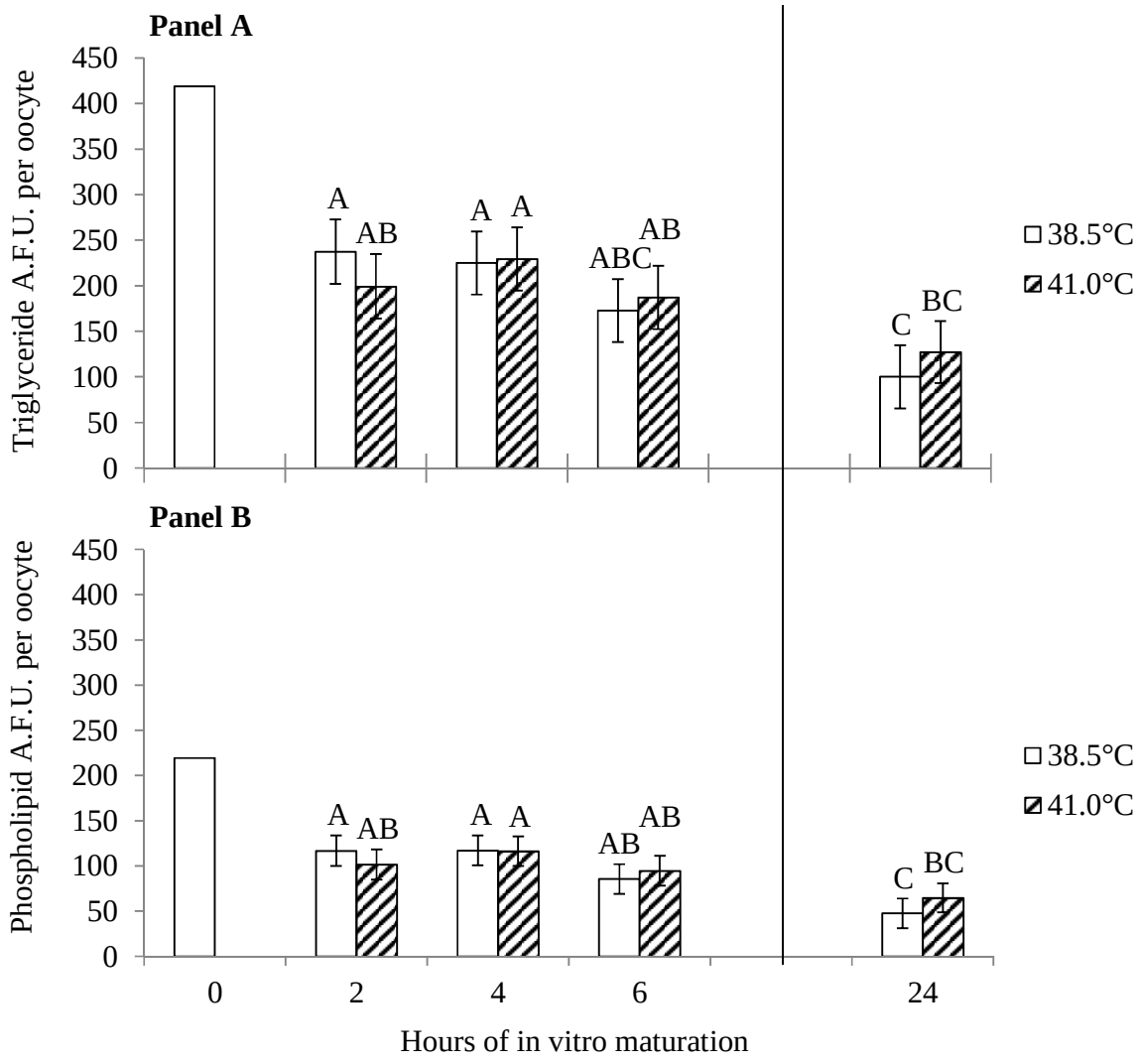


Figure 4.3.: Triglyceride (**Panel A**) and phospholipid content (**Panel B**) (average fluorescence units (A.F.U.) ± SEM) in bovine oocytes matured in vitro to 2, 4, 6 or 24 h at 38.5 or 41.0°C (heat stress exposure during the first 12 h of in vitro maturation only). $^{A-D}$Letters within a panel are indicative of differing means at $P < 0.05$. 0 h time point provided for visual comparison, but was not included in factorial treatment arrangement.

an intact GV was not different between control and heat stress treatments at 2 hIVM (Table 4.2). At 4 hIVM, fewer heat-stressed oocytes had an intact GV than did control oocytes (Table 4.2). A greater proportion of heat-stressed oocytes had undergone GVBD at 6 hIVM than had control oocytes as well (Table 4.2). However, by 24 hIVM there was again no difference in the proportion of heat-stressed and control oocytes to undergo GVBD (Table 4.2).

