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To the Graduate Council:

I am submitting herewith a thesis written by Julia Rowinski entitled "Impact of an Acute Heat Shock During In Vitro Maturation on Interleukin 6 and its Associated Receptor Component Transcripts In Bovine Cumulus-Oocyte Complexes." 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:

F. Neal Schrick, Kyle McLean

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

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

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Impact of an Acute Heat Shock During In Vitro Maturation on Interleukin 6 and its Associated Receptor Component Transcripts In Bovine Cumulus-Oocyte Complexes

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Julia Rose Rowinski

December 2020

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ABSTRACT

An acute heat stress event after LH surge increased intrafollicular interleukin 6 (IL6) levels in cows that became hyperthermic. To examine direct consequences of the physiologically-relevant elevated temperature of 41.0ºC on the cumulus-oocyte complex (COC), *IL6* transcript abundance and related receptor components were evaluated throughout *in vitro* maturation. Because IL6 and LIF share a signal transducer, *LIF* was also examined. Abundance of *IL6* was interactively influenced by temperature and time ($P < 0.0001$). Heat-induced increases in *IL6* were first noted at 4 hIVM; peak levels occurred at 4.67 versus 6.44 hIVM for 41.0 and 38.5ºC COCs, respectively (SEM = 0.23; $P < 0.001$). Abundance of *IL6R* ($P = 0.05$) and *IL6ST* ($P = 0.002$) were also interactively influenced by temperature and hIVM. Peak *IL6ST* levels occurred at 6.95 versus 8.29 hIVM for 41.0 and 38.5ºC, respectively (SEM = 0.23; $P < 0.01$). Transcript for *LIF* differed over time ($P < 0.0001$) but was not affected by 41.0ºC exposure. Blastocyst development after performing IVF was not affected by 41.0ºC exposure for 4 or 6 h. Progesterone released into maturation medium by COCs was interactively influenced by temperature and hIVM ($P = 0.0116$). When limiting analysis to when *IL6* was temporally produced, progesterone levels were only impacted by time and temperature. Heat-induced shift in the temporal production of *IL6* along with its impact on progesterone likely cooperate in heat-induced hastening of meiotic progression described by others.

Keywords:

Cumulus-oocyte complex, heat stress, Interleukin 6, oocyte maturation, progesterone

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CHAPTER ONE

INTRODUCTION AND REVIEW OF PERTINENT LITERATURE

Dairy cows lose the ability to maintain body temperature when temperature-humidity index approaches 72 [1]. In moderate to severe instances of heat stress, cow body temperature may reach or exceed 41.0°C [2-7]. This level of hyperthermia is problematic as for each 1°C increase in rectal temperature pregnancy rate decreases by ~25% [8]. Hyperthermia occurring at or near the time of breeding is especially problematic. Cows having elevated rectal temperatures before artificial insemination are more likely to return to service and have lower conception rates [9]. Hyperthermia related decreases in fertility are not limited to Holstein cows but are problematic in other breeds [10], including *Bos indicus* cattle [11].

Although mechanisms underlying reduced fertility are multifactorial, and to some extent relate to direct effects of elevated body temperature on the cumulus-oocyte complex (COC; [12-14]); heat-induced alterations in the maternal environment (e.g., ovulatory follicle components) are also problematic. Towards this end, Rispoli et al. [15] examined the follicular fluid proteome of lactating dairy cows that became hyperthermic as a result of an acute heat stress event occurring after a pharmacologically-induced LH surge. Hyperthermic cows had increased intrafollicular levels of IL6 in the ovulatory follicle [15]. Consequences specific to IL6 production were likely limited to ovulatory follicle components because levels in circulation were similar in cows maintained in thermoneutral and heat stress conditions [15]. Interleukin 6 is produced by the mural granulosa cells [murine: 16, porcine: 17] and the cumulus-oocyte complex [murine: 16, bovine: 18, ovine: 19, human: 20, 21].

Although functional significance of hyperthermia-induced increases in intrafollicular IL6 remains unclear, heat-induced increases in other cell types [22-24] along with noted effects to promote meiotic maturation [murine: 16, porcine: 17, bovine: 25] and cumulus expansion [murine: 16, 26] [porcine: 17, human: 27] prompted us to conduct the study described herein to examine *IL6*

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abundance in the cumulus-oocyte complex when directly exposed to the physiologically-relevant elevated temperature of 41.0ºC throughout *in vitro* maturation. Because functional significance of IL6 is dependent on having receptor components, relative abundance of its receptor and signal transducer was also evaluated. Signal transducer is shared by IL6 and LIF [18, 28] thus LIF transcript abundance was also examined. To determine the extent to which direct exposure of COCs to 41.0ºC for the short time periods of 4 or 6 h impacted embryo development, ability of oocytes to cleave and develop to the blastocyst stage after performing *in vitro* fertilization was also examined.

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CHAPTER TWO

MATERIALS AND METHODS

Collection and *in vitro* maturation of cumulus-oocyte complexes

Reagents and chemicals were obtained from MilliporeSigma (St. Louis, MO, USA) unless indicated otherwise. Oocytes were collected from abattoir-derived ovaries [13], with modifications noted below. Media were prepared per Rispoli *et al*. [29]. Folltropin-V (FSH) was obtained from Vetrepharm Canada, INC. (London, ON, Canada); same batch was used throughout. Cumulus-oocyte complexes (COCs) with compact cumulus cell vestments and homogenous ooplasm underwent *in vitro* maturation (Study 1: ~30 COCs per 0.5 ml maturation medium in polystyrene tubes; Sarstedt AG and Co., Nümbrecht, Germany; Study 2: 29 to 45 COCs (mean = 34.3 ± 0.66) per 0.5 mL in 4-well Nunc culture dishes; Thermo Fisher Scientific, Waltham, MA, USA). Incubator temperatures were verified before and during different studies using mercury thermometers sealed in media-filled bottles.

