The effects of oocyte heat stress on transposon expression in early bovine development

by

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ABSTRACT

THE EFFECTS OF OOCYTE HEAT STRESS ON TRANSPOSON EXPRESSION LEVELS IN BOVINE OOCYTE MATURATION AND EMBRYO DEVELOPMENT

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This thesis investigates the effects of the environmental stressor heat on the expression of Transposable Elements (TEs) during bovine oocyte maturation and early embryo development. The events associated with reprogramming in early development lead to low levels of DNA methylation, which normally constrains TE expression. Since heat exposure is known to affect TE expression in other organisms, we postulated that developing bovine embryos might be susceptible during this period. TEs can cause mutations and negative consequences through random insertion into DNA, and might contribute to negative effects of heat stress on fertility. The current study hypothesizes that exposure to heat during maturation induces changes in TE expression levels during development. Select TE transcripts were quantified in bovine oocytes and zygotes after exposure to heat (41°C for one hour). The results indicate a small number of significant changes in the expression levels between treatments, and decreased cleavage rates during development.

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DECLARATION OF WORK PERFORMED

I declare that I have carried out all of the work that I have presented in this thesis with the exception of procedures outlined below:

Bovine ovaries were collected by Saeid Ghiasi and Stephanie Hookey. Elizabeth St. John prepared all reagents and media for bovine *in vitro* oocyte maturation, *in vitro* fertilization and embryo culture. Reem Sabry and Solenn Bellion assisted in some ovary aspirations for biological replicates and running select PCR plates.

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ACTB – Beta actin
AI – Artificial Insemination
ANOVA – Analysis of variance
AR – Androgen Receptor
ART – Assisted Reproductive Technology
BSA – BOVINE SERUM ALBUMIN
CC – Cumulus Cells
cDNA – complementary DNA
COC – Cumulus-oocyte Complex
COVID-19 - Severe acute respiratory syndrome coronavirus 2, 2019
ddPCR – Digital Droplet Polymerase Chain Reaction
DNA – Deoxyribonucleic acid
EN – Endonuclease
Env – Envelope Protein
ERV – Endogenous Retrovirus
FBS – Fetal Bovine Serum
FOA – Fetal Oocyte Attrition
FSH – Follicle Stimulating Hormone
Gag – Group-Specific Antigen
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GV – Germinal Vesicle
HS – Heat Shock
Hsps – Heat Shock Proteins
ICM – Inner Cell Mass
IVC – In vitro Culture
IVF – *In vitro* Fertilization
IVM – In vitro Maturation
LH – Luteinizing Hormone
LINE – Long Interspersed Nuclear Element
MET - The maternal-to-embryonic transition
MII – Metaphase II, mature oocytes
miRNA – Micro Ribonucleic Acid
mRNA – Messenger RNA
NTC – No Template Control
ORF – Open Reading Frame
PCOS – Polycystic Ovarian Syndrome
PB – Polar Body
PBS – Phosphate Buffered Saline
PGBD – piggyBac-derived sequences
PLE – piggyBac-like element
Pol – DNA Polymerase
PolB – DNA Polymerase Beta
PPIA – Peptidylprolyl Isomerase A
PVS – Perivitelline Space
qPCR – Quantitative Polymerase Chain Reaction
RNA – Ribonucleic acid
ROS – Reactive Oxygen Species
RT – Reverse Transcription
SEM – Standard Error of the Mean
SINE – Short Interspersed Nuclear Element
siRNA – Small Interfering Ribonucleic Acid
S-IVM – Serum *In Vitro* Maturation Media
SOF – Synthetic Oviduct Fluid
TALP – Tyrode Albumin Lactate Pyruvate
TE – Transposable Element
TIR – Terminal Inverted Repeat
TLR – Toll-like Receptor
TSD – Target Site Duplication
TZP – Tranzonal Projection
UTR – Untranslated Region
YWHAZ – tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZFP – Zinc finger protein
ZP – Zona Pellucida

INTRODUCTION AND LITERATURE REVIEW

Introduction

The high fidelity transmission of genetic information through the replication of DNA is indispensable for the long-term survival of cells, organisms and entire species. This fidelity is potentially compromised by multiple, often repetitive, DNA sequences known as Transposable Elements (TEs) that frequently constitute a very high percentage of the genomes in most eukaryotic organisms. TEs are thought to be viral in origin and are present in almost all animal and plant genomes. They are often referred to as "genetic parasites" due to their reliance on host biology to complete their "life cycles" (Morrison, 2013; Parhad and Theurkauf, 2019). TEs are small mobile DNA elements that move from place to place in the genome through a variety of different mechanisms and can lead to potentially deleterious effects. Over many generations, these elements can induce profound changes in the structure, size and composition of eukaryotic genomes contributing to speciation (Sotero-Caio *et al.*, 2017). Additionally, the transposition of TEs has been linked to a variety of diseases such as cancer and fertility issues such as polyploidy (Belyayev, 2014; Parhad and Theurkauf, 2019). TE expression can occur in a variety of different cellular contexts, however, this review focus is on the expression of TEs during early embryogenesis. The current views and established TE classification schemes will also be presented in this review and some mechanisms of action by which they may affect development. The overall emphasis will be on one category of TEs known as LINE-1 and its role in bovine fertility.

Barbara McClintock first observed the behaviour of transposable elements (TEs), or "jumping genes" in the late 1940s. The genetic rearrangements and movement from one chromosome to another was visualized through cytogenetic analysis and pigmentation patterns in

maize and other plants (McClintock, 1950; Parhad and Theurkauf, 2019). Since then, a great deal of information has been collected about TEs and their behaviour in a variety of eukaryotic organisms. These elements are incredibly diverse and often make up a major portion of the genome. Within assembled vertebrate genomes, TEs can compromise up to 60% of the genome, with ray-finned fish and amphibious lineages exhibiting the highest TE abundance (Sotero-Caio *et al.*, 2017). In plants with larger genomes, such as *Lilium* and *Fritillaria* species, TE content can be as high as 85% (Lee and Kim, 2014). With such a high proportion and activity of TEs in eukaryotic genomes, it is important to understand how TEs are controlled to prevent their detrimental effects on the genome. It also presents larger questions regarding whether, and how, they are tolerated from an evolutionary perspective.

TE Classification

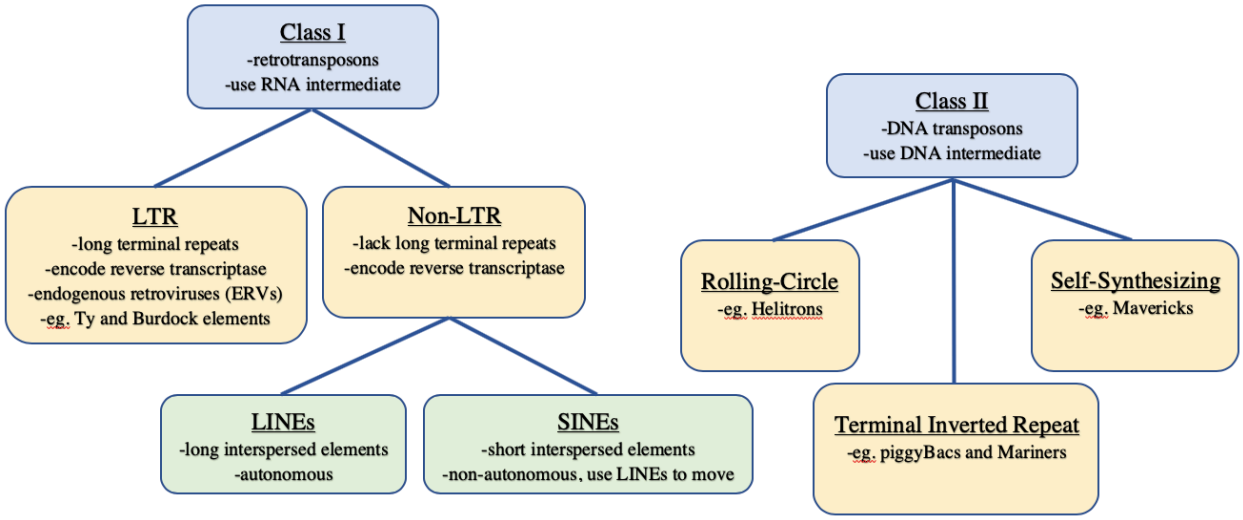

Figure 1 - Flow chart of the classification of Transposable Elements (TEs) into class I and class II elements. Class I or the retrotransposons with sub-families long terminal repeats (LTR) and non-LTR which include both the long interspersed elements (LINEs) and short interspersed elements (SINEs). Class II elements or the DNA transposons include the three sub-families rolling-circle, terminal inverted repeat and self-synthesizing elements.

TEs are divided into multiple classes based on structure and transposition mechanisms (Figure 1). These classes of TEs are rarely found in prokaryotes but can be identified in almost

all eukaryotic organisms (Sotero-Caio *et al.*, 2017). The schemes are based on whether a given TE uses RNA (Class I) or DNA (Class II) as intermediates for transposition, and whether they synthesize their own proteins to mobilize (autonomous) or require proteins from other TEs in order to do so (non-autonomous) (Beck *et al.*, 2011).

Class I TEs (Retrotransposons)

Class I transposable elements, or retrotransposons, mobilize through a copy-and-paste mechanism in which an RNA intermediate is generated by transcription, then reverse transcribed into DNA by a reverse transcriptase encoded by the TE itself or in another TE expressed concurrently (Finnegan, 1989; Parhad and Theurkauf, 2019). These Class I TEs can be further sub-divided into elements that have long terminal repeats (LTRs) retrotransposons, and a subset that lack long terminal repeats known as non-long terminal repeat (non-LTR) retrotransposons (Parhad and Theurkauf, 2019). This class of TEs are rarely found in prokaryotes, but are abundant in most eukaryotic organisms (Sotero-Caio *et al.*, 2017).

LTR retrotransposons include endogenous retroviruses (ERVs), which are unique in that, once mobilized, they can infect new cells within the host through the formation of virus-like particles. ERVs have long repeats at each end that are a few hundred base pairs in length (Alisch *et al.*, 2006). In between the long repeats are open reading frames similar to those of retrovirus *gag* and *pol* genes (Figure 2a). Examples of these LTR elements include Burdock, found in *Drosophila* and Ty, found in *Saccharomyces cerevisiae* (Parhad and Theurkauf, 2019). Some LTR retrotransposons have another open reading frame similar to the *env* retrovirus gene downstream of the *gag* and *pol* genes (Figure 2b) (Coffin *et al.*, 1997; Boeke, 2018). The *gag* gene is associated with transcripts that form a virus-like particle after transcription and RNA accumulation in the cytoplasm. The *pol* gene encodes a reverse transcriptase that is responsible

for copying the retrotransposon-encoded RNA into cDNA, an integrase that facilitates the cDNA integration into the genome, and a protease that cleaves the Pol protein (Havecker *et al.*, 2004; Boeke, 2018). The *env* gene encodes the envelope protein, allowing the retrovirus to penetrate and exit the cell membrane. (Coffin *et al.*, 1997; Garcia-Perez *et al.*, 2016). Most ERVs lack this functional *env* gene, except for select LTR retrotransposons including ZAM, which is expressed in the ovary but can also transpose and infect nearby germ cells (Parhad and Theurkauf, 2019).

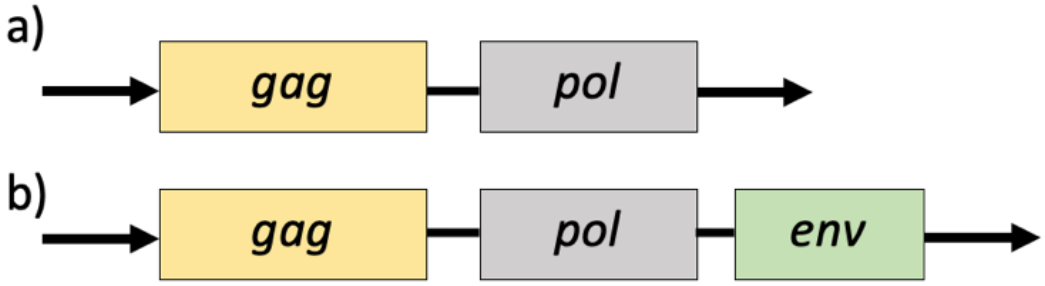

Figure 2 - Schematic diagram of LTR retrotransposon. The black arrows on either end represent the long-terminal repeats and the black lines in between the proteins represent the internal ribosomal entry site, or the site of translation initiation. These TEs are a few hundred base pairs in length. a) The simplified structure of most LTR retrotransposons. Encoding for structural proteins and enzymes are the *gag* and *pol* genes, which both aid in retrotransposition. b) Select LTRs contain an additional functional *env* gene which aids retroviruses in exiting the cell.

The non-LTR category of TEs includes Short Interspersed Nuclear Elements (SINEs) and the Long Interspersed Nuclear Elements (LINEs). SINEs are non-autonomous; they require encoded enzymes from LINE family TEs to mobilize and migrate within the genome (Parhad and Theurkauf, 2019). SINEs are short segments of DNA between 80-500 bp in length, in contrast to LINE elements that can reach several kb in size (Lee and Kim, 2014). LINEs are autonomous TEs that can freely move to different locations independent of other TE coding sequences. This occurs through the activity of reverse transcriptase and endonuclease that they encode (Figure 3) (Parhad and Theurkauf, 2019). One example of a LINE element is LINE-1, which is made up of a few thousand base pairs. LINE-1 accounts for the bulk of TE activity in

the mammalian genome, implicating them in genetic diversity (Beck *et al.*, 2010, 2011). LINE-1 is unique in that it encodes the open reading frame proteins (ORFs) that also participate in the mobilization of other retrotransposons such as *Alu* and SINE elements (Nazaryan-Petersen *et al.*, 2016).

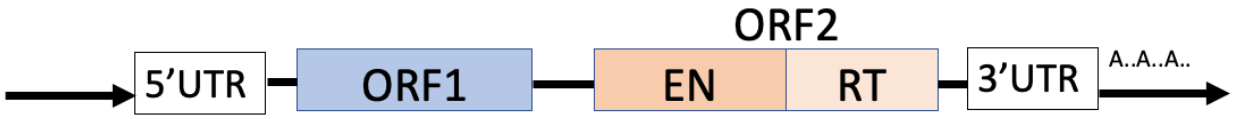

Figure 3 - Schematic diagram of LINE retrotransposons. LINE elements encode for two protein products, open reading frame (ORF) 1 (blue) and 2 (orange) that are needed for the mobilization of this TE. Without either of these proteins, the TE will not integrate within the genome. ORF1 is a RNA binding protein which has a nucleic acid-chaperone that is essential for retrotransposition. Because of this nucleic acid chaperone, it binds to its own transcript mobilizing the transcript from which they are encoded from. ORF2 is a multifunctional protein with endonuclease (EN, dark orange) activity allows for the nicks in the chromosomal target DNA allowing for LINE integration within the genome. The reverse transcriptase (RT, light orange) allows for target-primed reverse transcription of the LINE element mRNA. As well as other enzymatic activities. These proteins allow LINEs to "jump" from one chromosome to another. The black lines in between the proteins represent the internal ribosomal entry site, or the site of translation initiation. The two proteins are bordered by a 5' untranslated region (UTR), a 3' UTR and a poly-A tail (A..A..A..).