Lipolytic changes in triglyceride (R^2 = 0.2123; P = 0.0030) and phospholipid (R^2 = 0.2243; P = 0.0026) levels were associated with incidence of GVBD in bovine oocytes undergoing meiotic maturation (Figure 4.4). For instance, when lipid content was typically high at 0 hIVM, there was no evidence of GVBD; however, when lipid content was low at 24 hIVM, GVBD was high. The relationship of lipolytic change to GVBD was not influenced by IVM temperature (P = 0.5925 for triglyceride and P = 0.5041 for phospholipid; Figure 4.4).

There was a significant IVM temperature X time effect on oocyte ATP content (P = 0.0082; Figure 4.5). Oocyte ATP content was not different in control and heat-stressed oocytes matured for 2 to 6 h after placement in maturation media. At 24 hIVM, ATP content was increased, with heat-stressed oocytes containing more ATP than controls (P = 0.0500; Figure 4.5).

Lipolytic changes in triglyceride (R^2 = 0.1086; P = 0.0184) and phospholipid (R^2 = 0.1252; P = 0.0096) were associated with ATP content in bovine oocytes undergoing meiotic maturation. For example, when lipid content was typically high at 0 hIVM, ATP content was low; however, when lipid content was low at 24 hIVM, ATP content was high. This association was not influenced by IVM temperature (P = 0.2589 for triglyceride and P = 0.2069 for phospholipid).

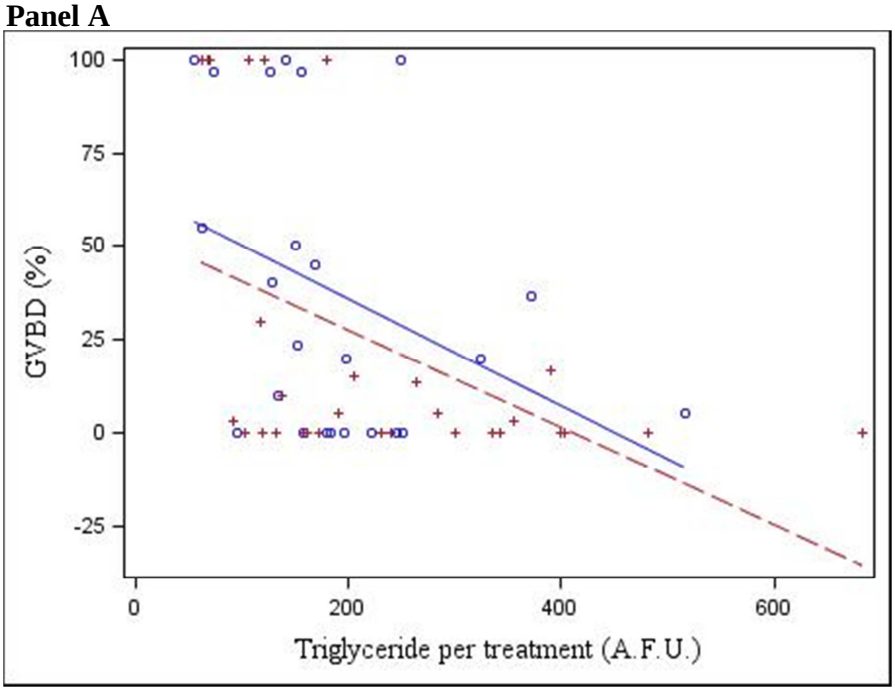
Table 4.2. Meiotic progression of bovine oocytes undergoing IVM at 38.5 or 41.0°C