Study one: Interleukin 6, IL6 Receptor, Signal Transducer and LIF transcripts during in vitro maturation in COCs matured at 38.5 or 41.0ºC

Cumulus-oocyte complexes were matured at 38.5 or 41.0ºC (exposure to 41.0ºC was restricted to first 12 h only; thereafter COCs were transferred to 38.5ºC). At 2, 4, 6, 8, 10, 12, 16, 20 and 24 h *in vitro* maturation (hIVM) subsets of COCs were removed from culture and kept separate by treatment (Figure 1; 2 x 9 factorial treatment arrangement). Per each time period, COCs were washed twice in Dulbecco's phosphate buffered saline containing 0.1% polyvinyl alcohol and pelleted (600 x g, 5 min). After supernatant removal, COCs were lysed in extraction buffer (Quick-RNA Kit; Zymo Research, Irvine, CA, USA) and stored at -80°C until RNA isolation. A subset of COCs was also processed soon after removal from ovary to provide a 0 hIVM group. Maturation medium that was conditioned by COCs during culture was centrifuged (5 min, 3000 x g);

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supernatant was stored at -20ºC. Cumulus-oocyte complexes were collected from ovaries on four different days with 3,840 total COCs being utilized. On a given days collection, two different pools of 30 COCs per each temperature were evaluated at 0, 4, 8, 12, 16, 20, and 24 hIVM resulting in a total of 8 observations per these treatment combinations. Related to 2, 6, and 10 hIVM, only one group of 30 COCs was utilized per these treatment combinations resulting in a total of 4 observations for these time periods.

Total RNA isolation, cDNA synthesis, primer design and ddPCR

Total COC RNA was isolated using the Quick-RNA Microprep Kit (Zymo Research, Irvine, CA, USA) with on-column DNAse treatment per manufacturer. Quantity (Nanodrop ND-1000; NanoDrop Technologies, USA) and quality (RNA Nano LabChip; Bioanalyzer 2100, Agilent, USA) of total RNA were determined (RIN values ranged from 7.2 to 10; median of 8.9). Reverse transcription with oligo (dT) and random primers (500 ng per 20 ul reaction; iScript Reverse Transcription Supermix, Bio-Rad, Hercules, CA, USA) was performed per manufacturer and diluted 5-fold with 1 mM Tris-HCl (pH 8.0) and 0.01 mM EDTA (0.1X TE) before performing digital droplet polymerase chain reactions (ddPCR) analyses. A pool resulting from all samples within each collection day was sham-transcribed (iScript No-RT Control Supermix, Bio-Rad) as an additional control.

Primer-BLAST (National Center for Biotechnology Information; U.S. National Library of Medicine, Bethesda, MD, USA) was utilized to design primers spanning exon-exon junction and/or introns (Table 1). Resulting amplicons were sequenced to ensure specificity. Digital droplet PCR was performed in duplicate using 10 ng of nucleic acid per reaction per manufacturer’s instructions. No template controls (NTC; 0.1X TE) were analyzed to assess background signal and control for exogenous contamination. Samples were amplified for 40 cycles, 30s per conditions in Table 1

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followed by signal stabilization (4°C for 5 min, 40°C for 5 min, hold at 12°C). Acquired data were analyzed using QuantaSoft Analysis Pro (ver. 1.0, Bio-Rad) to calculate number of copies per µl.

Transcript abundance was normalized to succinate dehydrogenase A (SDHA). Succinate dehydrogenase A has been used as a normalizer for *in vivo* and *in vitro* matured COCs [30-33] and for heat-stressed COCs [34]. It is stably expressed in cumulus during maturation [30, 35] and not altered by elevated temperature exposure (JL Edwards & J Klabnik, unpublished data).

Progesterone production

Progesterone released into the maturation medium by COCs matured at 38.5 or 41.0ºC (Figure 1) was analyzed by radioimmunoassay per manufacturer's instructions (Double Antibody RIA; MP Biomedicals, Santa Ana, CA., USA). Assay sensitivity was 0.02 ng/mL; inter- and intra-assay coefficients of variation were 7.6 and 6.0%, respectively.

Study Two: Embryo development after COC exposure to 41.0ºC for 4 or 6 hIVM

Because heat-induced increases in *IL6* levels were noted by 4 hIVM and by 4 and 6 hIVM for *IL6ST,* a second study was performed to evaluate consequences of a 41.0ºC exposure for 4 or 6 h on embryonic development. Cumulus-oocyte complexes meeting criteria described above were randomly allocated to three different treatment groups: 38.5ºC for 24 hIVM, 41.0ºC for 4 hIVM, or 41.0ºC for 6 hIVM. After 4 or 6 hIVM at 41.0ºC, COCs were transferred to 38.5ºC for remainder of *in vitro* maturation. After a total of 24 hIVM, a combination of frozen-thawed-washed sperm from two bulls was added at ~500,000 motile sperm/ml to each well of COCs. Presumptive zygotes were denuded of cumulus and associated sperm at ~16 to 18 h after addition of sperm. Embryonic cleavage was assessed 66 to 70 h after addition of sperm at which point essential amino acids were added to culture medium. At 172 to 178 h after addition of sperm, blastocyst development was recorded. Blastocyst stage and quality scoring was performed as described by Schrock et al. [36]. Nuclei were enumerated using fluorescent microscopy (40X magnification using a Nikon Eclipse

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TE300; UV-2A filter: ex 330 to 380 nm, em 400 to 420 nm; Nikon Instruments, Melville, NY, USA) after fixation in 3% paraformaldehyde. Thereafter, embryos were stained using 5 µg/ml Hoechst 33342, washed, and then mounted on glass slides in Dulbecco's phosphate buffered saline containing 50% glycerol and 0.5 µg/ml Hoechst 33342.