Class II TEs (DNA Transposons)

Class II elements are also involved in generating genomic diversity in organisms. In contrast to Class I TEs however, they do not have an RNA intermediate to transpose, instead utilize a DNA intermediate (Finnegan, 1989). This class of elements has been found in both prokaryotic and eukaryotic organisms (Sotero-Caio *et al.*, 2017). Class II TEs are integrated into new genomic sites through the binding of the enzyme transposase to the element's terminal inverted repeat (TIR). These elements are classified based on their shared structural features, their TIR sequence and the length of their target site duplication (TSD), which flanks the TIR. They are classified based on their mechanisms of transposition (Han *et al.*, 2013). The three

major subclasses of these elements include TIR DNA Transposons or “cut-and-paste”, rolling-circle transposons, and self-synthesizing DNA transposons (Figure 1).

TIR TEs operate on a “cut-and-paste” mechanism where the sequence self-duplicates or moves from its initial location or donor site to a different location or acceptor site. As mentioned previously, the transposition intermediate is DNA and the protein responsible for its movement is a transposase. Examples of this sub-family include piggyBac and mariner elements (Sabot *et al.*, 2004). The piggyBac elements are functional in a wide range of organisms including invertebrates, yeast, protozoa, plants and vertebrates. Due to their high transposition activity, piggyBac elements are often used as genetic tools for germline transformations and gene tagging (Bouallègue *et al.*, 2017). The piggyBac-derived sequences (PGBD) are a species of piggyBac elements that contain the sequences derived from a piggyBac transposase. Two of these elements, PGBD1 and PGBD2 were present in the common ancestor of mammals and can be found today in the human genome (Sarkar *et al.*, 2003). Mariner elements have the highest number of horizontal transposon transfer events reported among Class II elements, highlighting their capacity to effectively transfer genetic material between species (Bertocchi *et al.*, 2017). Through data analysis, at present, mariner-like elements can be separated into two groups, Hsmar1 and Hsmar2 (Pace and Feschotte, 2007). Hsmar1 element entered the primate lineage over 50 million years ago, and maintained its activity until about 37 million years ago. At this time, Hsmar1 in the mammalian genome lost its ability to encode a functional transposase protein, after which no further evidence of movement and amplification is found (Miskey *et al.*, 2007). Genetic disorders such as Charcot-Marie-Tooth, Prader-Willi/Angelman and Williams syndromes have been associated with the Hsmar2 transposon in hotspots for homologous

recombination (Carvalho and Lupski, 2016). Interestingly, Hsmar2 can be active in humans and bacteria, emphasizing its role in the evolution of the human genome (Gil *et al.*, 2013).

Rolling-circle transposons, which include Helitron elements, have an unusual structure and mechanism of transposition. These elements were the first group of TEs to be discovered through whole genome analysis, and subsequentially were found in the genomes of plants, animals and fungi (Pritham *et al.*, 2007). The origins for Helitrons were suspected to be from viruses specific to prokaryotes or eukaryotes, however, recent evidence favors prokaryotic and plasmid origins (Heringer and Kuhn, 2018). The rolling circle mechanism through which they replicate and migrate is as follows: the Helitron transposase covalently binds to the 5'-end of the element (leading strand) where it is displaced and the lagging strand is synthesized. A catalytic tyrosine nicks the 3'- end forming a double-stranded circular intermediate and transposase cleaves the leading strand from the circular intermediate. Cleavage occurs from the host's genome which leads to a cascade of events including a free 3'-OH joining the first 5'-phosphotyrosine linkage and the 3'- end of the circular intermediate joining the free 5'-end. These events form a single stranded loop that is integrated into the genome for a period of time. This new loop appears to be resolved during the hosts genome replication (Heringer and Kuhn, 2018). The protein Rep/Helicase, for which these elements encode, has both endonuclease and helicase activity (Kapitonov and Jurka, 2001; Thomas and Pritham, 2015). These elements also include sequences homologous to those in single-stranded DNA viruses (circo- and geminivirus) that have been seen to infect plants and animals (Pritham *et al.*, 2007).

The final group of Class II transposable elements to be discussed here are known as the self-synthesizing or Maverick/Polinton elements. These elements are relatively large in size (~15-20 kb) and share structural features with a mobile element (*Tlrl*) found in *Tetrahymena*

thermophila, a free-living ciliate. These elements have been identified in various eukaryotic genomes including nematodes and zebrafish (Pritham *et al.*, 2007). Maverick elements are bound by inverted repeats and encode up to ten genes, including an integrase and DNA polymerase which aid in replication (Fedoroff, 2013). The integrase and protein-primed family B DNA polymerase (PolB) that is encoded within their sequence, incorporates within the host DNA through retroviral-like transposases (Arkhipova *et al.*, 2019). Interestingly, these elements also encode a protein that is homologous to the jelly-roll fold structure in viral capsid proteins (Krupovic and Koonin, 2015). It is speculated that they may form virions under specific, limited conditions, but this has not been proven experimentally.

Genetic Consequences of TE expression

As described above, the defining feature of TEs is their propensity to move readily throughout the genome. In the course of this process, transposition can occur into exons, introns and regions of "junk" DNA. Although TE activation is normally tightly repressed, activation of TEs followed by insertion into coding and regulatory regions can have significant deleterious consequences for the genome and the organism. These consequences arise both from alterations in encoded proteins, and from changes in gene transcription and/or in post-transcriptional regulation (Garcia-Perez *et al.*, 2016; Parhad and Theurkauf, 2019). TE insertion within coding regions is likely to change the sequence, structure and function of the encoded protein. Insertion into regulatory regions in promoters and untranslated regions of genes may result in increased or decreased expression of the associated gene and gain or loss of function (Morrison, 2013). Furthermore, transposition can lead to secondary DNA strand breaks, mutations, and genetic instability leading to duplications, deletions and inversions in chromosomes (Parhad and Theurkauf, 2019). While beneficial genomic changes can very occasionally result, leading to

positive outcomes through selection, by far the most common outcomes of TE transposition are negative for the host cell and organism.

While TE activation in somatic cells can lead to negative consequences for that cell and any cellular progeny, activation within the germ line or developing embryo is far more problematic as it is likely to have lasting consequences for all offspring. Genome modification leading to altered patterns of inheritance, gene expression, amplification of DNA sequences and changes to the gene sequences can all seriously affect long term viability. Dysregulated patterns of inheritance can include alterations to the chromosomes through inversions, translocations and deletions as mentioned previously (Finnegan, 1989). Arguably, one of the most important altered patterns of inheritance occurred during the formation of the human Y chromosome. The Y chromosome has lost genetic material when compared to the X chromosome, but has also gained a series of repetitive sequences including many SINE and ERV elements (Herrera and Garcia-Bertrand, 2018). While this example highlights the possibility that TE mobilization has the potential to contribute to genetic variation and, ultimately, to the emergence of new species, the most common outcomes at the level of an individual animal are decreased and/or loss of viability.

One example of a mobilization event that compromises viability was first observed in 1988 when LINE-1 elements were found to be inserted into exon 14 of the factor eight gene in several patients diagnosed with hemophilia A (Kazazian *et al.*, 1988) leading to altered clotting function. Insertions of TEs into other loci can cause other genetic disorders such as specific types of immune deficiencies and muscular dystrophies (Agrawal *et al.*, 1998; Taniguchi-Ikeda *et al.*, 2011).

Transposable element amplification within the genome can occur if they are recognized as intermediate elements for replicative events, or if they are duplicated from recombination (Finnegan, 1989). When amplified within the genome, TEs can elicit genomic changes including evolutionary diversification (Finnegan, 1989; Manthey *et al.*, 2018). For example, in rats and mice, studies have revealed that approximately 10 million years ago a member of the LINE-1 TE family was broadly amplified, causing species separation from a common ancestor into the modern murine genera (Pascale *et al.*, 1990). In other mammalian species, *Myotis lucifugus* (vesper bats), it is seen that amplification of the DNA transposons *Helitrons* within the last 30-36 million years has influenced their genomic organization and has led to the overall diversification of the species (Pritham and Feschotte, 2007).

Another example of potentially beneficial consequences of TE activation can be found in observations that TEs can modify regulatory sequences in the genome through the generation of sequence variation when regulatory elements from the TE transpose into regulatory domains (eg. enhancers) of a gene. These changes are more likely to be advantageous and the resulting variation may produce a more highly regulated expression in a given allele, or may even result in a "fusion" that encodes a functionally different gene product (Finnegan, 1989). A beneficial LINE-like element known as the telomere-associated DNA element has been observed in some Drosophila lines. When present, this element can bind to damaged chromosomal ends protecting the organism's telomeres. This element is expressed in germ cells, subject to positive selection that can lead to heritable expansions in number (Sheen and Levis, 1994; Malone *et al.*, 2009). Through these examples of changed patterns of inheritance, gene expression, amplification of DNA sequences and changes to gene sequences, TEs can be seen to represent a significant

participant in the mechanisms that underlie changes in the genomic landscape that help generate genetic diversity in many eukaryotic organisms.

TEs clearly participate in events that ultimately, through mutation and the selection of beneficial mutations, improve survival of the species as a whole. The immediate and potentially beneficial roles for TEs at the level of the individual organism have not been widely studied. Numerous TEs are known to be expressed (Bui *et al.*, 2009; Barrera *et al.*, 2017; Khazaee *et al.*, 2018) and appear essential for development. However, the specific roles they play in this context, and whether normal variation in the expression of individual TEs positively or negatively affects development remains unknown. Overall, it appears that some TE expression is necessary, but excessive expression is detrimental.

Genome Defense Pathways

Epigenetic reprogramming is a cellular event in which changes and specific modifications to DNA (methylation), histones (methylation, acetylation) and chromatin conformation of an individual cell are removed so that the underlying patterns of gene expression can be altered to that of a different cell type or fate (Bui *et al.*, 2009; Garcia-Perez *et al.*, 2016). Prior to reprogramming, these modifications normally constrain TE expression. After their removal in the reprogramming process, TEs are less effectively suppressed in the cell and become more likely to undergo transcription and subsequent transposition when they previously could not.

Specific examples of repressive epigenetic marks removed in reprogramming include DNA methylation, which helps repress the over-expression of human LINE-1 elements and many endogenous retrovirus (ERV) elements in mice (Karimi *et al.*, 2011; Yang and Wang, 2016). Similarly, H2K9me3 modifications to histones are present in heterochromatin and repress

TE activity (Karimi *et al.*, 2011). As explained in the previous section, TE activity such as insertions and mutations are normally deleterious to the host genome, therefore, epigenetic TE suppression is an extremely important component of genome defense against TE mobility (Lee and Kim, 2014). Since reprogramming normally occurs during gametogenesis and early embryonic development, and involves the removal of these epigenetic constraints, the genome is particularly vulnerable during this period (Barrera *et al.*, 2017). Any factor that alters the homeostasis of a cell or gene expression during reprogramming, is likely to have significant effects on development and long-term effects on the individual organism (Figure 4) (Carmignac *et al.*, 2019), in part by changing the pattern of TE expression in this vulnerable period. An example of such factors could be environmental stressors that have the ability to alter the levels of TE expression before or during these periods (Morgan *et al.*, 2005; Duan *et al.*, 2017)

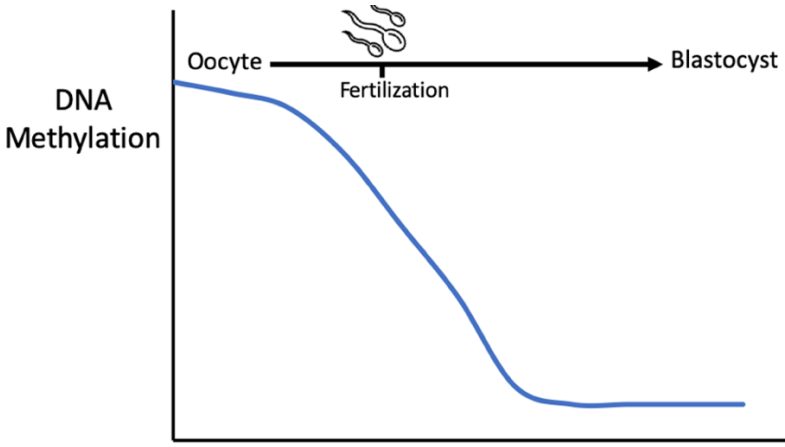

Figure 4 - DNA Methylation levels throughout development. One of the most important aspects of DNA methylation is that it represses TE expression. However, during development there are 2 vulnerable time-frames where this methylation is stripped away. Representative, here, is during early embryogenesis.

To help mitigate problems associated with TE activation during reprogramming, an additional defense mechanism, known as the PIWI pathway, has evolved. The PIWI pathway is an RNA silencing pathway that functions during reprogramming events in mammalian germ cells to control TE activity and expression (Vandewege *et al.*, 2016). This pathway has been

previously reviewed (Russell and LaMarre, 2018), and only a brief overview will be presented here. In this pathway P-element induced wimpy testis (PIWI) proteins, which are members of the larger Argonaute family, form complexes with 26-32 small RNAs, known as piRNAs, that are targeted to expressed TEs through complementarity (Fejes Tóth *et al.*, 2016). This leads to destruction of the TE transcript (preventing re-integration) and may actively direct the re-establishment of epigenetic suppression at the originating TE loci (Russell and LaMarre, 2018). Originally described in *Drosophila* (Lin and Spradling, 1997), four different PIWI proteins have been shown to be expressed in gametes, embryos and some somatic cells (stem cells, natural precursors, etc.) of human and bovine species (van Wolfswinkel, 2014; Roovers *et al.*, 2015; Fernandes *et al.*, 2018). The specific PIWI/piRNA complex recognizes the target TE transcript RNA once it has been exported from the nucleus through antisense complementarity (Luo and Lu, 2017). The PIWI complexes then cleave the TE transcripts through RNAse III activity. In some cases, cleavage generates novel "secondary" piRNAs that then target additional TE sequences (Malki *et al.*, 2014). This aspect of the pathway is known as the "ping-pong" amplification cycle, where the abundance of piRNAs increase, and the number of TE transcripts decreases (Aravin *et al.*, 2008). In addition to their transcript cleavage activity PIWI/piRNA complexes also have the ability to bind to specific sites on nascent (newly transcribed) TE RNA and recruit repressive histone methylation marks which further suppress TE transcription (Rojas-Rıós and Simonelig, 2018). It has been postulated that loci adjacent to targeted TEs, as well as other loci transcribing RNAs that contain complementary TE sequences can also be suppressed. To add further complexity, it has been demonstrated in *Caenorhabditis elegans* that environmental stressors can decrease piRNA levels during periods of elevated temperatures and that piRNA levels can increase during bacterial infections (Belicard *et al.*, 2018). This suggests

that TE expression may be “permissively” regulated by environmental factors and collectively illustrate the complexity of defense mechanisms against TE mobility and expression.