Treatment Combinations		Nuclear Stage (%)						
hIVM[1]	Temperature (°C)	GV[2]	CC[3]	MI[4]	GVBD[5]	AI[6]	TI[7]	MII[8]
2	38.5	99.26[a]	0.73[d]	0[b]	0.74[d]	-	-	-
2	41.0	100.00[a]	0[d]	0[b]	0[d]	-	-	-
4	38.5	95.40[a]	4.63[cd]	0[b]	4.59[d]	-	-	-
4	41.0	88.76[b]	11.27[b]	0[b]	11.24[c]	-	-	-
6	38.5	88.76[b]	9.94[bc]	1.27[b]	11.24[c]	-	-	-
6	41.0	58.76[c]	36.60[a]	4.60[a]	41.24[b]	-	-	-
24	38.5	0.14[d]	0[d]	4.75[a]	99.85[a]	2.07[a]	2.09[a]	91.06[a]
24	41.0	0.65[d]	0[d]	5.18[a]	98.07[a]	0.65[a]	0.64[a]	91.59[a]

[a – d] means differ within a column ($P < 0.05$).
[1]hIVM = hours of in vitro maturation
[2]GV = Germinal Vesicle stage
[3]CC = Condensed Chromatin
[4]MI = Metaphase I stage
[5]GVBD = Germinal Vesicle Breakdown
[6]AI = Anaphase I stage
[7]TI = Telophase I stage
[8]MII = Metaphase II stage
– = nuclear stage not present at these time periods

Figure 4.4. Relationship of triglyceride (**Panel A**) and phospholipid content (**Panel B**) (average fluorescence units (A.F.U.) ± SEM) to GVBD (%) in bovine oocytes matured in vitro at 38.5 or 41.0°C (heat stress exposure during the first 12 h of in vitro maturation only) (R^2 = 0.2123 and P = 0.0030 for triglyceride; R^2 = 0.2243 and P = 0.0026 for phospholipid). Symbols correspond to different IVM temperatures: + = 38.5°C and ○ = 41.0°C.

Panel A



Panel B

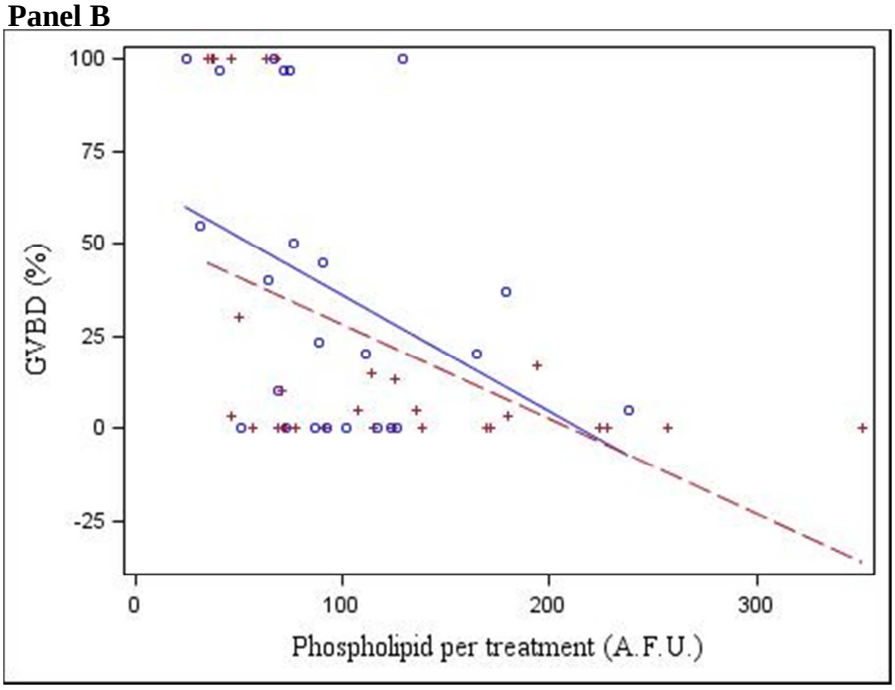


Figure 4.4. cont.