Statistical analyses

For study one, data were analyzed using generalized linear mixed models (PROC GLIMMIX, SAS 9.4, SAS Institute, Cary, NC, USA) as a randomized block design, blocking on replicate (i.e., day of COCs collection). Mindful of the 2 x 9 factorial treatment arrangement, fixed effects in the model included IVM temperature (38.5 and 41.0ºC), IVM time (2, 4, 6, 8, 10, 12, 16, 20 and 24 h), and respective interaction (IVM temperature x IVM time; 18 treatment combinations). Treatment differences were determined using F-protected least significant differences and are reported as least squares means ± standard error.

Multisource nonlinear mixed model regression (JMP PRO 14, SAS Institute, Cary, NC, USA) was performed *a posteriori* to determine the extent to which maturation of COCs at 41.0ºC shifted the timing of changes in transcript abundance compared to levels observed in COCs matured at 38.5ºC. The nonlinear prediction model was $a*Exp(-(0.5*((hIVM-b)/c)^2))$ where a is peak (highest) value, b is when peak value occurred, and c is growth rate (width of temporal production).

Study two was analyzed as a randomized complete block, blocking on day of oocyte collection with main effect of treatment. Mixed model analysis of variance (PROC GLIMMIX; SAS 9.4) was utilized to determine treatment differences in relation to responses in embryo development including rates of cleavage, 8 to 16 cells, and blastocysts as stage and quality scores. Treatment differences were determined using F-protected least significant differences.

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CHAPTER THREE
RESULTS

Study One: Interleukin 6, IL6 Receptor, Signal Transducer and LIF transcripts during *in vitro* maturation in COCs matured at 38.5 or 41.0ºC

Relative abundance of IL6 in COCs matured at 38.5 and 41.0ºC

The differences in *IL6* transcript abundance differed depending upon IVM temperature and hIVM (Temp x hIVM, $P < 0.0001$; Figure 2A). While barely detectable soon after COC collection (0 hIVM), *IL6* abundance at 2 hIVM was similar between COCs matured at 38.5 and 41.0ºC. By 4 hIVM, acute exposure to 41.0ºC increased relative abundance of *IL6* transcript compared to 38.5ºC. Interestingly, by 6 and 8 hIVM the relative abundance of *IL6* in COCs exposed to 41.0ºC was lower than that observed in COCs matured at 38.5ºC. By 12 hIVM, relative abundance of *IL6* was similar between 41.0 and 38.5ºC COCs, levels remained low for remainder of maturation.

Use of multisource nonlinear mixed model regression showed that the major consequence of 41.0ºC exposure at the beginning of maturation was to shift the temporal production of *IL6*. To this end, *IL6* levels peaked at 4.67 hIVM in COCs directly exposed to 41.0ºC, whereas *IL6* levels peaked at 6.44 hIVM when COCs were matured at 38.5ºC ($P < 0.001$; Figure 2A, Table 2). Peak values and growth rate were similar in COCs matured at 38.5 and 41.0ºC (Table 2).

Relative abundance of IL6 receptor (IL6R) in COCs matured at 38.5 and 41.0ºC

Differences in the relative abundance of *IL6R* differed depending on hIVM and IVM temperature (Temp x hIVM, $P = 0.05$; Figure 2B). Abundance of *IL6R* was highest during the first 8 hIVM with abundance decreasing thereafter and reaching lowest levels by 20 to 24 hIVM. Except for the 10 hIVM time period, *IL6R* levels were similar in COCs matured at 38.5 and 41.0ºC (Figure 2B, Table 2).

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Relative abundance of IL6 signal transducer (IL6ST) in COCs matured at 38.5 and 41.0ºC

Relative abundance of *IL6ST* transcripts differed depending on IVM temperature and hIVM (Temp x hIVM, P = 0.002; Figure 2C). While barely detectable in COCs soon after collection from antral follicles, *IL6ST* abundance at 2 hIVM was similar between COCs matured at 38.5 and 41.0ºC. However, by 4 and 6 hIVM, 41.0ºC exposure resulted in higher levels of *IL6ST* compared to 38.5ºC counterparts. At 8 hIVM relative abundance of *IL6ST* in COCs matured at 41.0ºC was similar to COCs matured at 38.5ºC. By 10 hIVM, *IL6ST* was lower in COCs matured at 41.0ºC compared to those matured at 38.5 ºC. By 16 hIVM, relative abundance of *IL6ST* was similar between 41.0 and 38.5ºC and equivalent to levels observed at the onset of maturation (i.e., 2 hIVM).

Use of multisource nonlinear mixed model regression showed that the major consequence of 41.0ºC exposure at the beginning of maturation was to shift the temporal production of *IL6ST*. To this end, *IL6ST* peak levels occurred at 6.95 hIVM when COCs were matured at 41.0ºC, whereas peak values were noted at 8.29 hIVM when COCs were matured at 38.5ºC (P < 0.01; Figure 2C, Table 2). Peak values and growth rates for *IL6ST* were similar in COCs matured at 38.5 and 41.0ºC (Table 2).

Relative abundance of leukemia inhibitory factor (LIF)

Relative abundance of *LIF* transcript was affected by hIVM (time, P < 0.0001, Figure 3) but not by maturation temperature. Soon after collection and placement of COCs in maturation medium, *LIF* levels increased up through 8 hIVM. After 10 hIVM *LIF* levels decreased. By 12 hIVM, relative abundance of *LIF* was similar to values obtained at 2, 4 and 6 hIVM.

Progesterone production

Progesterone released per COC into the maturation medium differed depending on IVM temperature and hIVM (Temp x hIVM, P = 0.0116; Figure 4A). When all time points were included

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in the model (2 to 24 hIVM), heat-induced increases in progesterone produced per COC were most prominent at 20 and 24 hIVM (Figure 4A). When including only the time periods when *IL6* levels were shifted by direct exposure to 41.0ºC (i.e., 2 to 8 hIVM), progesterone produced per COC was only affected by IVM temperature and hIVM (i.e., no interaction; heat induced differences were not influenced by time; Figure 4B). To this end, progesterone per COC was 45.7 vs 54.9 pg when COCs were matured at 38.5 and 41.0ºC ($P = 0.002$). Independent of temperature but related to time (hIVM), progesterone per COC was 12.7, 40.7, 63.2 and 84.5 pg at 2, 4, 6 and 8 hIVM, respectively ($P < 0.0001$).