Environmental Influence on Physiology and Gene Expression

It has been widely documented that different environmental stressors can alter the developmental competence of an oocyte and can elicit response pathways to help aid in cellular survival during these periods of stress. Many of these studies have used mammalian models, including mouse, bovine and human cells. In response to heat stress, bovine and mouse granulosa cells elicit a heat shock protein response (Li *et al.*, 2016a; Khan *et al.*, 2020) and in response to oxidative stress, elicit an oxidative response (D. Cetica, L. N. Pintos, G. C. Dalv, 2001; Lord *et al.*, 2015). Granulosa cells are necessary for oocyte growth and nutrition, and any impairment to these cells may lead to negative outcomes for the oocyte.

In order for mammals to maintain homeostasis with their environment, they need to sustain internal thermal equilibrium with temperature and humidity of the external environment (Kadzere *et al.*, 2002). Any divergence from this equilibrium at a cellular or whole organism level, will lead to heat stress and internal processes will be altered (Kadzere *et al.*, 2002). During periods of heat stress, vital organs including the brain and heart must be protected in order to sustain life. In order to do this, blood flow can be reduced to less immediately vital areas including the reproductive organs. In bovine, this can lead to oocytes, embryos and the developing fetus not receiving enough nutrients resulting in a decrease in their growth, in addition to an overall decrease in fertility and pregnancy success (Savsani *et al.*, 2015). Although many organisms have physiological mechanisms in place to help maintain thermal equilibrium during periods of heat stress, studies have shown, in particular within the bovine uterus, that heat stress can concurrently increase the uterine temperature, affecting fetal growth and overall

fertility (Alejandro *et al.*, 2014). In addition to increased bovine uterine temperatures, increased environmental temperatures can also lead to increased rectal and blood temperatures (Khan *et al.*, 2020). Arguably the most important ovarian structure, the follicle, is negatively impacted by heat stress. During such periods, the number of dominant ovarian follicles, the length of time the follicle remains in a dominant state, and the number of follicular waves are all significantly decreased *in vivo* (Wolfenson *et al.*, 1995; Wilson *et al.*, 1998).

In addition to physiological alterations, genes and proteins, including heat shock proteins are induced in periods of heat stress to help cellular damage. Heat shock proteins (HSPs) were originally grouped together and discovered to be upregulated in *Drosophila* when heat-stressed (Ritossa, 1962). Since then, they have been found to play essential roles in a variety of stressful environmental conditions including pathogen infections, UV light and cold temperatures (Swindell *et al.*, 2007). These proteins are often coupled with co-chaperones to prevent protein aggregation and protein quality control (Miller and Fort, 2018). Newer studies have characterized the specific genes involved in the bovine heat shock response, including *HSPA8*, *HSPA14*, *HSP90AA1* and *HMOX1*, all of which have higher expression rates when cattle or their cells are exposed to higher temperatures (Khan *et al.*, 2020). Experimentally, in addition to the upregulation of HSPs during periods of heat stress in cattle, reproductive hormone changes are observed with significant reductions in estradiol production and stenedione synthesis (Roth *et al.*, 2001). Heat stress in swine can lead to functional cellular changes including induced apoptosis and inhibited proliferation in granulosa cells (Sirotkin, 2010); these results were confirmed in additional cellular studies using mouse models (Liu *et al.*, 2015).

Although these pathways are in place to help offset the environmental stress the oocyte is exposed to, they are often insufficient, leading to a significant decrease in developmental rates

and an overall decrease in fertility (Lapa *et al.*, 2011). Proper identification and understanding of genes and pathways that participate in the response to heat are necessary to fully understand the negative effects of environmental stressors, specifically on early embryo development in various mammalian species and in the subsequent development of strategies to ameliorate them.

Environmental Influences on TE Expression

In addition to the genes mentioned above, many other factors are likely to participate in the responses to thermal stress in the pre-implantation embryo. While no specific positive or negative roles have been ascribed to TEs, their expression is known to be environmentally influenced in other species. In plant species such as *Arabidopsis thaliana*, two adaptive TEs (a LINE and Copia element) were likely targets of positive selection, contributing to the ability of the plant to adapt (Li *et al.*, 2018). In addition to plants, environmental influences change TE expression levels in *Drosophila,* leading to downstream changes on genes and phenotypic changes associated with olfaction, behaviour and pigmentation traits (Rech *et al.*, 2019). In mammals, evidence for environmental influences on TE expression during development can be found in a study by Barrera et al. (2017) using bovine oocytes, which found that LINE-1 expression levels and markers for DNA methylation were altered by the composition of the culture media. In another study, TE expression was increased in a synthetic *in-vitro* environment compared to *in-vivo* in mouse embryos (Carmignac *et al.*, 2019). These results suggest that early developing embryos are highly sensitive to changes to their external environment which can lead to changes in the pattern of TE expression. Another study looking at the influence of oxidative stress on maturing bovine embryos, found that oxidative stress significantly decreased global DNA methylation within the 4-cell embryos and blastocysts. In turn, a significant increase in specific TE expression was seen at the blastocyst stage (Li *et al.*, 2016b). Developing bovine

embryos displayed significant changes to methylation and TE expression in response to oxidative stress, illustrating their vulnerability to mutations and other events that may lead to detrimental consequences to the embryo. A delicate balance exists in organisms as the drive for genetic diversity and adaptation meets with the need to enable silencing mechanisms to aid in genome stability.

LINE-1 in the Oocyte

The high proportion of LINE-1 sequences present in most mammalian genomes, coupled with their potential importance in reproduction and development has generated a number of key studies on the distribution, diversity and evolution of this TE class in mammals. They are the most active TEs in placental mammals (Eutherians) (Sotero-Caio *et al.*, 2017). Despite their abundance and implication in disease, relatively little is known about LINE-1 involvement in normal regulatory processes within the mammalian genome.

In humans, LINE-1 elements are the only active elements in the autonomous retrotransposon class of TEs and they comprise about 17% of the human genome (Kano *et al.*, 2009; Garcia-Perez *et al.*, 2016). Research examining TEs and LINE-1 in reproduction, often do not use human samples, however, some studies have examined LINE-1 in human oocytes including one revealing that LINE-1 is transcriptionally active in human oocytes as early as the germinal vesicle (GV) stage of development. LINE-1 was detected in both the nucleus and ooplasm of the oocytes (Georgiou *et al.*, 2009). This was the first and only study of its kind to provide evidence that LINE-1 is transcriptionally expressed in human oocytes.

LINE-1 biology has been much more widely studied using murine models. In mice, LINE-1 elements are slightly more abundant than in humans, comprising almost 19% of the genome. Specifically, in the non-LTR retrotransposon family, LINE-1 is the most abundant type

accounting for about 57% of all non-LTR TEs expressed in the mouse oocyte (Fadloun *et al.*, 2013). One particularly interesting study in early mouse development implicates LINE-1 in fetal oocyte attrition (FOA). FOA is the elimination of over 80% of the initial pool of oocytes before birth. Mammal species such as primates, rodents and humans all experience this phenomenon. In an important recent study it was observed that increased LINE-1 retrotransposon expression in mice resulted in up to a 3-fold reduction in the number of fetal oocytes at birth. In that study, the authors postulated that the mechanisms involved in FOA somehow select oocytes with the lowest LINE-1 activity (Malki *et al.*, 2014). The consequences of increased LINE-1 expression may therefore be the elimination of a greater number of oocytes during FOA, causing a decrease female reproductive lifespan. Other roles for this TE are likely but as yet unidentified. As mentioned previously, the environment in which an oocyte matures can be highly influential in driving altered, often higher, TE expression levels.

LINE-1 in the Embryo

In humans, LINE-1 elements are thought to become expressed and mobilize most readily during early embryo development (Garcia-Perez *et al.*, 2016). There has been some speculation that expression may also be high during germ cell development, however, studies have suggested that the majority of the retrotransposition of and by these elements occurs in the embryo itself (Kano *et al.*, 2009). LINE-1 expression levels have been documented throughout various stages of development and localized to specific tissues and cells. Using single-cell genomics, the expression and mobilization of LINE-1 within embryos has been identified in both trophectoderm cells and the inner cell mass. Additionally when analyzing placental tissues from newborns, LINE-1 insertion events were also documented (Muñoz-Lopez *et al.*, 2019). This suggests that transposition events early in embryo development have the potential to influence

trophectoderm development and function throughout placentation and thereby influence fetal growth.

Similar to humans, the majority of LINE-1 mobilization in mice occurs during early embryonic development (Kano *et al.*, 2009). This occurs during the pre-implantation development period, primarily within the trophectoderm of the blastocyst (Muotri *et al.*, 2005; Reichmann *et al.*, 2013). The relative levels of LINE-1 and other TEs are dynamic and are often observed to be stage-specific throughout development. At the 2-cell stage, the mouse embryo exhibits a high proportion of LINE-1 expression (57%) in the transcriptome compared to other TEs in the non-LTR family. At the 8-cell stage, there is a slight decrease (49%) in expression and at the blastocyst stage, LINE-1 expression accounts for the highest proportion (68%) of a non-LTR TE in the transcriptome (Fadloun *et al.*, 2013). This suggests that LINE-1 may play a more important role than previously thought during embryonic development. A recent study has shown that LINE-1 expression in mice was required to silence *Dux*, synthesizing rRNA (and stimulating proliferation), which then helps the embryo exit from the 2-cell stage. These events support a model where LINE-1 leads to an increase in ribosomal biogenesis, which facilitates the growth of the early embryo (Percharde *et al.*, 2018). A recent study examining the nuclear exosome targeting complex, Zcchc8 showed that suppression of this factor in mouse embryos leads to elevated LINE-1, developmental defects and infertility, linking high TE expression with compromised reproduction (Wu *et al.*, 2019). These examples demonstrate how LINE-1 serves as an important influence on oocyte development and embryo growth. These examples also circumstantially implicate controlled, LINE-1 expression with multiple developmentally important processes.

TE Implications for Bovine Fertility

Agricultural practices have promoted the commercial development of IVF in cattle which has drastically changed the dairy industry (Sirard, 2018). This technology has allowed farmers to make informed selection of offspring with optimal traits based on production and fertility markers with functionally annotated data (Abdollahi-Arpanahi *et al.*, 2017). This has led to dramatic growth in the bovine fertility industry, yet has somewhat increased the potential for some genetic and developmental problems. Farmers have therefore been forced to choose between the benefits of an increased milk or meat yield, with consequences of reduced fertility. This may be attributed to a variety of different factors including inadequate nutrition, environmental stress, and reduced genetic diversity (Sheldon and Dobson, 2003). As mentioned earlier in the literature review, environmental stress has the potential to alter the transcriptional setting and modify the levels of TE expression with downstream effects on fertility. This has not been widely investigated in cattle. Cattle are an excellent model to study environmental stressors on because they can be a reliable translational model to humans having similar developmental characteristics, and similar defense pathways such as the PIWI pathway/proteins which are dissimilar in other experimental organisms such as mice (Ernst *et al.*, 2017).

LINE-1 elements are the most abundant LINE elements found within the bovine genome (Adelson *et al.*, 2009). LINE-1 is important in many developmental processes, therefore, the examination of this TE is a reasonable place to start when investigating potential causes of some fertility problems. Bovine models will not only directly generate knowledge relevant to the agriculture industry, but due to many developmental similarities, reflect many aspects of human fertility as well. The bovine oocyte and embryo has many phenotypic similarities to the human (Table 1). Oocyte diameter, and timing of embryo events is more closely matched between

humans and cattle compared to mice (Betteridge and Rieger, 1993). Additionally, in pre-implantation embryos, both biochemical and regulatory processes are more similar between cattle and humans (Ménézo and Hérubel, 2002).

Table 1. Comparison of various phenotypic parameters of the mouse, human and cattle oocyte and embryo (Adapted from Betteridge and Rieger, 1993).

	Human	**Cow**	**Mouse**
Oocyte Diameter (µm)	150-180	150-180	90-100
Time to reach (hrs)…			
2-cell stage	30	36	12
Blastocyst	120	150	70
Hatching Blastocyst	150	200	100

Future studies benefitting the agriculture sector could involve the analysis of gene and TE expression levels and the determination of differences between breeds of cattle in southern, warmer climates. *Bos indicus* (South Asia) cattle are a better-adapted breed than *Bos Taurus* (European) to thrive in warmer climates and are less likely to suffer from hyperthermia (Kringer III et al., 2003). There was early evidence from this study indicating that a greater proportion of *B. indicus* embryos reached more advanced developmental stages (morula and blastocyst) than *B. Taurus* when heat stressed. In addition, Pantaneira cattle (*B. indicus, B. Taurus* hybrid) had twice as much expression of HSP (HSP70) compared to Girolando cattle (*B. Taurus*), suggesting that this may be an adaptive advantage to protect reproductive processes such as oocyte maturation and embryo growth in high heat environments (Souza-Cácares *et al.*, 2019). This early evidence suggests that environmental adaptive advantages may occur as early as the oocyte and embryo pre-implantation developmental stages. Further studies will be necessary to determine whether, in addition to HSPs, TEs may influence these differences in fertility and result in an adaptive advantage between bovine species. Investigating TE pathways that have evolved in *B. indicus*,

may contribute to higher thermotolerance may help develop strategies to improve fertility in heat stressed cattle from other species such as *B. taurus*.

Conclusions

Transposable elements make up a significant portion of the genome of virtually every eukaryotic organism and have recently been a focus of research in different fields. A variety of different TE sub-families including SINEs, LINEs, Helitrons and Maverics have been identified. All have the ability to alter the genomic landscape of eukaryotes through mutation, altering patterns of inheritance, gene expression, amplification of DNA sequences or changes to the gene sequences. Potential roles in adaptive responses are emerging but the underlying mechanisms are not clear. Multiple pathways, including histone modification and DNA methylation, help protect an organism against widespread TE expression and deleterious effects. As outlined here, LINE-1 is one important TE that is expressed during many stages of both oocyte maturation and embryo development. Many studies examining LINE-1 have been performed in mice and it will be beneficial to investigate other mammal species, such as bovids, due to their similarities with human embryogenesis and their importance in the agriculture industry.

Despite the high abundance of LINE-1 in the mammalian genome, many aspects of the biology of this TE remain unexplored. Future areas of study could include other external factors that affect LINE-1 expression levels such as heat stress, oxidative stress, and whether stress occurring during periods of vulnerability when DNA methylation is removed (ie. during reprogramming in early embryogenesis and gametogenesis), affects fertility.