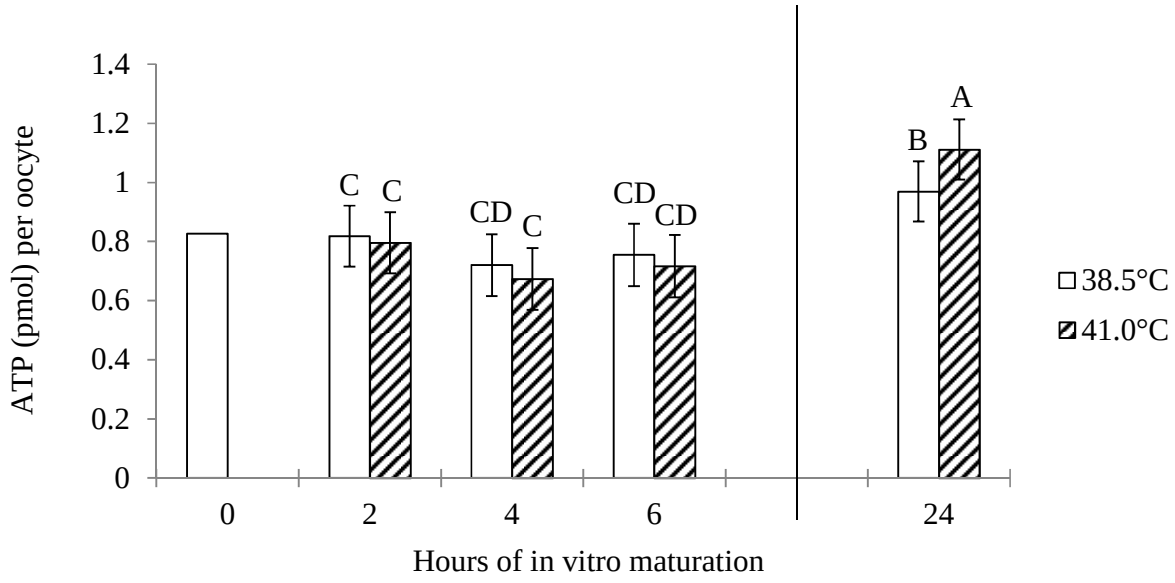


Figure 4.5. ATP content (pmol ± SEM) in bovine oocytes matured in vitro to 2, 4, 6 or 24 h at 38.5 or 41.0°C (heat stress exposure during the first 12 h of in vitro maturation only). In vitro maturation temperature X time interaction; P = 0.0082; SEM = 0.10. $^{A-D}$Letters within a panel are indicative of differing means. 0 h time point provided for visual comparison, but was not included in factorial treatment arrangement.

CHAPTER 5

DISCUSSION

This study confirmed that exposure of bovine oocytes to heat stress hastens meiotic maturation, as incidence of GVBD was higher in heat-stressed oocytes by 4 and 6 hIVM compared to controls. We also observed a marked decrease in oocyte triglyceride and phospholipid levels by 2 hIVM, after which lipid levels decreased again at 24 hIVM. Maturation at an elevated temperature did not alter this lipolytic pattern. Both prevalence of GVBD and oocyte ATP content were associated with lipolytic changes of triglyceride and phospholipid regardless of maturation temperature. Application of heat stress during the first 12 hIVM, however, increased ATP content of oocytes at 24 hIVM.

Heat-stressed oocytes undergo GVBD earlier than control oocytes; this effect was first evident at 4 hIVM and was even more pronounced at 6 hIVM. Our findings extend beyond what has been previously reported for heat stress-induced hastening of bovine oocyte meiotic maturation; Edwards *et al.* (2005) did not observe differences in the proportion of oocytes with an intact GV between control and heat-stress treatments at 4 hIVM. The results herein further support the notion that heat-stressed oocytes may be aged at the time of fertilization due to an accelerated rate of meiotic maturation, thus explaining some of the reduction in developmental competence of heat-stressed oocytes (Edwards *et al.* 2005; Schrock *et al.* 2007; Rispoli *et al.* 2011).

Heat stress-induced hastening of GVBD was not related to lipolytic changes of triglyceride and phospholipid as measured herein. However, this does not preclude lipolysis from being a prerequisite for GVBD given that stimulation of downstream fatty acid β-oxidation (fatty acids derived from lipolysis of triglyceride are converted by mitochondria into ATP)

promotes progression to MII (reviewed by Dunning & Robker 2012). Furthermore, inhibition of fatty acid β-oxidation during IVM in bovine, porcine, and murine oocytes decreased the proportion that progressed to MII (Paczkowski *et al.* 2013).