Study Two: Embryo development after COCs exposed to 41.0ºC for 4 or 6 hIVM

Ability of COCs to cleave and develop to the blastocyst stage after being exposed to an acute, short-term heat shock of 41.0ºC during the first 4 or 6 hIVM was similar to COCs matured at 38.5ºC (Table 3). Stage and quality of blastocyst stage embryos from COCs matured at 38.5 or 41.0ºC were similar. Number of nuclei in blastocyst stage embryos did not differ when originating from COCs matured at 38.5 ºC for 24 h, 41.0 ºC for 4hIVM or 41.0ºC for 6 hIVM (Table 3).

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CHAPTER FOUR
DISCUSSION

Novel findings described herein provide further insight related to *IL6* and its receptor signaling component transcripts in the bovine cumulus-oocyte complex as it undergoes maturation. Examination at frequent time intervals confirmed temporal expression of *IL6* and its associated *IL6ST* to the first 12 hours of *in vitro* maturation. Interestingly, the major impact of 41.0ºC was to shift the timing of expression for *IL6* and its associated signal transducer. Specific to this end, peak *IL6* levels in COCs exposed to an acute heat shock of 41.0ºC occurred ~2 hours *earlier* than thermoneutral COCs. Regarding impact on *IL6ST* expression, peak levels in COCs exposed to 41.0ºC occurred ~1.5 hours *earlier* than thermoneutral COCs. Functional significance of these findings along with heat-induced increases in progesterone remain unclear, but heat-related changes may be important to affect/promote meiotic maturation.

Interleukin 6 is a pleiotropic cytokine produced by numerous different cell types [37-39]. Specific to the COC, both the oocyte and its associated cumulus produce IL6 (bovine: [18], human: [20, 21], murine: [16], ovine: [19]. Our study demonstrated that the *IL6* transcript is minimally detectable in germinal vesicle (GV) stage COCs soon after removal from antral follicles. Transcript levels are higher by 2 hIVM and peak 6.44 hours in COCs matured 38.5ºC; levels decrease thereafter and remain low for remainder of maturation. Collectively these findings document temporal expression of *IL6* in the bovine during the first part of maturation and extend the findings of Tscherner et al. [18] who examined *IL6* abundance in bovine COCs at 0, 7, and 24 hIVM. In that study [18] *IL6* was abundant at 7 hIVM but was not apparent in COCs soon after removal from antral follicles (0 hIVM) or after 24 hIVM. Temporal expression of IL6 during the first part of maturation occurs in other species. Consistent with our findings in the bovine, *IL6* was detectable but present in low levels in ovine COCs soon after removal from antral follicles [19]. Peak *IL6* was noted at 4 hIVM; levels decreased thereafter. Use of Western blot confirmed presence of the IL6

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protein in ovine COCs soon after removal from the antral follicle with highest levels at 4 and 8 hIVM [19].

Regarding the potential for COC-derived *IL6* to be impactful at the level of the cumulus-oocyte complex, outcomes of study one show that *IL6* receptor abundance is greatest when *IL6* levels are markedly increasing during the first 6 hIVM. Furthermore, receptor levels are lowest during the latter half of maturation (~12 h to 24 hIVM) when *IL6* transcripts remain unchanged and are at low levels. Transcript abundance for the *IL6* receptor relates well to protein levels in ovine COCs [19] which has been localized to the oocyte surface [19]. Although receptor binding is important, intracellular signaling related to IL6 is dependent on forming a complex with IL6 signal transducer [40-43]. Like *IL6*, *IL6ST* was temporally expressed during the first half of maturation, except peak levels occurred ~2 hours later. In murine COCs and hybridoma cells, addition of IL6 increases IL6ST transcript and protein levels [16, 44]. Whether or not COC-derived increases in *IL6* affect *IL6ST* expression in the bovine is unclear. Because of its importance after receptor binding, it is intuitive for *IL6ST* levels to peak and persist a bit longer than *IL6*, which is what was observed in study one.

Nonetheless, the presence of this pleiotropic cytokine, its receptor, and associated signal transducer set the stage for IL6 to play an active role within the maturing COC. Towards this end, Liu [16] showed that the addition of IL6 to murine COCs during in vitro maturation improved success of embryo transfers by increasing number of pups born [16]. Other efforts using ovine [19], bovine [abstract: 25] and porcine [abstract:17] COCs noted improvements in meiotic progression (i.e., metaphase I or metaphase II) with the IL6 addition to the maturation medium. "Pro"-effects of IL6 are likely "dose" dependent. Zhao et al. [19] showed that a lower dose of IL6 (10 ng/mL) increased maturation rates, whereas a higher dose (100 ng/mL) reduced maturation rates and impaired embryo development when IL6 was added to ovine COCs during *in vitro* maturation.

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Although the specific factor(s) underlying the beginnings of maturation are not yet fully elucidated, an increase in COC-derived *IL6* with levels peaking at or around 6 hIVM is likely a significant promotant of GVBD. In other cell types, IL6 reduces gap junction permeability [45] which is requisite for GVBD. When murine COCs are cultured in a hypoxanthine-containing medium to inhibit spontaneous breakdown of the GV, addition of IL6 and its soluble receptor *induced* GVBD, despite COCs being held in the continued presence of a meiotic inhibitor [16]. Marked increases in COC-derived *IL6* peaking at or around 6 hIVM in study one overlap with time period leading up to and when GVBD occurs [46-49]. Interestingly and consistent with consequences of adding IL6 to meiotically inhibited oocytes, direct exposure of COCs to an elevated temperature of 41.0ºC increases IL6 in other cell types [22-24] and *induces* GVBD in bovine COCs [46, 47, 49]. Heat-induced hastening of this developmentally-critical event is detectable as early as 4 hIVM and more prominent by 6 hIVM (Hooper et al., 2015). Recent efforts of Campen et al. [46] demonstrated changes in maternal chromatin at the beginnings of GVBD coincided with heat-induced reductions in gap junction permeability. Although factors triggering accelerated GVBD when activated by 41.0ºC remain unclear, novel findings in this study clearly indicating a heat-induced *shift* in the timing of *IL6* expression and its associated signal transducer by ~1.5 to 2 hours *earlier*, attests to the notion for IL6 to be an underlying contributive factor in the heat-induced hastening of GVBD previously reported by others [46, 47, 49].