Environmental stressors will continue to influence mammals, hence, the importance to establish mitigation measures to improve bovine and human fertility. The more we understand the conditions under which TEs are expressed, the more likely we are to develop and implement

strategies that prevent some unfavourable consequences that they can cause, such as infertility, genetic disorders and cancers. Additionally, as further genetic sequencing is undertaken, we have the opportunity to learn more about the biology of these unusual, and intriguing segments of DNA known as TEs.

RATIONALE & HYPOTHESIS

Transposable elements (TEs) are repetitive DNA sequences within the genome. Many TEs are derived from ancient viruses such as retroviruses and were not originally considered to have functional roles, instead representing a group of parasitic elements that resided in the "junk" or non-functional regions of the DNA. Many copies of individual TEs and several different types and classes exist within the genome, comprising up to 60% of the DNA in some vertebrate species. One key feature of these TEs is that they can freely move around the genome and insert themselves into regions of DNA causing mutations and breaks. These events can lead to harmful downstream consequences including developmental disorders and cancer. More recently, scientists have determined that TEs may be implicated in a number of fertility problems (Fadloun *et al.*, 2013; Percharde *et al.*, 2018; Wu *et al.*, 2019), which represents the focus of this thesis.

Despite the observation that TEs represent a significant portion of the genome in some mammals, it is important to note that under normal circumstances the expression of TEs is suppressed by epigenetic characteristics of DNA, histones and chromatin, and they do not cause harmful effects. There are, however, two key periods when these protective mechanisms, including DNA methylation are reduced: germ cell development and during early embryogenesis (Figure 4). During these periods, TEs are more likely to become expressed, and the patterns and levels of expression may be significantly influenced by environmental factors that can influence the transcriptional machinery, activating the expression of TEs. These previous observations concerning the biological behaviour of TEs suggest that environmental influences on fertility may involve changes in the expression and activity of TEs.

Heat stress is a known environmental factor affecting fertility and likely influenced by TE expression. Heat stress is a significant challenge faced by many species globally, particularly, in tropical climates. In domestic species, fertility and productivity of livestock is especially important and agriculture workers often struggle with fertility and production in these climates. Unfortunately, due to climate change and warmer summer temperatures, more northern latitudes are now also experiencing this problem in livestock, specifically cattle, during the increased summer temperatures. The normal body temperature for an adult cow is around 38.5^{O}C and in severe heat stress, body temperature can reach up to 41^{O}C. During times of heat stress, cattle can experience a change in reproductive hormones and a decrease in their overall fertility. Some epigenetic pathways and mechanisms for this loss remain largely unexplored, and for the reasons described above, bovine embryos are an excellent species for a deeper examination of mechanisms of response to heat stress.

Therefore, this project aims to test the principle hypothesis that: **Heat, as an environmental stressor, induces changes in the expression levels of TEs in early bovine embryo development with downstream effects on fertility.**

To test this hypothesis, the following objectives were investigated:

1. Characterize the expression patterns and levels of specific Transposable Elements (LINE-1, BovB and ERV1) in bovine denuded GV and MII oocytes, and corresponding mature cumulus cells in response to a heat treatment (41^{O}C for 1 hr) during *in vitro* oocyte maturation.
2. Quantify Transposable Element (LINE-1, BovB and ERV1) expression levels in bovine preimplantation embryos during the following developmental stages: 2-4 cell, 8-16 cell,

and day 8 blastocyst stage after subjecting the oocyte to heat stress (41^{O}C for 1 hr) during *in vitro* oocyte maturation.

3. Quantify and compare embryo cleavage rates after *in vitro* heat stress on oocytes during maturation (41^{O}C for 1 hr).

CHAPTER ONE: THE *IN VITRO* EFFECTS OF A HEAT STRESS ON THE EXPRESSION LEVELS OF TRANSPOSABLE ELEMENTS IN BOVINE OOCYTES AND EARLY EMBRYOS

1.1 Abstract

Transposable Elements represent a large group of mobile DNA sequences in the genome of most eukaryotes. Under most circumstances, they remain "silent" in the genome and are not transcribed, although they can become activated in a multistep process and jump to different locations in the genome, disrupting normal gene function at sites where they randomly insert. Transposable Element subtypes including Long Interspersed Element-1 (LINE-1) and Endogenous retrovirus (ERV) have been identified as potential drivers of interindividual and interspecies genetic variation (Sheen *et al.*, 2000; Grandi and Tramontano, 2018). These long-term effects are usually a direct result of their unique ability to change their position within DNA, leading to many potential consequences such as mutations, duplications, gene fusions, DNA breaks, translocations and other genetic alterations. In the short-term, however, evidence suggests that the abnormal expression of these elements can lead to fertility problems. Normally, epigenetic factors and small RNA pathways protect organisms against these harmful effects of TE expression, however, during gametogenesis and early embryo development, the dynamic reprogramming of the epigenetic environment facilitates increased TE mobilization. Importantly, environmental stressors can alter the transcriptional environment, which may alter the expression of TEs. Species around the world, including livestock, experience various environmental stressors daily. Due to rising global temperatures, cattle are experiencing heat stress more frequently and this may be responsible for some fertility declines (Gangwar *et al.*, 1965; Cavestany *et al.*, 1985). Understanding how TE expression levels and patterns change in response to environmental stressors may provide significant insight into the mechanisms

underlying fertility changes. We investigated developmental parameters and expression levels of important TEs after heat exposure during bovine oocyte maturation and in subsequent early embryo development. Cleavage rates were significantly decreased in embryos from heat-treated oocyte groups. In general, TE levels displayed dynamic and stage-specific expression throughout development. Expression of the TE L1 was significantly suppressed at the 8-16 cell stage of embryo development after oocyte heat treatment compared to controls. In addition, trends towards variable TE (LINE-1, BovB, ERV1) expression at other stages were also observed. Unfortunately, our inability to obtain additional samples due to the COVID-19 shutdowns precluded further assessment of the potential significance of altered TE expression under these conditions. These results support the hypothesis that the environment plays a significant role in TE expression of bovine oocytes and embryos which may have significant consequences for later development.

1.2 Introduction

By the time of birth most female mammals have reached a relatively fixed number of oocytes that are capable of contributing to reproduction over their lifetimes. At puberty, this number is drastically decreased into a small pool of viable oocytes that can be ovulated (De Felici *et al.*, 2005). Some studies have suggested that germinal stem cells in mice and humans may also have the ability to produce more oocytes after the female is born (Virant-Klun, 2015), however, this is highly controversial (Johnson *et al.*, 2004) and it is generally accepted that the majority of oocytes are formed during fetal development. These oocytes remain arrested in the GV stage in primordial follicles for the majority of their existence (Markholt *et al.*, 2012). During this time, the oocytes remain susceptible to DNA damage from various environmental factors. Over time, the oocytes lose their ability to effectively repair DNA, and damage accumulates within the genome of the remaining oocytes (Stringer *et al.*, 2018). These factors contribute to the general decrease in oocyte quality and higher infertility in older individuals (Astbury *et al.*, 2020). After puberty, GV oocytes are episodically "reawaken" by a gonadotropin signal and waves of follicles regularly mature from the primordial to the primary, secondary and antral follicle stages throughout the organism's reproductive life. After maturation, one or more oocytes are then ovulated whereupon they remain in an arrested state, the metaphase II (MII) stage, awaiting fertilization (Mrazek and Fulka, 2003; Monniaux *et al.*, 2014).

During follicular and oocyte maturation, a coordinated series of important biological events imperative for subsequent developmental steps occurs. These events include the production and accumulation of RNA and proteins needed for later embryogenesis. Additionally, during maturation from the GV stage to the MII stage, a significant amount of chromatin remodelling and nuclear re-positioning takes place (Marlow, 2010). Although their specific roles

are unknown, Transposable Elements (TEs) often become expressed during this period, likely as a result of widespread epigenetic changes. A substantial body of research in several species has demonstrated that this TE expression is necessary for successful embryo development, however, the specific TE roles are unknown (Kano *et al.*, 2009; Fadloun *et al.*, 2013; Garcia-Perez *et al.*, 2016).

TEs are sequences of DNA that can readily mobilize and insert at other locations in the genome; they can comprise over half of the genome in some mammals (Garcia-Perez *et al.*, 2016). The movement and insertion of TEs into DNA sequences is known as transposition. Transposition frequency is highest during the early stages of development and within some somatic tissues (Elbarbary *et al.*, 2016). As described in the literature review, some TEs mediate their own transposition throughout the genome, but can also support the transposition of other TEs and occasionally, portions of nearby genes, particularly regulatory domains, through mRNA intermediates (Elbarbary *et al.*, 2016). Oocytes and supporting somatic cells can interact during maturation, exchanging information and coordinating the maturation of follicles and of the developmentally competent oocyte (Chang *et al.*, 2018). In addition, the cumulus cells surrounding immature and mature oocytes provide essential nutrients for cellular growth such as metabolites and regulatory molecules. This transfer is facilitated by gap junctions connecting the cumulus cells to the oocyte (Zhou *et al.*, 2016). These exchanges could include TE transcripts, which would then accumulate within the oocyte.

Following the process of maturation, the oocyte undergoes fertilization. Fertilization occurs when a spermatozoan makes contact with the oocyte, which, initiates a cascade of essential internal processes within the zygote (Valadão *et al.*, 2019). The spermatozoan penetrates through the oocyte's zona pellucida, where the two plasma membranes fuse. The

oocyte that was previously arrested in the MII stage of meiosis, can then complete this meiotic division and continue with development.

After fertilization, the zygote undergoes repeated mitotic cell divisions, increasing the total number of cells (blastomeres). The division sequence commences with the separation of two blastomeres, which then divide into four cells, then eight cells, and continues exponentially (Gilbert and Barresi, 2017). Once the 8-cell stage is reached, the cells are all in close contact with one another, forming tight clusters (compaction), which continue to divide to form a morula. The cells within the morula then specialize to form the inner cell mass and trophectoderm which later result in the embryonic tissues and placenta respectively. Six to eight days post-fertilization (in bovine), the morula becomes a blastocyst in which the inner cell mass will develop into the fetus and the outer shell (trophoblast) will form the fetal placenta (Khan and Ackerman, 2020). The blastocyst then breaks out or hatches from the zona pellucida and will then implant into the uterus or posterior wall during natural ovulation, or be frozen for later use during artificial implantation (Valadão *et al.*, 2019).

Preimplantation embryo development is a crucial period of development during which essential processes take place that determine multiple aspects of the overall growth and success of an embryo. All of the tested stages in the current study (2-4 cell, 8-16 cell, blastocyst) represent important milestones for embryonic development, with the blastocyst stage as the final stage of development before implantation. Cleavage rates were determined from embryos collected 48 hrs post-fertilization during what is normally the 2-4 cell developmental stage. The cleavage rates were determined because they can be a key indicator of embryo viability as well as overall fertility (Lundin *et al.*, 2001). Human studies have documented that early cleavage rates have led to a higher success in IVF transfers and an increase chance of viable pregnancy

when compared to later cleaving embryos (Lundin *et al.*, 2001). Cleavage rates usually are around 70-85% using normal bovine IVF procedures (Ferré *et al.*, 2020). With few other reliable, non-invasive measures to indicate an embryo's potential to establish a pregnancy, these rates, in addition to the overall morphology are important factors to document and consider when assessing embryo health (Kropp and Khatib, 2015).

The 8-16 cell stage serves as an important developmental stage where the bovine genome becomes fully activated and the maternal-to-embryonic transition (MET) takes place (Lagutina *et al.*, 2010). Although the majority of genomic and thermotolerance mechanisms are activated during the 8-16 cell stage in bovids, some activation and RNA transcription is seen earlier in the 2-4 cell stage (Wrenzycki *et al.*, 1998). During the 2-4 cell stage, specific gene activation that can increase thermotolerance includes increased transcription of *hsp70*, which prevents thermal cell damage (Chandolia *et al.*, 1999). Importantly, increased apoptotic responses are observed during the 8-16 cellular stage in bovine embryos and play a significant role in homeostasis and overall development of the cell (Paula-Lopes and Hansen, 2002). Early stressors (before fertilization) on the developing oocyte are less likely to produce readily detectable changes in the fetus if it manages to survive implant, instead, stress-induced insults incurred after fertilization are more likely to lead to functional and morphological changes at the cleavage stage (Gardner and Kelley, 2017). Throughout all stages of early development, there is significant potential for TE transcript accumulation and degradation within the cell and tissues. Emerging evidence suggests that TE accumulation may be a dynamic process, in which TE levels may be stage-specific throughout development (Fadloun *et al.*, 2013) suggesting unknown roles in development.

In other contexts, TE transcript levels are known to be affected by environmental factors (Makarevitch *et al.*, 2015; Roy *et al.*, 2020). As such, environmental changes leading to altered TE transcription could lead to outcomes such as novel proteins, DNA breaks and mutations (Garcia-Perez *et al.*, 2016). While most of the resulting diversity would be expected to have primarily negative consequences, a small fraction may lead to beneficial evolutionary outcomes. In support of this, TEs might be involved in horizontal gene transfer (between cells in the embryo), which is linked to several important speciation events (Serrato-Capuchina and Matute, 2018). To help mitigate against some of these potentially damaging effects, the oocyte has several potent epigenetic defense systems that prevent excessive TE activation (Russell and LaMarre, 2018). These include DNA methylation, repressive histone modification and small non-coding RNA defence pathways. During maturation and early zygote development, some of these epigenetic features are transiently lost, leaving the oocyte particularly vulnerable to these environmental influences.

The environmental factors to which oocytes are exposed are known to influence their quality and overall offspring health and can include nutrition, stress, hormones and follicular fluid composition (Krisher, 2013; Souza-Cácares *et al.*, 2019). Normally, these factors work together to provide the optimal environment for oocyte maturation and growth by regulating gene expression through different signalling pathways, however, maturation and early embryo development represent periods of vulnerability when the maturing oocyte's epigenetic environment is altered (ie. Reprogramming) (Krisher, 2013). Exposure to environmental stressors during this period has clear potential to both changing normal gene expression and altering the pattern of TE expression, with significant downstream consequences for the host (Wrenzycki and Stinshoff, 2013).

One stressor that is very important in the agriculture sector is heat stress. Maintaining livestock such as cattle in warmer climates has long been a struggle due to rapid overheating leading to widespread heat stress in herds. Recently, warmer summer temperatures at higher latitudes have increased the prevalence of this issue for cattle in otherwise temperate regions (Bishop-Williams *et al.*, 2015). Heat stress is associated with a variety of health consequences including changes in reproductive hormones and an overall decrease in fertility (Dash *et al.*, 2016). Many of the potential mechanisms behind this decrease remain unstudied. Efficient, year-round reproduction is an important element of most dairy farming enterprises in temperate zones. Investigating mechanisms that underlie lower fertility under heat stress is likely to inform strategies to help mitigate or improve this issue. We postulate that one key element of altered fertility under heat stress is changes in the pattern of TE expression during oocyte maturation.