The marked decline in triglyceride and phospholipid levels by 2 hIVM was unexpected. Previous researchers measured lipid at 0 hIVM and after 22 to 24 hIVM and theorized that the decline was gradual (Ferguson & Leese 1999; Kim *et al.* 2001; Auclair *et al.* 2013). Similar to those studies, the triglyceride content of oocytes matured for 24 h herein was significantly decreased compared to immature oocytes. The significance for the bovine oocyte to decrease lipid content by almost half within the first 2 hIVM is unknown. However, during maturation the oocyte is tasked with quickly undergoing important nuclear and cytoplasmic changes in preparation of becoming a zygote after fertilization. This may include a decrease in lipid content such that the oocyte would more closely resemble the functionality of a zygote, as triglyceride content significantly decreases after fertilization when the zygote begins cleaving (Ferguson & Leese 1999). Another possible role of rapid lipid breakdown during the first 2 hIVM may be to release important proteins or histones required for oocyte meiotic maturation. Bovine cumulus oocyte-complexes require a 1 to 2 h transcriptional phase once maturation is initiated to synthesize necessary proteins for driving meiosis (reviewed by Hyttel *et al.* 1997), and greater than 60% of total oocyte/embryonic histones were detected in association with oocyte lipid droplets in Drosophila (Cermelli *et al.* 2006). Further evaluation has determined that associated histones are capable of translocation to the nucleus for packaging and regulation of DNA transcription, and these became unavailable for use in transcriptional regulation if lipid droplets did not properly redistribute during oocyte and early embryonic development (Cermelli *et al.* 2006).

Although our study and others have reported that oocyte triglyceride and phospholipid levels decreased during IVM, it is unknown where or how the fatty acids released from the breakdown of these lipids are utilized. As ATP content did not differ within the first 6 hIVM, it does not appear that the oocyte itself is oxidizing the fatty acids during early maturation. However, when lipolytic changes are most pronounced bovine oocytes are intimately associated with the surrounding cumulus cells (Hyttel *et al.* 1986). Intimate associations via gap junction-complexes allows for a bidirectional flow of signals and metabolites between the oocyte and the cumulus (reviewed by Eppig 1991). Though cumulus cells were not evaluated as a part of our study, we cannot preclude the potential for these cells to receive, and possibly utilize, the by-products released from lipolytic breakdown occurring in the oocyte. Auclair *et al.* (2013) reported that lipid droplet breakdown was greater in bovine oocytes matured with intact cumulus cells compared to those matured without surrounding cumulus. Furthermore, fatty acids are commonly packaged into vesicles to allow for transportation to membrane surfaces in other cell types (Winawer 2006), and Kruip *et al.* (1983) observed small vesicles surrounding many of the cumulus cell processes in bovine oocytes during early maturation.

Nonetheless, fatty acid β-oxidation is important for oocyte maturation and embryo development after fertilization (Ferguson & Leese 2006; Dunning *et al.* 2010; Paczkowski *et al.* 2013). Depending upon the extent to which the fatty acids are utilized by the mitochondria present within the oocyte, subsequent production of ATP could be increased. In fact, we observed greater ATP content in oocytes matured for 24 hIVM than at 0 hIVM, which agreed with other studies (Stojkovic *et al.* 2001; Iwata *et al.* 2011).

Interestingly, ATP content in bovine oocytes exposed to heat stress during the first 12 hIVM was greater at 24 hIVM when compared to non-stressed controls. This finding is similar

to results previously reported by our laboratory (Nagle 2011). Elevated ATP content has been observed in other cell types after application of elevated temperatures, and some researchers theorize that this greater ATP availability may be a marker of cellular stress (reviewed by Streffer 1985). Specifically, it may indicate a certain level of mitochondrial dysfunction, which is further supported by the observed alteration of mitochondrial transcript abundance in heat-stressed oocytes (Payton 2009). Reasons for altered mitochondrial ATP production in stressed cells include a potentially greater energy (ATP) requirement as they must combat stress-related apoptosis (reviewed by Streffer 1985) or possible alteration of metabolic pathways resulting in a surplus of unused ATP (reviewed by Welch 1992).

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VITA

Leah Marie Hooper was born in Atlanta, Georgia. She graduated from Riverdale High School in Murfreesboro, TN in 2008. She attended the University of Tennessee at Knoxville, from which she graduated with a B.S. in Animal Science and Biology minor in May of 2012. She began her M.S. with Dr. Lannett Edwards the following semester after having worked in her laboratory for a little over two years as an undergraduate student. In December of 2014, Leah graduated with a M.S. degree in Animal Science with a concentration in reproductive physiology and embryology.