Leukemia inhibitory factor is a member of the IL6 family [50-53]. Because it is known to positively impact oocyte maturation in multiple species [54-56], examination of transcript abundance throughout maturation complimented efforts described herein examining COC levels of *IL6*. Unlike *IL6*, however, there was no impact of 41.0ºC on *LIF* expression at any time period examined. Although *LIF* levels increased during the first part of maturation (up through 8 to 10 hIVM) and decreased thereafter, highest levels were reached ~2 hours *after IL6* peaked which is more consistent with changes in *IL6ST*. Interleukin 6 and LIF share the same signal transducer

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with IL6 having higher affinity when both ligands are present [18, 28]. Depending on the importance of IL6 as a GVBD promotant (peaks earlier than IL6ST), temporal production of *LIF* to better coincide with signal transducer may be important to influence other developmentally important events for maturation success (e.g., metaphase I and metaphase II progression).

Associated cumulus cells revert back to granulosa cell-like state while the oocyte undergoes maturation [57]. Towards this end and within a short order of time, cumulus-derived progesterone released into the maturation medium increases and continues to do so throughout maturation [46, study one: 58]. When examining just the time periods when *IL6* levels were shifted by direct exposure to 41.0ºC (i.e., 2 to 8 hIVM), COCs released more progesterone into maturation medium (45.7 vs 54.9 pg for control and heat stress, respectively). Similar findings were previously reported by Campen et al. [46]. Mindful that levels are likely much higher in the COC, the presence of genomic and non-genomic progesterone receptors in bovine oocytes [59] highlights opportunities for progesterone to be acting in a paracrine manner at the level of the oocyte. Although significance of heat-induced "heightening" of progesterone in the COC remains to be determined, progesterone stimulates GVBD [60] and affects meiotic progression [61] attesting to functional significance during this early time period. In porcine COCs, suppression of progesterone production with aminoglutethimide almost entirely prevented GV-breakdown; inhibitory effects on GV-breakdown were overcome by additional progesterone [62]. Regarding the potential interplay of progesterone and IL6, blocking progesterone's ability to bind to its receptor using RU486 prevented FSH-induction of *IL6* in murine oocytes [16] suggesting that progesterone may be a contributory factor helping modulate IL6 production.

Exposure to 41.0ºC at the onset of maturation may be "shocking" but is unavoidable when striving to understand consequences of elevated temperature(s) when occurring during oocyte maturation. Collection of COCs during late fall, winter, spring, first part of summer before summer heat stress attests to the relevance of our model for gaining an understanding of the consequences

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of elevated body temperature(s) that may occur in sexually active females. Depending on management, it is not uncommon for the body temperature of sexually active females to become elevated (up to 1.3ºC; [63-70]) with peak levels occurring at or around the time of the LH surge [70-72]. In two different studies described in a manuscript that is in the final stages of preparation for peer review (Edwards et al., unpublished), rectal temperatures of *Bos taurus* (n = 427) and *Bos indicus* (n = 1,278) females submitted for fixed timed artificial insemination ranged from 37.8 to 41.8ºC and 37.0 to 40.9ºC, respectively. Use of logistic regression showed that per each ºC increase in rectal temperature at insemination odds for pregnancy increased by 1.6 and 1.9 times (Edwards et al., unpublished). Mindful of this, but also expecting that prolonged exposures are detrimental to embryo development [12-14, 36, 49, 73-78], additional effort was put forth to examine consequences of 41.0ºC exposure when occurring during the first 4 or 6 hIVM. Study two outcomes clearly illustrate that blastocyst development after performing IVF was not affected by 41.0ºC exposure for 4 or 6 h. In fact, blastocyst development, stage and quality scores, and nuclei numbers were numerically higher when COCs were matured at 41.0ºC versus thermoneutral temperature of 38.5ºC. Because the major consequence of 41.0ºC was to shift temporal production of *IL6* (study one) and hasten onset of GVBD by an equivalent amount of time, it is unclear if performing IVF earlier than 24 hIVM would have been beneficial as it is when 41.0ºC exposure persists for 12 h [36].

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CHAPTER FIVE
RESEARCH ACKNOWLEDGMENTS

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Table 1: Primer sequences and annealing conditions used for ddPCR

Gene	GenBank Accession Number	Amplicon Location (bp)	Primer Set	Primer Concentration (nM)	Annealing Temperature (°C)
IL6	NM_173923.2	349-568	3'-GCATCTTCTCCAGCAGGTCAG 5'-CAATCTGGGTTCAATCAGGCGAT	250	56
IL6R	NM_001110785.3	343-666	3'-TCGGGCTGTAGGAGTTTGTAGC 5'-GCGCTTGGTGGTGGATGTTC	125	56
IL6ST	XM_010816769.3*	1136-1355	3'-CGCGTCTGATTTGCCAACAA 5'-GTCTCATGCTCACGGCACTA	250	58
LIF	NM_173931	157-359	3'-CTGGGCCGTGTAATAGAGGAT 5'-TCTTGGCGGCAGGAGTTGT	250	58
SDHA	NM_174178	1433-1646	3'-TCCGTAGAGGCTGCTGATCT 5'-GTCCTGCAGACCCGGAGATA	250	58

* Wooldridge et al. (2019)

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Table 2: Impact of 41.0ºC exposure on IL6, IL6R and IL6ST levels in COCs during *in vitro* maturation using multisource nonlinear mixed model regression.