Previous studies have documented the adverse effects of environmental stressors during embryo development on the overall fertility and success of the embryo. Using oxygen concentration as an environmental stressor in developing bovine and sheep embryos, the amount of 2-4 cells and 8-16 cells reaching the morula stage was decreased in conditions below and above the standard IVM conditions (5% O_2) (Thompson *et al.*, 1990). In addition to the effects of environmental stressors on fertility during early development, TE have been expressed during these sensitive time-points. High oxygen tension did not affect LINE-1 and ERV1 TE expression levels throughout early embryo development (Li *et al.*, 2016b).

Previous studies have recognised TE expression levels during embryo development when exposed to environmental stressors, however, few studies have acknowledged TE levels in bovine embryos exposed to a heat stress. Furthermore, few reports have investigated LINE-1 or any TE expression patterns in cumulus cells. One study examining LINE-1 levels in polycystic

ovary syndrome (PCOS) patients did find higher LINE-1 levels in the cumulus cells of mature (MII) oocytes in PCOS patients with no change in the immature (GV) stage oocytes (Pruksananonda *et al.*, 2016). No studies have quantified TE expression differences in cumulus cell changes exposed to environmental stressors.

Due to our incomplete understanding of TE expression levels during development, any data on the patterns of expression or their effects on fertility would substantially improve our understanding of these ubiquitous participants in embryo development. It is crucial in assisted reproductive biotechnologies for both animals and humans to select the highest quality embryos with the greatest potential for success, specifically pertaining to IVF procedures. For the dairy and livestock industry, IVF is a costly procedure where any decline in fertility or inefficiencies may lead to economic losses. It is imperative to minimize these losses to maximize the success of transferred embryos to produce a viable pregnancy. Overall, such studies should provide insight into when TE expression changes occur and whether such changes correlate with any significant fertility problems.

For the present study, TEs were chosen from the most widely expressed and studied families to gain a better understanding of their expression levels during development and the influence of environmental stressors. The chosen TEs represent those most often implicated in reproduction in order to develop a clearer understanding of their expression levels within and between these families in normal and stressed conditions. LINE elements were among those selected in this study for a number of reasons. First, they are autonomous, meaning that they can freely move to different locations around the genome without the mechanistic assistance provided by the activation of other TEs. This relatively unique property is attributed to sequences within the TE encoding the endonuclease (EN) and reverse transcriptase (RT) proteins (Figure

3). These TEs are among the most active in the mammalian genome. Additionally, they are the most widely implicated in reproduction. Two primer sets, “LINE-1” and “L1”, were designed to examine the same LINE-1 TE during development, but at different locations on the element. This was done to determine whether long stretches of the LINE-1 TE are transcribed during periods of heat stress, or whether expression is from shorter segments. BovB is a related TE (member of the LINE family) and specific primer sets were designed to examine TE expression from this family. This element was first identified in ruminants and marsupials, however, recent studies have shown the presence of these elements in other mammalian genomes suggesting an ability to transfer horizontally between species (Ivancevic *et al.*, 2018).

The ERV family has many similar characteristics to the LINE family, including their autonomy of expression. The machinery they encode allowing them to transpose is distinct from the LINE family and includes the *gag*, *pol* and *env* proteins (Figure 2). Although, they are not as abundant in mammalian genomes as the LINEs, many copies of ERVs are still present. These elements are considered very old, and are linked in evolutionary terms with significant events. ERVs initially found their way into the vertebrate genomes through retroviral infections (Garcia-Perez *et al.*, 2016). ERV1 is one example of a TE found in the mammalian genome that is present in numerous species and can be dated back millions of years ago (Kapusta *et al.*, 2013).

Although TEs have been investigated in the context of reproduction, their origin, roles in evolution and the impact that normal and aberrant TE expression might have during development are still unknown. In particular, there are very limited numbers of studies examining effects of environmental stressors, specifically heat, on TE biology (expression, transposition, integration) during early oocyte maturation and early embryo development. Additionally, no downstream developmental effects on fertility of stress-induced changes in TE expression have been reported.

The present study aims to facilitate the development of a better understanding of when and which TEs are expressed during bovine oocyte maturation and early embryo development. We have compared the differences in expression levels between heat stressed bovine oocytes/embryos and control oocytes/embryos when stress is applied during the maturation phase. Overall, this should provide insight into whether TE expression is altered by heat stress and help establish the roles and importance of TEs in any subsequent fertility changes.

In addition to the important, basic mechanistic questions, this research may contribute to the development of strategies to improve management of bovine fertility in warmer climates and animals prone to heat stress. This may have long-term effects on the improvement of bovine IVF procedures, and the implementation of practices to maximize the potential for a successful pregnancy. Quantifying the expression levels of TEs during normal oocyte maturation and embryo development may be important in future studies on their larger roles in biological pathways. By documenting the developmental rates, we can shed light to any downstream consequences for fertility that heat or TE expression may have. Ultimately, this project may increase our understanding of assisted reproductive biotechnologies in other mammalian species, including humans.

1.3 Materials and Methods

Experimental Design

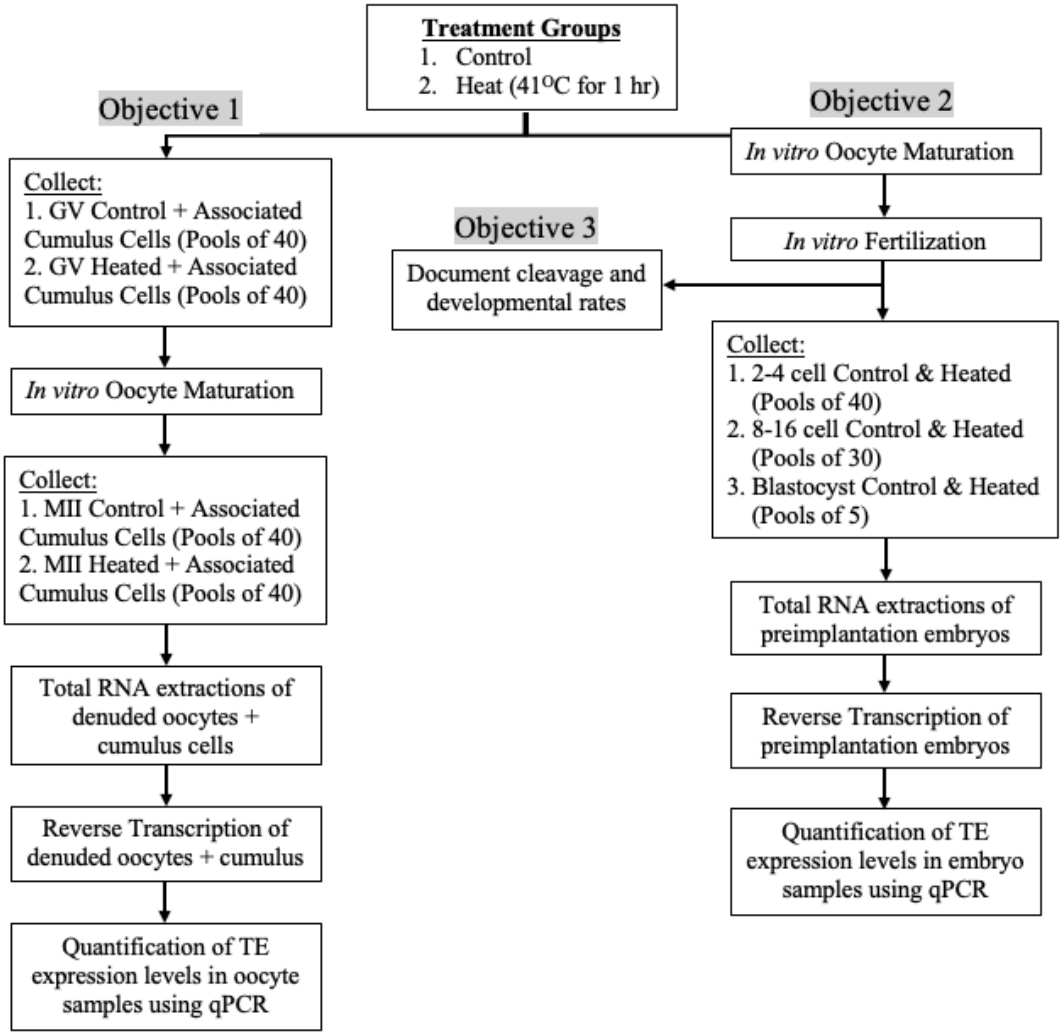

Figure 5 - Oocyte Experimental Design Outline. A flow chart describing the experimental design for Objectives 1, 2 and 3.

Oocyte aspiration and *in vitro* maturation

Bovine (*Bos taurus*) ovaries were collected from a local abattoir (Cargill Meat Solutions, Guelph, ON, Canada) and transported to the University at 34°-36°C, washed in sterile saline and stored in an incubator until follicles were aspirated. Cumulus oocyte complexes (COCs) were collected into a 1 mL vacutainer tube containing warmed (35- 37°C) collection medium., consisting of 1M HEPES (SIGMA H3375)-buffered Ham's F-10 (SIGMA N6635) supplemented

with 2% steer serum (Cansera), Heparin (2 IU/mL) (SIGMA H3149), Sodium Bicarbonate, and Penicillin/Streptomycin (1%) (Gibco, 15140-122).

Using a 5 µL wiretrol micro dispenser and under a dissecting microscope, COCs were collected following aspiration and were randomly separated into two groups, Germinal Vesicle (GV) and Metaphase II (MII) collection periods (Figure 6). Good quality oocytes were selected based on phenotypic characteristics including a dark cytoplasm and tightly packed cumulus cells. In a 4-well Nunc dish, the GV group was washed four times in 600 uL of sterile phosphate buffered saline (PBS) (Multicell 311-010) with 0.01% polyvinyl alcohol (PVA) (SIGMA P8136). Preliminary studies in our laboratory had previously been conducted to test and calibrate the heating equipment and to identify conditions (time and temperature) over which maturing oocytes can be exposed to prior to development and still retain some ability to cleave and develop into an embryo. In these studies, exposure to 41° C for one hour was identified as a suitable condition for further study (Matwee *et al.*, 2001). Pools of 40 COCs from the GV heated group were placed in a laboratory oven pre-heated to 41^{O}C for one-hour, and the GV control group waited for one hour in one of the IVF incubators at 38.5^{O}C. After one hour, pools of 40 COCs from the GV groups were denuded of their cumulus cells using a micropipette and mechanical disruption. The oocytes then underwent examination to check for absence of polar bodies in the perivitelline space (PVS) to confirm that they were still in the GV stage of development. Pools of GV oocytes were then snap frozen in a 1.5 mL microcentrifuge tube using liquid nitrogen and stored at a temperature of -80^{O}C for RNA extractions. Cumulus cells from the heated and control denuded GV oocytes were collected from the wells in the 4-well Nunc dish and centrifuged in 1.5 mL microcentrifuge tubes at 10,000 rpm for five-minutes. The supernatant was discarded and the pellet resuspended in 600 uL of PBS/PVA. The cells were

spun again and the supernatant discarded. The pellet was then snap frozen in liquid nitrogen and stored at -80^{O}C, if needed for later experimentation.

The MII oocyte groups were washed twice in 35 mm Nunc dishes containing synthetic *in vitro* HEPES buffered TCM199 (SIGMA M4530) maturation media (S-IVM) with 2% steer serum and sodium pyruvate (SIGMA P4562). All media was equilibrated to 38.5^{O}C and 5% CO_2 and made at least 2 hrs prior to use. The MII oocyte groups were then placed in a third S-IVM wash containing hormones (S-IVM + H) supplemented with 10 uL LH (1 µg/mL – NIH), 12.6 uL FSH (0.5 µg/mL – Follitropin V), 10 uL Estradiol (1 µg/mL – SIGMA E2785), and 800 uL Fetal Bovine Serum (FBS) (10% - Gibco 12483-020) to 10 mL of S-IVM. The COCs were then transferred into 80 uL SIVM + H micro drops under mineral oil (SIGMA M5310), 20 COCs per drop and the control group was incubated for 24 hrs at 38.5^{O}C in 5% CO_2. The heated MII treatment group was then placed in a laboratory oven pre-heated to 41^{O}C for one-hour, then returned to the incubator for 24 hrs at 38.5^{O}C in 5% CO_2. After *in vitro* oocyte maturation for 24 hrs, COCs were washed four times in 600 uL of PBS/PVA and pools of 40 COCs from the MII control and heated groups were denuded of their cumulus cells using a micropipette and mechanical disruptions. The oocytes then underwent microscope examination to check for evidence of at least one polar body in the PVS to confirm their MII stage of development. The pools of 40 oocytes were then placed in 1.5 mL centrifuge tubes, snap frozen in liquid nitrogen and stored at -80^{O}C for RNA extractions. Cumulus cells from the heated and control denuded MII oocytes were collected from the wells in the 4-well Nunc dish and centrifuged in 1.5 mL centrifuge tubes at 10,000 rpm for five-minutes. The supernatant was discarded and the pellet washed in 600 uL of PBS/PVA before being snap frozen in liquid nitrogen and stored at -80^{O}C,

for later RNA extraction. These protocols were repeated to achieve at least three biological replicates.

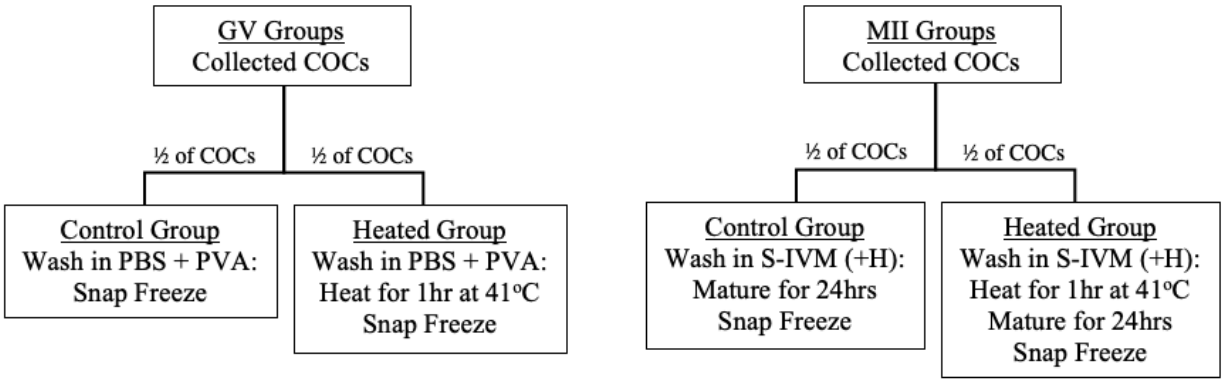

Figure 6 - Oocyte Collection Methods. A visual representation of the methods used to collect the GV and MII oocytes in both heated and controlled groups.

In vitro embryo production and developmental rates

Oocytes were collected, treated and matured utilizing the same methods as described above, without splitting into GV and MII groups. COCs were separated, with half placed into a treatment group and half into a control group. Both groups were washed twice in 35 mm Nunc dishes containing S-IVM and once in S-IVM + H. COCs were then placed in the S-IVM + H drops, 20 COCs per drop in 35 mm Nunc dishes, and the control group was incubated for 24 hrs at 38.5^{O}C in 5% CO_2. All media was equilibrated to 38.5^{O}C and 5% CO_2 and made at least 2 hrs prior to use. The heated group was placed in a laboratory oven pre-heated to 41^{O}C for one-hour, then incubated for 24 hrs at 38.5^{O}C in 5% CO_2. After *in vitro* oocyte maturation was achieved following 24 hrs, the oocytes were not frozen but fertilized using frozen-thawed *Bos taurus* semen (Semex, Guelph, ON). If possible, semen samples were used from the same bull to minimize sources of variation.

Before fertilization occurred, COCs were removed from the S-IVM drops using a 5 µL wiretrol and washed three times in a 4-well Nunc plate in 600 µL of HEPES Sperm Talp (Contents listed in Appendix) and 15% Bovine Serum Albumin (BSA) (SIGMA A8806). The

COCs were then placed in a fourth wash containing 600 µL of IVF Talp media (Contents listed in Appendix) and 15% BSA. After washing the COCs were placed in groups of 20 in 35 mm Nunc dishes comprised of IVF Talp + 15% BSA 80 µL drops covered in mineral oil.

The swim up method was used to isolate the highest quality of sperm from each sample. Semen was thawed in a water bath (36-38^{O}C) for one-minute, then pipetted to the bottom of a 5 mL tube containing 1.5 mL of HEPES + 15% BSA. Tubes were then placed in an incubator (38.5^{O}C in 5% CO_2) for 45 minutes. Following this, the highest motility sperm, in the upper layer of the tube was taken and centrifuged for 7 mins at 200xg to precipitate the sperm. Using IVF Talp + 15% BSA, the pellet was resuspended.

Using a pipette, 1 x 10^6 sperm cells/mL/drop was used to fertilize the COCs. The motile sperm and COCs were incubated for 18 hrs at 38.5^{O}C and 5% CO_2. Following IVF, in a 4-well Nunc plate the presumptive zygotes (PZs) were denuded using a micropipette and mechanical disruption and washed three times in 600 µL of HEPES + 15% BSA and once in Synthetic Oviduct Fluid (SOF) media. Added into the SOF media was Sodium pyruvate (NaPy), essential amino acids (SIGMA M5550), non-essential amino acids (SIGMA M7145), Gentamicin (SIGMA G1272), 15% BSA and 2% FBS. After washing, the PZs were then transferred in groups of 25-30 to 30 µL IVC SOF drops covered in mineral oil and incubated at 38.5^{O}C in 5% O_2.

Cleavage rates were calculated 48 hrs post-fertilization, comparing the number of cleaved zygotes to the total number fertilized per group. Embryos were then collected at three developmental stages: 2-4 cell stage (pools of 40 embryos) collected 28-45 hrs post fertilization, 8-16 cell stage (pools of 30 embryos) collected 80-100 hrs post fertilization and blastocyst (pools of 5 embryos) collected at day 7-9 post fertilization. The number of pooled oocytes needed for

each developmental stage was determined in a previous study (Sabry *et al.*, 2020), or in a pilot study prior to experimentation to determine the optimal amount needed for qPCR quantification. Embryos were collected at these time points, washed three times in 600 µL of sterile PBS/PVA, then snap frozen in a 1.5 mL plastic tube using liquid nitrogen and stored at a temperature of -80^{O}C for RNA extractions.

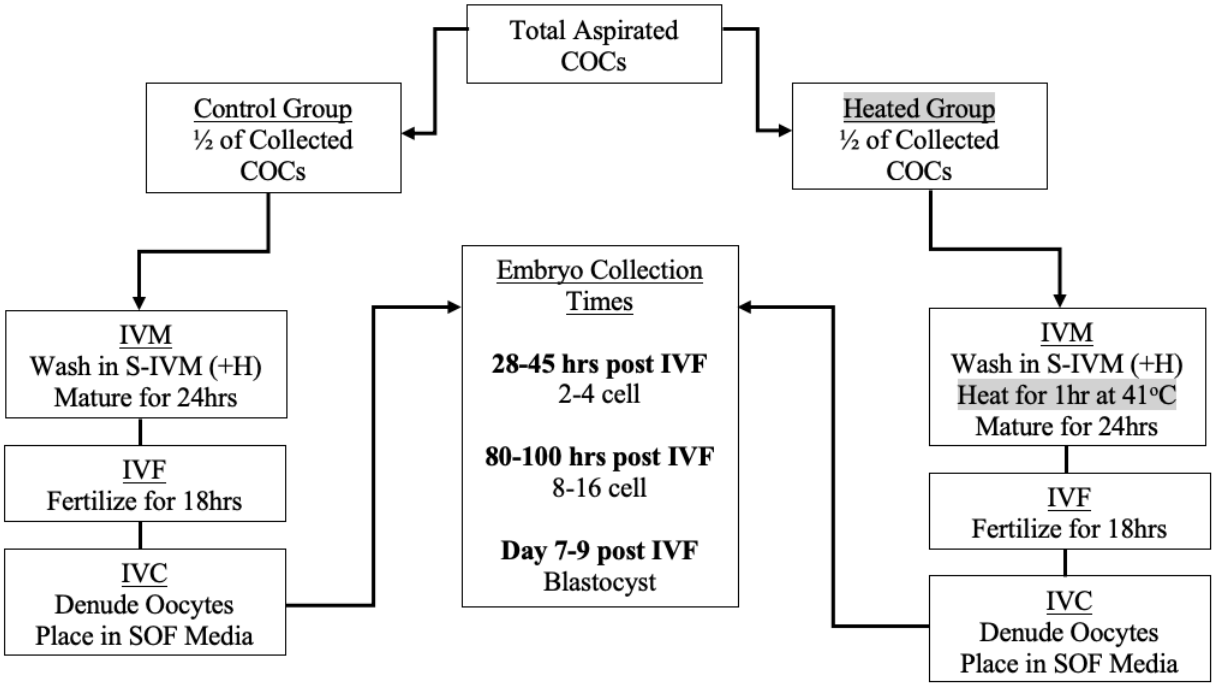

Figure 7 - Embryo Collection Methods. A visual representation of the methods used to collect embryos in heat treated and controlled groups.

RNA extraction and cDNA synthesis

The total RNA in the samples was extracted using the miRNeasy Micro Kit (Qiagen, Toronto, ON) according to the manufacturer's protocol. In brief, cells were lysed using the QIAzol lysis reagent and vortexed to homogenize the content. Chloroform was added to aid in separation before being centrifuged at 4^{O}C to separate contents. The upper aqueous layer was removed and combined with 100% ethanol before adding to the RNeasy MinElute spin column. DNAse digestion enzyme was then placed on the membrane in the column for 15 minutes.

Washes with buffers and 80% ethanol were performed. The column membrane was dried by centrifuging for 5 minutes and then the RNA was removed from the membrane using 19 µL of RNase-free water. The denuded oocytes and early embryos did not have enough RNA to quantify, therefore, to avoid wasting sample, the identical number of oocytes and embryos were used in each pool (GV and MII=40 oocytes) to compare amongst treatments and replicates. 7 µL of extracted mRNA was used to RT so that the resulting complementary DNA (cDNA) could be diluted (1:2.5) to provide enough cDNA for qPCR amplification. RNA from cumulus cells of the mature oocytes were quantified using a Nanodrop 2000c (ThermoFisher Scientific, Waltham, MA). RNA concentration (ng/uL) was documented (Table 2) and the amount of RNA needed to reverse transcribe was calculated using the lowest RNA concentration from the replicates. The amount of cumulus cell cDNA and water to dilute the replicates varied depending on initial RNA concentration. A final concentration of 2.5ng/uL was used to calculate the amount of cDNA needed by each cumulus cell sample (Figure 8) and was sufficient to attain accurate qPCR measurements of TE expression levels.

7 µL of sample mRNA was revered transcribed (RT) using the qScript cDNA Synthesis Kit (Quantabio 95047, Mississauga, ON) under the manufacturer's instructions. 7 µL of sample mRNA, 4 µL of qScript Supermix, 1 µL of qScript Reverse Transcriptase and 8 µL of nuclease-free water were added together and placed in a T100 Thermal Cycler (BioRad, Mississauga, ON) and the following protocol was performed: 1 cycle of 25^{O}C for 5 mins, 1 cycle of 42^{O}C for 30 mins and 1 cycle of 85^{O}C for 5 mins. The cDNA was then stored at -20^{O}C for further qPCR analysis. A group of 200 MII oocytes was also extracted and reverse transcribed to act as a calibrator for qPCR and to perform standard curves on each primer set.

RNA was extracted from embryos using the protocols described above, however, similar to the oocytes, the early stage embryos did not have enough RNA to quantify, therefore, to avoid wasting sample, an identical number of oocytes and embryos were pooled (2-4 cell=40 embryos, 8-16 cell=30 embryos, Blastocyst=5 embryos) to compare amongst treatments and replicates. 7 µL of extracted mRNA was used for the RT procedure so that the resulting complementary DNA (cDNA) could be diluted sufficiently to provide enough cDNA for qPCR amplification. A dilution of 1:2.5 was sufficient during all stages of embryo development measured, to attain accurate qPCR measurements of TE expression levels.

Table 2. Raw data output from spectrophotometer. Included is calculated RNA and water needed per sample with lowest spectrophotometrically determined concentration.

Sample Name	RNA Concentration (ng/uL)	260/280	260/230	RNA Volume Needed (uL)	Water Volume Needed (uL)
Control Rep. 1	25.5	1.89	0.91	11	5
Heat Rep. 1	35.4	1.65	0.79	8	8
Control Rep. 2	19.0	1.81	0.51	15	1
Heat Rep. 2	29.8	1.73	0.84	10	6
Control Rep. 3	57.9	1.60	0.81	5	11
Heat Rep. 3	51.3	1.76	0.78	6	10
Control Rep. 4	23.0	1.80	0.57	13	3
Heat Rep. 4	30.0	1.52	0.38	10	6
Control Rep. 5	27.7	1.59	0.76	11	5
Heat Rep. 5	57.7	1.60	0.71	5	11
Control Rep. 6	34.2	1.56	0.56	9	7
Heat Rep. 6	17.7	1.73	0.74	16	0

Maximum amount of RNA can RT X Lowest spec'd RNA Concentration = Total cDNA Concentration
16uL of RNA X **17.7 ng/uL** = **280ng**

Total cDNA Concentration / Total amount of cDNA per sample after RT = cDNA Concentration/uL
280ng / **20uL** (amount of cDNA after RT) = **14ng/uL**

$C_1V_1 = C_2V_2$
C_1= cDNA Concentration/uL, V_1=Amount of cDNA needed for dilution, C_2= Concentration of cDNA after dilution, V_2= Amount of sample needed for all 6 primer sets during PCR
14ng/uL X V_1 = **5ng/uL** X **50uL**
V_1= **17.9uL** of cDNA and (50uL - 17.9uL) **32.1uL** of Water

Figure 8 – Sample calculation of how cumulus cell cDNA was diluted for a final concentration of 2.5ng/µL.

Primer Sets and Design

Primers including “LINE-1”, “L1” and “BovB” were designed and chosen from the LINE family and “ERV1” from the ERV family to further study in the current experiments (Table 3). Two primer sets, “L1” and “ERV1” were used from a previous study analyzing oxygen concentrations as an environmental stressor in developing bovine embryos (Li *et al.*, 2016b). New primers (“LINE-1” and “BovB”) were designed targeting the BovB TE using the *Bos taurus* BovB genomic sequence and an additional primer was designed targeting the L1 TE using the *Bos taurus* ORF II gene sequence. Primer sequences and information can be found in **Table 3**. Sequences were found in the NCBI database and primer sets were designed using the Primer3 online software (http://primer3.ut.ee).

Quantitative PCR and Reference Gene Selection

Oocyte TE RNA and “housekeeping” mRNA expression profiles were analyzed in six biological replicates with two treatment groups by Quantitative real-time PCR (qPCR) using a CFX96 Touch Real-Time PCR Detection System (BioRad). mRNAs were amplified using SensiFAST SYBR Mix (Bioline 98050) using forward and reverse primers. The RT-PCR master mix consisted of 5 µL of SensiFAST SYBR, 1 µL of working dilution of forward and reverse primer and 2 µL of RNase free water. This led to a volume of 8 µL of master mix added per well, then 2 µL of sample cDNA added for a total volume of 10 µL per well. The samples were analyzed using the following protocol: 95^{O}C for 5 mins (activate SYBR), 44 cycles of 95^{O}C for 10 seconds (denaturation), 60^{O}C for 10 seconds (annealing) and 72^{O}C for 10 seconds. Following these cycles, 95^{O}C for 10 seconds (acquisition of fluorescence) and 72-95^{O}C in 0.5^{O}C increments (melt curve acquisition). All qPCR samples were run in technical triplicates on each plate. Agarose gels were initially run after the PCR protocol on oocytes using the products of the PCR

wells to ensure accuracy of the primers in sample types (See appendix II for gel images). Primer size (bp) was compared with the gel images to ensure the primer specificity, importantly, with the newly designed "BovB" and "LINE-1" primer sets.

A standard curve was completed for each primer set using the 200 MII oocytes described above. 4-5 serial dilutions were used to determine the efficiency of each primer, which were then later used in the gene expression analysis. Calculated efficiencies between 90-110% were deemed acceptable. Primer sequences and efficiencies can be found in **Table 2** and **Table 3** below.

Previous studies have illustrated a number of reliable reference genes (Li *et al.*, 2016b; Barrera *et al.*, 2017). Four candidate housekeeping genes for relative quantification were utilized for the oocyte group using qPCR and the same protocol described above. The geNorm algorithm within the qBase application software was used to identify a minimum of two stable reference genes for each data set in bovine oocytes. Beta-actin (ACTB) and Peptidylprolyl isomerase A (PPIA) mRNAs showed stable expression between oocyte and cumulus cell treatment groups were selected. An additional GeNorm analysis was run with the different groups and stages of embryo development, and the following genes, ACTB, PPIA and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) showed the most stable expression (results shown below). GeNorm analysis was run on both the control and heat-treated samples for each developmental stage.