Transcript		Peak Value*	Peak Time (hIVM)	Growth Rate**
IL6	38.5ºC	0.32 ± 0.03[a]	6.44 ± 0.23[a]	2.43 ± 0.28[a]
	41.0ºC	0.33 ± 0.03[a]	4.67 ± 0.23[b]	1.82 ± 0.22[a]
	P-value	P > 0.05	**P < 0.001**	P > 0.05
	R^2	0.67		
	SSE	0.41		
IL6R	38.5ºC	0.01 ± 0.00[a]	5.02 ± 1.49[a]	6.33 ± 2.01[a]
	41.0ºC	0.01 ± 0.00[a]	5.16 ± 0.70[a]	4.42 ± 0.82[a]
	P-value	P > 0.05	P > 0.05	P > 0.05
	R^2	0.33		
	SSE	0.001		
IL6ST	38.5ºC	2.66 ± 0.19[a]	8.29 ± 0.23[a]	2.66 ± 0.22[a]
	41.0ºC	2.77 ± 0.20[a]	6.95 ± 0.23[b]	2.63 ± 0.23[a]
	P-value	P > 0.05	**P < 0.01**	P > 0.05
	R^2	0.69		
	SSE	22.7		

*Peak value: highest obtained level
**Growth rate: Full Width Half Maximum (3 standard deviations from the mid-point at half maximum)
[a,b] means differ P < 0.05

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Table 3: Impact of an acute exposure to 41.0ºC for the first 4 or 6 h of *in vitro* maturation

Treatment	No. OMM[1]	Cleaved (%)	8 to 16-cell (%)	Blastocysts (%)	Stage	Quality	Nuclei
38.5ºC-24 h	454	68.87 ± 3.48	74.64 ± 3.47	23.81 ± 2.77	6.69 ± 0.12	1.79 ± 0.12	101.68 ± 11.39
41.0ºC-4 h*	444	74.77 ± 3.19	75.19 ± 3.39	29.61 ± 3.08	6.75 ± 0.12	1.96 ± 0.12	118.76 ± 11.39
41.0ºC-6 h*	440	75.62 ± 3.07	65.88 ± 3.78	27.73 ± 2.92	6.64 ± 0.12	1.74 ± 0.12	116.07 ± 11.39
	P-value	0.1426	0.0714	0.2558	0.6006	0.1586	0.4313

*Exposed to 41.0ºC for 4 or 6 h at onset of maturation period followed by 38.5ºC for a total of 24 h

[1]Number of COCs placed in maturation medium (OMM) for indicated treatment

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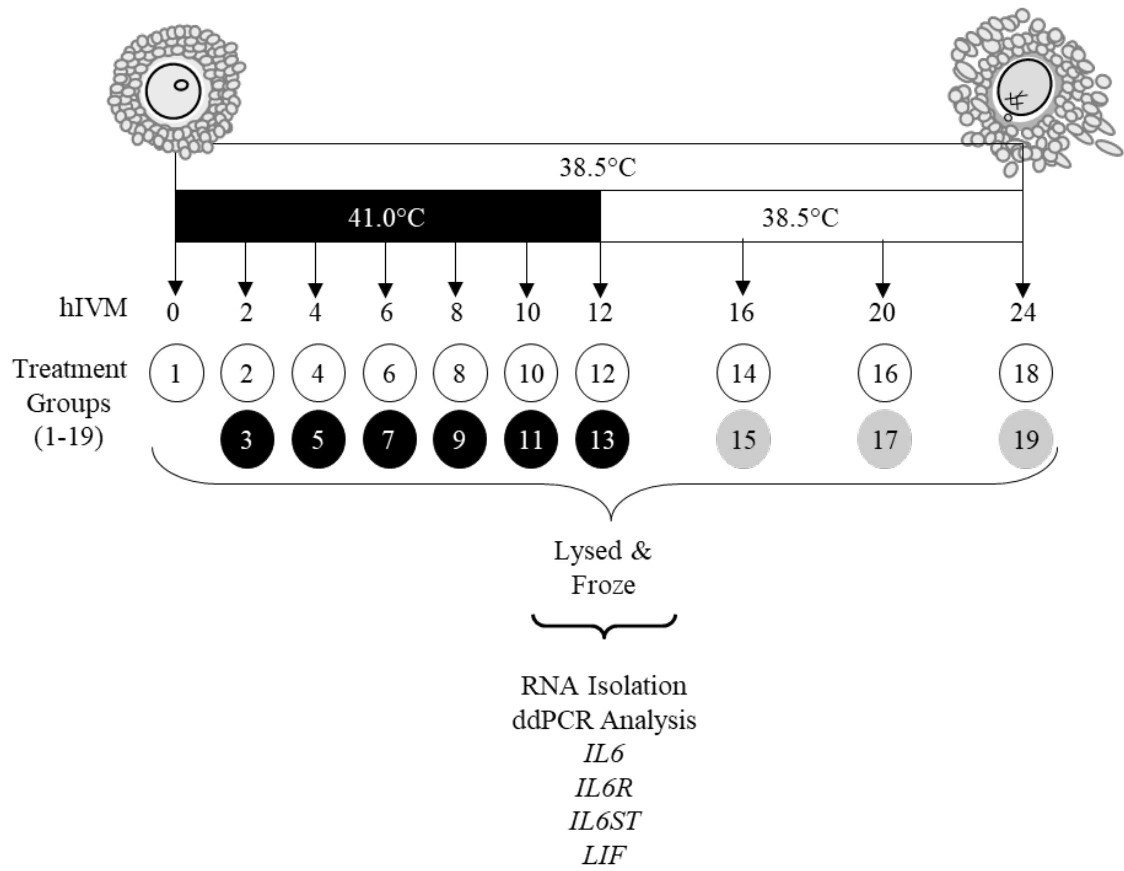

Figure 1: Schematic of study design. At 2, 4, 6, 8, 10, 12, 16, 20, or 24 hIVM subsets of COCs matured at 38.5ºC (designated by white circles) or 41.0ºC (first 12 designated by black circles, then moved to 38.5ºC designated by gray circles) were removed from culture, washed, lysed before storage at -80ºC until RNA extraction and subsequent RNA analyses.