Table 3. qPCR Reference Gene Primer Sets

Gene Symbol	Gene Name	Product Size (bp)	GenBank Accession #	Primer Sequence Sets (5'- 3')	Efficiency (%)	Source
ACTB	*Beta-actin*	186	NM_173979.3	F: CACCATTGGCAATGAGCGGTTC R: AGGTCTTTGCGGATGTCCACGT	93.0	Reichmann et al., 2012
PPIA	*Peptidylprolyl isomerase A*	111	NM_178320.2	F: CCTTCCTGGGCATGGAATCCT R: TCTTCATTGTGCTGGGTGCC	100.0	Carmignac, et al., 2019
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*	153	NM_001034034.2	F: TTCCTGGTACGACAATGAATTTG R: GGAGATGGGGCAGGACTC	103.2	Ferris et al., 2016

Table 4. qPCR Transposon Primer Sets

Transposon Target	Primer Name	Product Size (bp)	GenBank Accession #	Primer Sequence Sets (5'- 3')	Efficiency (%)	Source
LINE-1	LINE-1	200	DQ273009.1	F: TCCCTATCAAGCTACCAACGG R: TCTGTCTTTGTGCCAGTACCA	99.3	Designed using Primer 3 software
LINE-1	L1	133	-	F: GCAATCCCTATCAAGCTACCA R: TGTTCCTCCAGTTCCATTCTT	93.5	Li et al., 2016
BovB	BovB	147	DY223095.1	F: TCTTCGTGGACACTGGCTTC R: CGAGGACGTCTTTGGGAACA	99.3	Designed using Primer 3 software
ERV1	ERV1	277	-	F: TGTTAAGCTCAAAGACCCACAC R: CCGTTCAGGAATTTCAGACAA	95.9	Li et al., 2016

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 6 statistical software (GraphPad Software). Housekeeping genes (ACTB, PPIA) were used to normalize oocyte qPCR data and determine relative oocyte expression levels. Data was analyzed using a two-way analysis of variance (ANOVA), which was followed by a Tukey's post-hoc test to determine the significance of differences between the heated and control groups. A minimum of three biological replicates was analyzed for each condition. Data shown represent by the mean +/- standard error of the mean (SEM) for TE expression. Statistical significance was defined as having a p-value less than 0.05 ($P<0.05$).

An unpaired 2-tailed t-test was run in GraphPad Prism 6 statistical software on each of the embryo data sets and cumulus cells to determine the significance between the heated and control groups of the analyzed developmental stage. A minimum of three biological replicates was analyzed for each condition. Data shown represent by the mean +/- standard error of the mean (SEM) for TE expression. Statistical significance was defined as having a p-value less than 0.05 ($P<0.05$).

Cleavage and blastocyst rates were analyzed in GraphPad Prism 6 statistical software. Rates underwent an unpaired 2-tailed t-test to determine any statistical significance. A minimum

of three biological replicates were used for each data set. Significance was defined by a p-value less than 0.05 ($P<0.05$).

1.4 Results

Reference Gene Selection

In order to properly normalize and quantify RNA expression data using qPCR, stable housekeeping genes need to be identified and run with each biological replicate. The computer software geNorm was used to calculate the stability of the reference genes. The system uses ranked stability values (M) and pairwise variations (V) to determine the most suitable reference gene where M values less than 0.5 rare present for stably expressed genes and V values below 0.15 indicate the minimum number of genes needed (Sarker *et al.*, 2018).

For the oocyte maturation phase (GV & MII), four candidate genes were considered including ACTB, PPIA, YWHAZ and GAPDH. These candidates were chosen as they have been used in previous studies analyzing transposon expression in oocytes and embryos (Reichmann *et al.*, 2013; Li *et al.*, 2016b; Carmignac *et al.*, 2019). Candidates were run using qPCR on the two treatment groups in two developmental stages (GV & MII). Analysis was run identifying the most stable reference genes within each individual developmental stage and amongst different developmental stages. ACTB (M=0.23 , V=0.103) and PPIA (M=0.265 , V=0.103) were ultimately selected and used to normalize the RNA expression levels in the denuded oocytes as shown in **Figure 9** below.

For the mature cumulus cells, the same four candidate genes were considered including ACTB, PPIA, YWHAZ and GAPDH. Candidates were run using qPCR on the two treatment groups in the three developmental stages. Analysis was run identifying the most stable reference genes within each individual developmental stage. ACTB (M=0.27 , V=0.133) and PPIA

(M=0.235 , V=0.133) were ultimately selected and used to normalize the RNA expression levels in the mature cumulus cells as seen in **Figure 10** below.

For the embryo development phase (2-4, 8-16, blast cell), five candidate genes were considered including ACTB, PPIA, YWHAZ, GAPDH, and SDHA. These candidates were chosen as they have been used in previous studies analyzing transposon expression in oocytes and embryos (Reichmann *et al.*, 2013; Li *et al.*, 2016b; Carmignac *et al.*, 2019). Candidates were run using qPCR on the two treatment groups in the three developmental stages. Analysis was run identifying the most stable reference genes within each individual developmental stage. A GeNorm analysis was run independently for each developmental stage because different references were suggested for each subsequent stage. For the 2-4 cell stage, ACTB (M=0.26 , V=0.112) and GAPDH (M=0.19 , V=0.112) were ultimately selected and used to normalize the RNA expression levels in the embryos as seen in **Figure 11** below. For the 8-16 stage, ACTB (M=0.13 , V=0.062) and PPIA (M=0.1 , V=0.062) were selected and used as seen in **Figure 12**. Lastly, for the blastocyst stage, ACTB (M=0.059 , V=0.035) and PPIA (M=0.1 , V=0.035) were selected and used as seen in **Figure 13**.

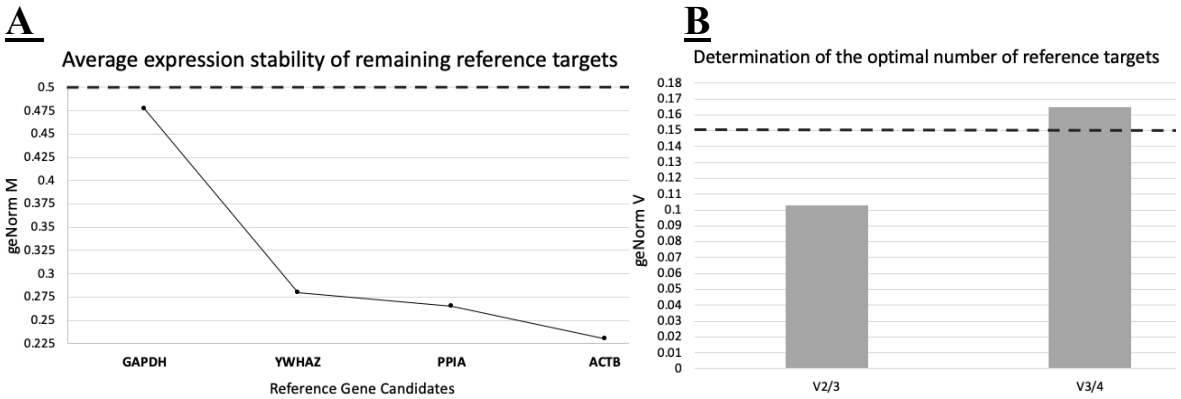

Figure 9 - Optimal reference gene selection for denuded oocytes using the software geNorm. A) Using samples from denuded oocytes from both GV and MII developmental stages, PPIA and ACTB are the most stable candidate genes to use. **B)** Two reference genes are indicated as the most suitable number to use with these samples. The black dashed line indicates the stable threshold values (M=0.5 and V=0.15).

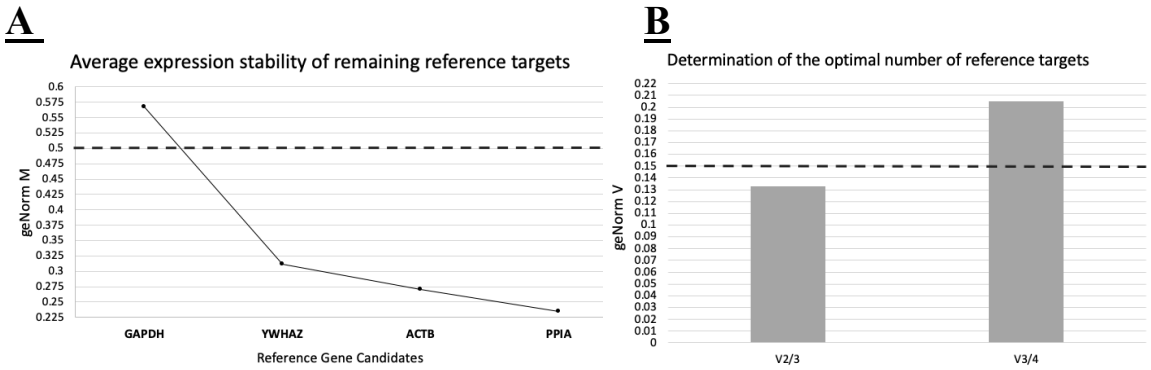

Figure 10 - Optimal reference gene selection for mature cumulus cells using the software geNorm. A) Using samples from mature cumulus cells, ACTB and PPIA are the most stable candidate genes to use. **B)** Two reference genes are indicated as the most suitable number to use with these samples. The black dashed line indicates the stable threshold values (M=0.5 and V=0.15).

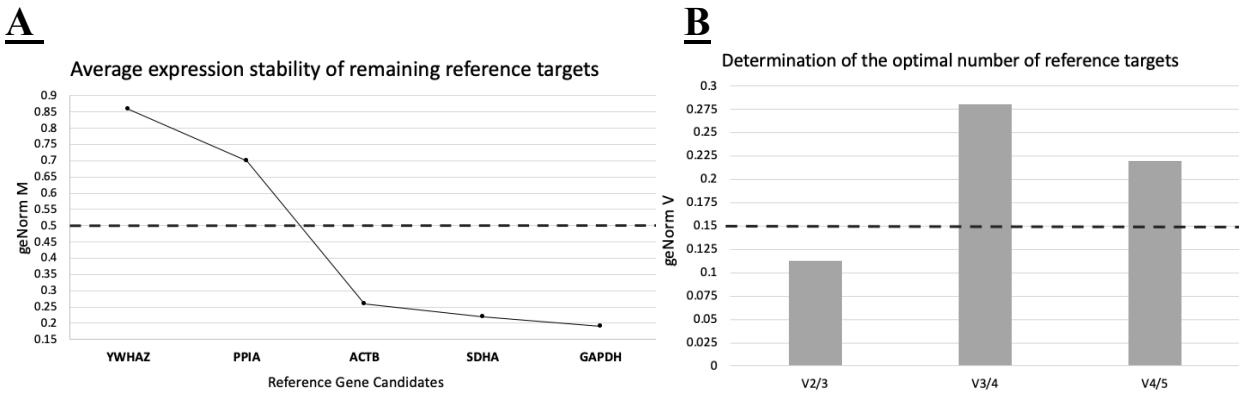

Figure 11 - Optimal reference gene selection for 2-4 cell embryos using the software geNorm. A) Using samples from mature cumulus cells, ACTB and GAPDH are the most stable candidate genes to use. **B)** Two reference genes are indicated as the most suitable number to use

with these samples. The black dashed line indicates the stable threshold values (M=0.5 and V=0.15).

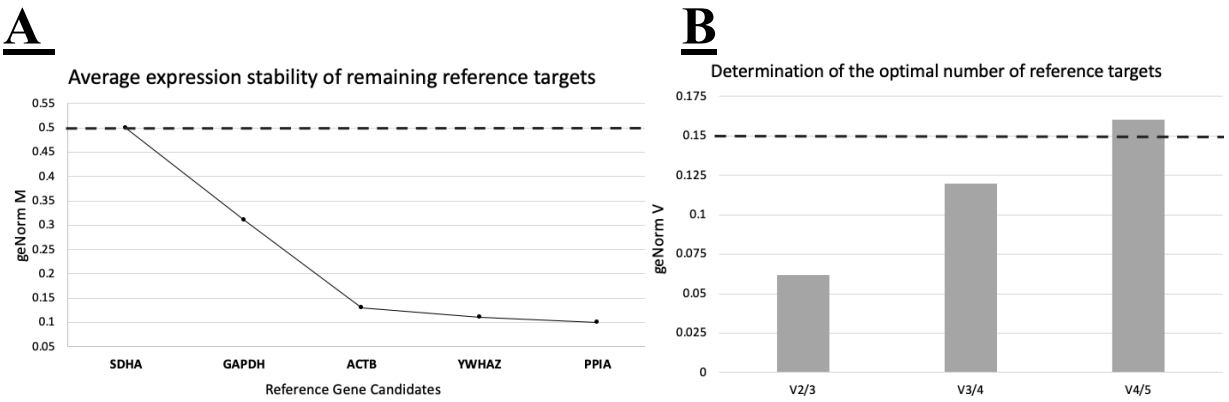

Figure 12 - Optimal reference gene selection for 8-16 cell embryos using the software geNorm. A) Using samples from mature cumulus cells, ACTB and PPIA are the most stable candidate genes to use. **B)** Two reference genes are indicated as the most suitable number to use with these samples. The black dashed line indicates the stable threshold values (M=0.5 and V=0.15).

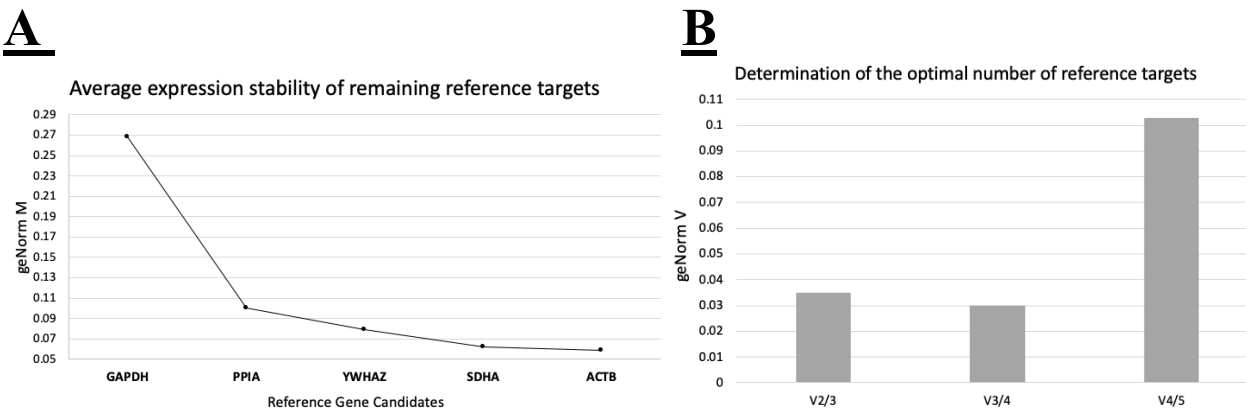

Figure 13 - Optimal reference gene selection for blastacyst embryos using the software geNorm. A) Using samples from mature cumulus cells, ACTB and PPIA are the most stable candidate genes to use. **B)** Two reference genes are indicated as the most suitable number to use with these samples. The black dashed line indicates the stable threshold values (M=0.5 and V=0.15).