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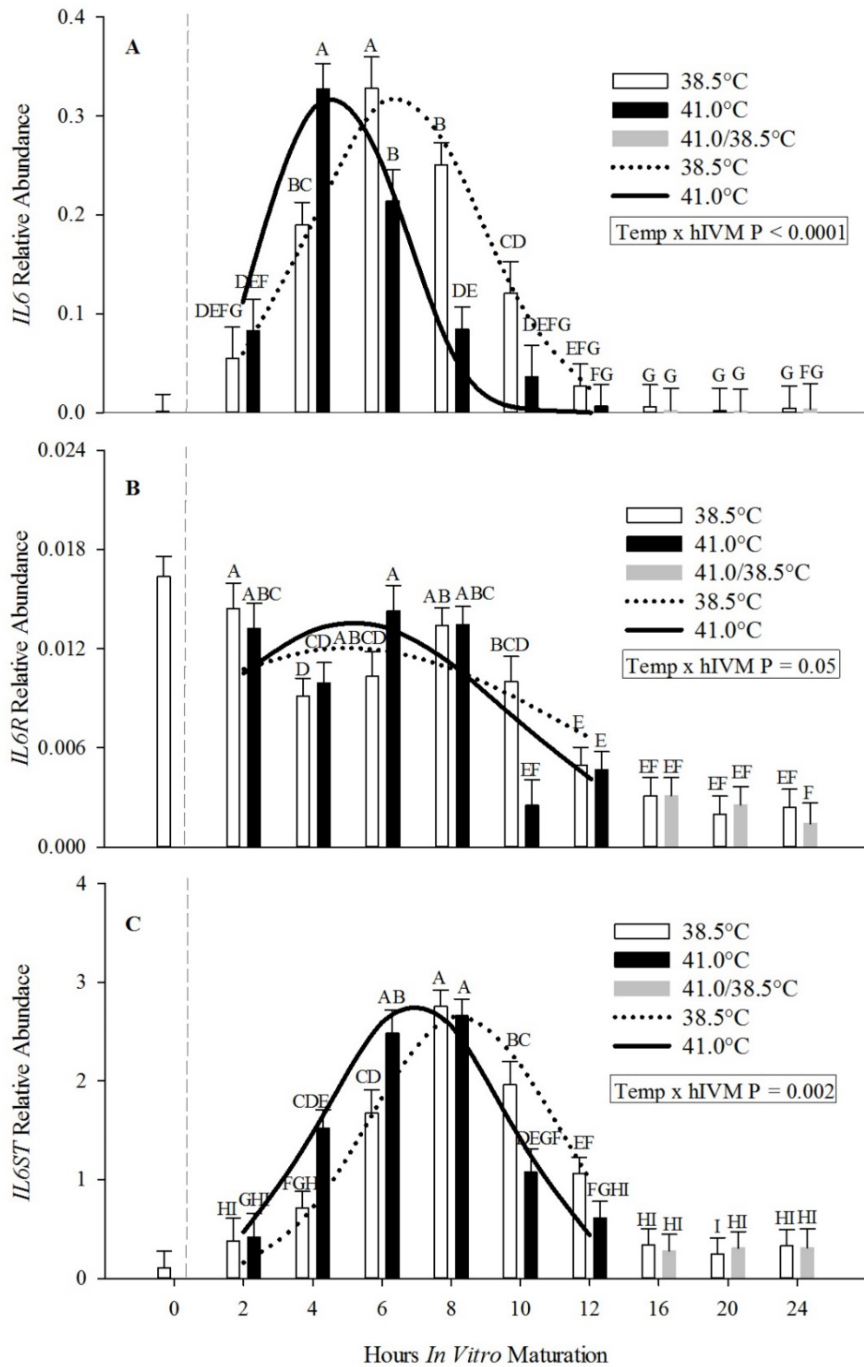

Figure 2: Relative abundance of interleukin 6 and signaling molecules in cumulus-oocyte complexes matured for up to 24 h at either 38.5ºC or 41.0ºC (first 12 h; 38.5ºC thereafter). Interaction of temperature x hIVM between 38.5ºC and 41.0ºC for *IL6* (Panel A), *IL6R* (Panel B), *IL6ST* (Panel C). Bars (least squares means ± SEM) having $^{A\text{-}I}$ different letter designations differ at indicated *P* (Temp x hIVM). Dashed (38.5ºC) and solid (41.0ºC) lines indicate relative abundance curves over the first 12 hIVM.

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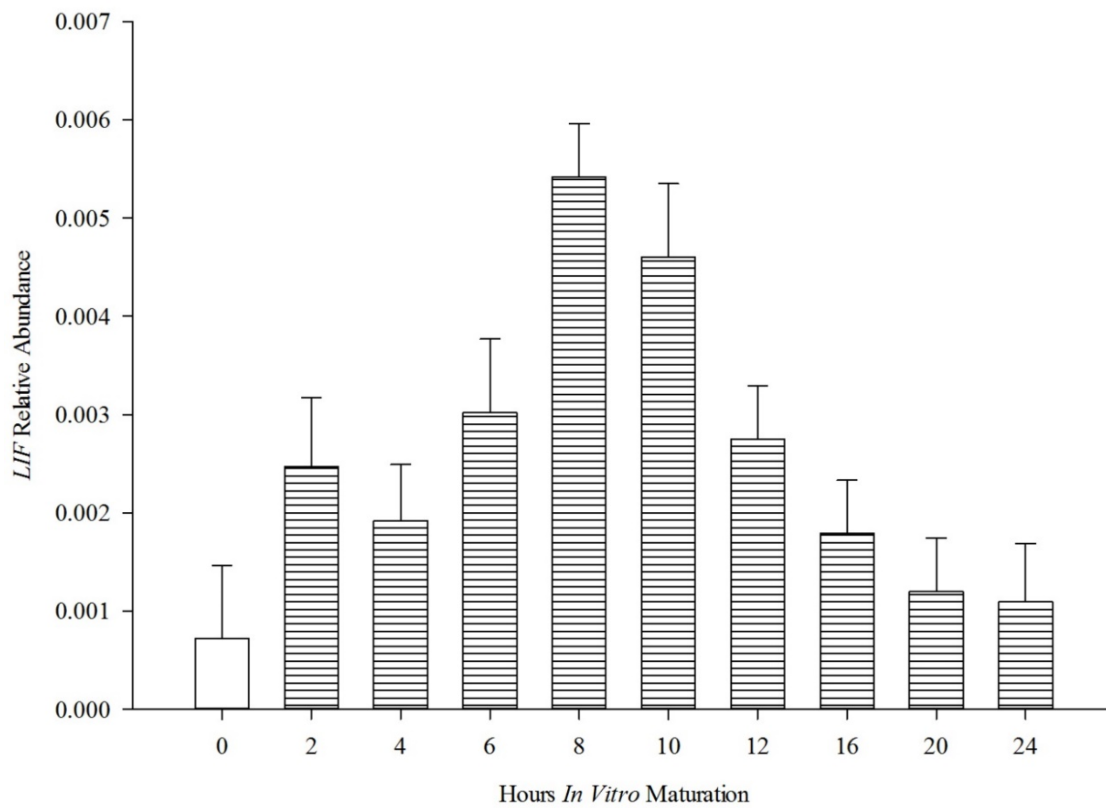

Figure 3: Relative abundance of *LIF* in cumulus-oocyte complexes during maturation, 0 hIVM not included in analysis but as a visual representation of a starting point, averaged across maturation temperatures presented as least squares means ± SEM.

[A-D] means differ $P < 0.05$

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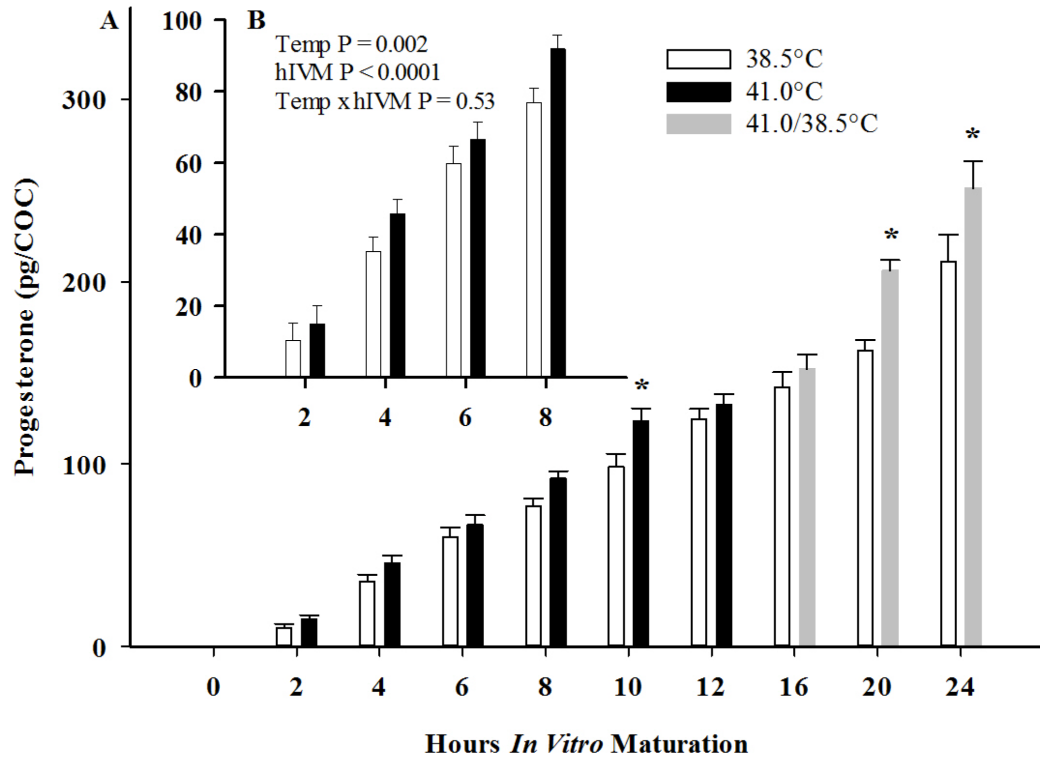

Figure 4: Average progesterone produced per cumulus-oocyte complex (COC) during *in vitro* maturation (IVM) at 38.5ºC or 41.0ºC as measured in conditioned medium. COCs underwent IVM for up to 24 h at 38.5ºC or 41.0ºC (first 12 hours; 38.5ºC thereafter). (A) Temperature x hIVM P = 0.012; * denotes heat-induced increase in progesterone at indicated time point (B) Impact of 41.0ºC on COC on progesterone production during time period when relative abundance of *IL6* was altered by 41.0ºC.

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VITA

Julia Rose Rowinski was born in Lemont, Illinois. She attended University of Kentucky where she received her B.S. in Animal Science with a minor in Biology. She was a member of Sigma Alpha, block and bridle, and UK shotgun team at University of Kentucky. Julia was raised going to work with her mother at an human infertility clinic and that is where her interest for IVF and reproduction stemmed from. In the fall of 2018, she arrived at the University of Tennessee to pursue her M.S. in reproductive physiology under the mentorship of Dr. Edwards.

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