TE expression levels in control and heat stressed oocytes and cumulus cells

Quantitative RT-qPCR was used to determine the relative expression levels of TE transcripts using the four TE primer sets (LINE-1, L1, ERV1, BovB). At least three biological replicates were used and relative expression was quantified against the two optimal reference genes (PPIA and ACTB). Denuded oocytes from two stages of oocyte maturation (GV and MII) and corresponding cumulus cells from mature oocytes were used in order to determine the effects of heat on TE expression levels over the course of maturation. LINE-1 transcript expression was slightly lower in denuded oocytes in both heated GV and MII developmental stages compared to the control group whether these changes were assessed with the "LINE-1" or "L1" primers (Figure 14A, B). Similarly, BovB expression was lower in denuded GV oocytes in the heated group compared to the control (Figure 14C), however, control L1 expression levels trended higher in the MII stage (Figure 14B & C) relative to unheated controls. ERV1 expression levels demonstrated a different pattern of expression differences, with the heated group trending to higher expression compared to the control groups in both stages of oocyte maturation (Figure 14D). Similar patterns of expression were seen across all measured TE sub-types in cumulus cell samples (Figure 15). Trends towards lower expression of TEs in heated cumulus cells was observed across all TEs tested. No statistically significant changes were observed in oocyte expression levels for all four TEs in response to heat.

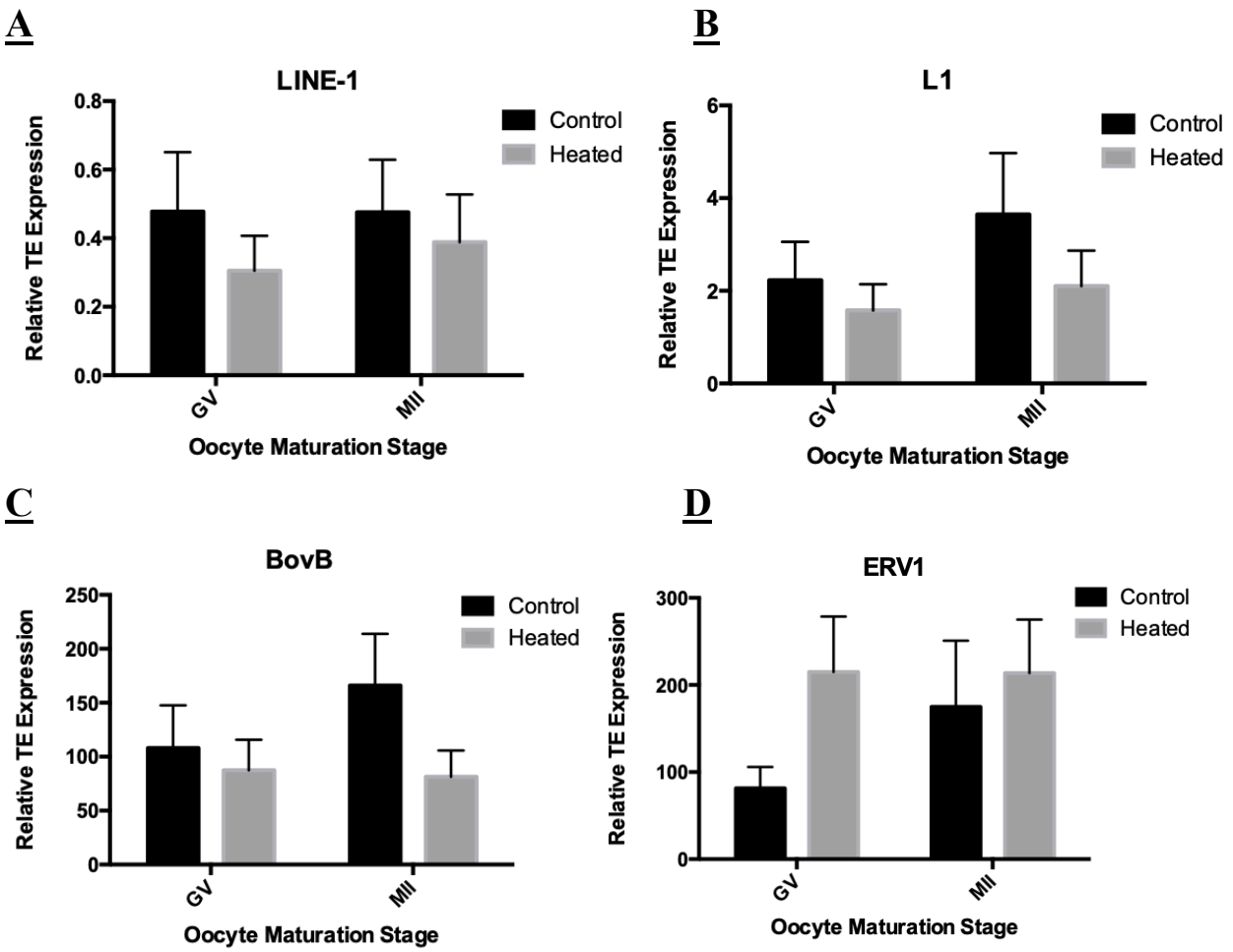

Figure 14 – Relative TE expression levels in GV and MII stage oocytes. A) LINE-1 **B)** L1 **C)** BovB **D)** ERV1 expression levels from oocytes exposed to heat (1 hr at 41^{O}C), and control. Error bars represent +/- SEM and P<0.05. *P<0.05. n=5 for **B** & **C** and n=6 for **A** and **D**.

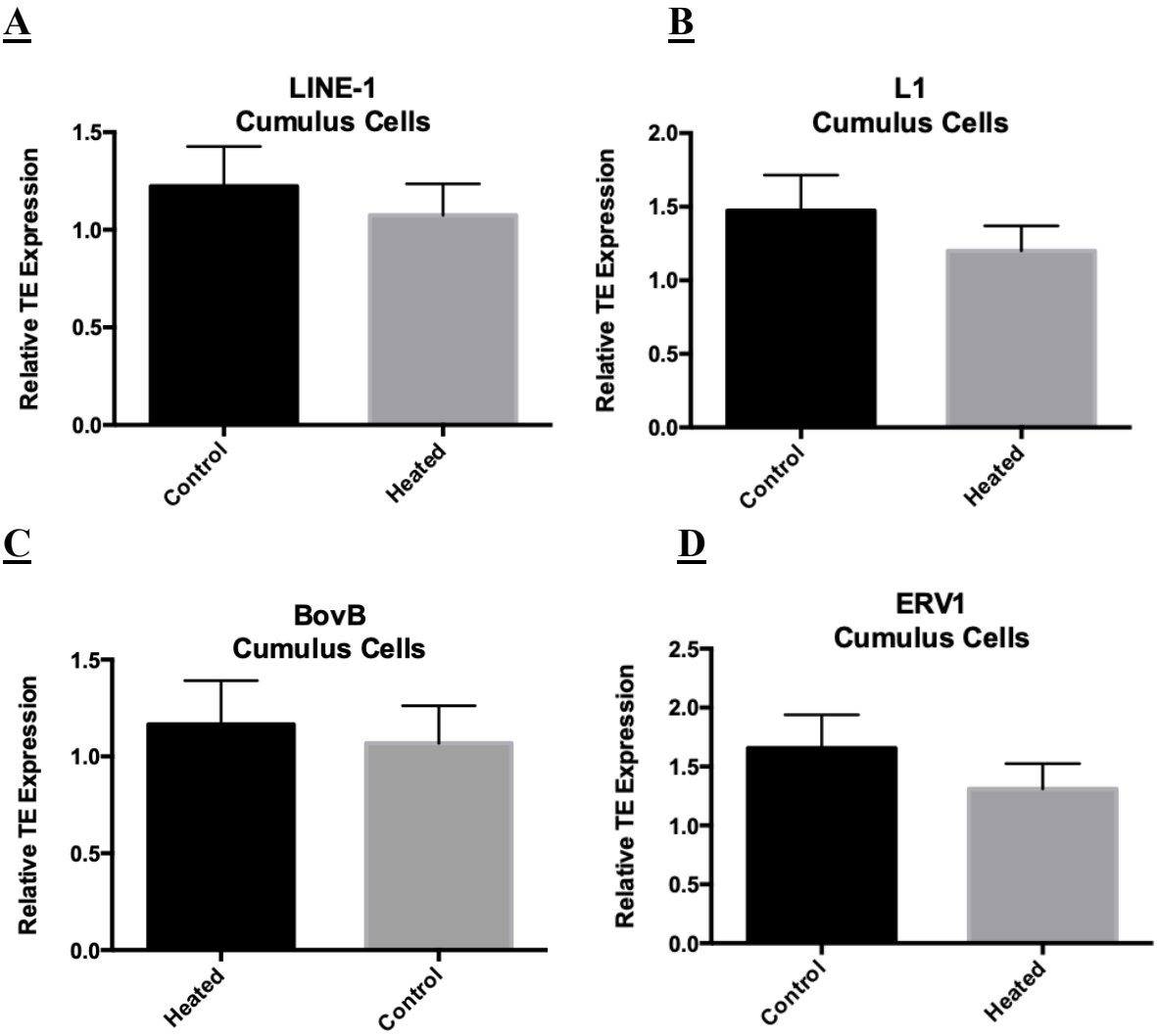

Figure 15 - Relative TE expression levels in mature oocyte cumulus cells. A) LINE-1 **B)** L1 **C)** BovB **D)** ERV1 expression levels from oocytes exposed to heat (1 hr at 41^{O}C), and control. Error bars represent +/- SEM and $P<0.05$. *$P<0.05$. n=4.

TE expression levels in control and heat stressed embryos

Quantitative RT-qPCR was used to determine the relative expression levels of TE transcripts using the four TE primer sets (LINE-1, L1, ERV1, BovB). At least three biological replicates were used and relative expression was quantified for 2-4 cells against the two optimal reference genes (GAPDH and ACTB). A decrease in LINE-1 and L1 expression levels was seen in the heated group compared to the controls (Figure 16A and B). ERV1 remained relatively unaffected at this stage of development between treatment groups (Figure 16 D). BovB displayed differing results, showing an increase in expression levels in the heated group compared to the control group (Figure 16 C). Although trends towards lower expression were seen in L1 and higher expression levels observed in BovB (P=0.09), no statistically significant changes were observed in expression levels in response to heat at the 2-cell stage of development.

Relative expression was quantified for 8-16 cells against the two optimal reference genes (PPIA and ACTB). A decrease in all measured TEs was seen at the 8-16 cell stage of development (Figure 17). A significant decrease (P=0.048) was seen in L1 at this stage of development (Figure 17 B). Similar trends were seen in the 8-16 cell stage of development as the 2-4 cell stage of development, except for BovB which demonstrated a decrease in expression in the 8-16 cell stage (Figure 17 C) in contrast to its expression during other stages.

Relative expression was quantified for TEs in blastocysts against the two optimal reference genes (PPIA and ACTB). At the blastocyst stage of development, all measured TEs displayed an increase in expression level in the heated group compared to the control group (Figure 18). ERV1 (Figure 18D) displayed an upwards trend (P=0.09), however, no statistically significant changes were observed in expression levels for all four measured TEs in response to heat.

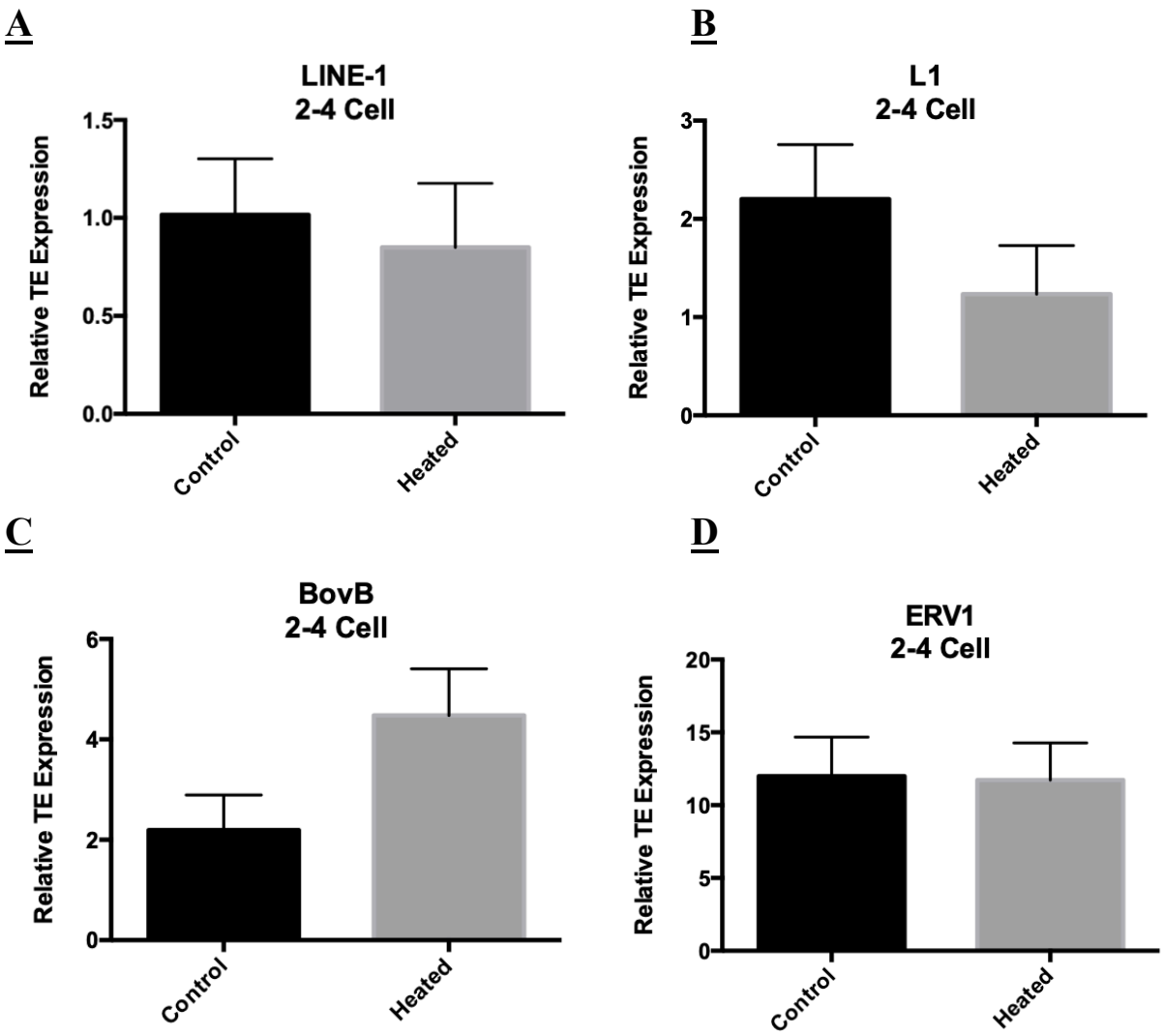

Figure 16 – Relative TE expression levels in 2-4 cell embryos. A) LINE-1 **B)** L1 **C)** BovB **D)** ERV1 expression levels from embryos exposed to heat (1 hr at 41^{O}C) immediately after aspiration, and control. Error bars represent +/- SEM and P<0.05. *P<0.05. n=4.

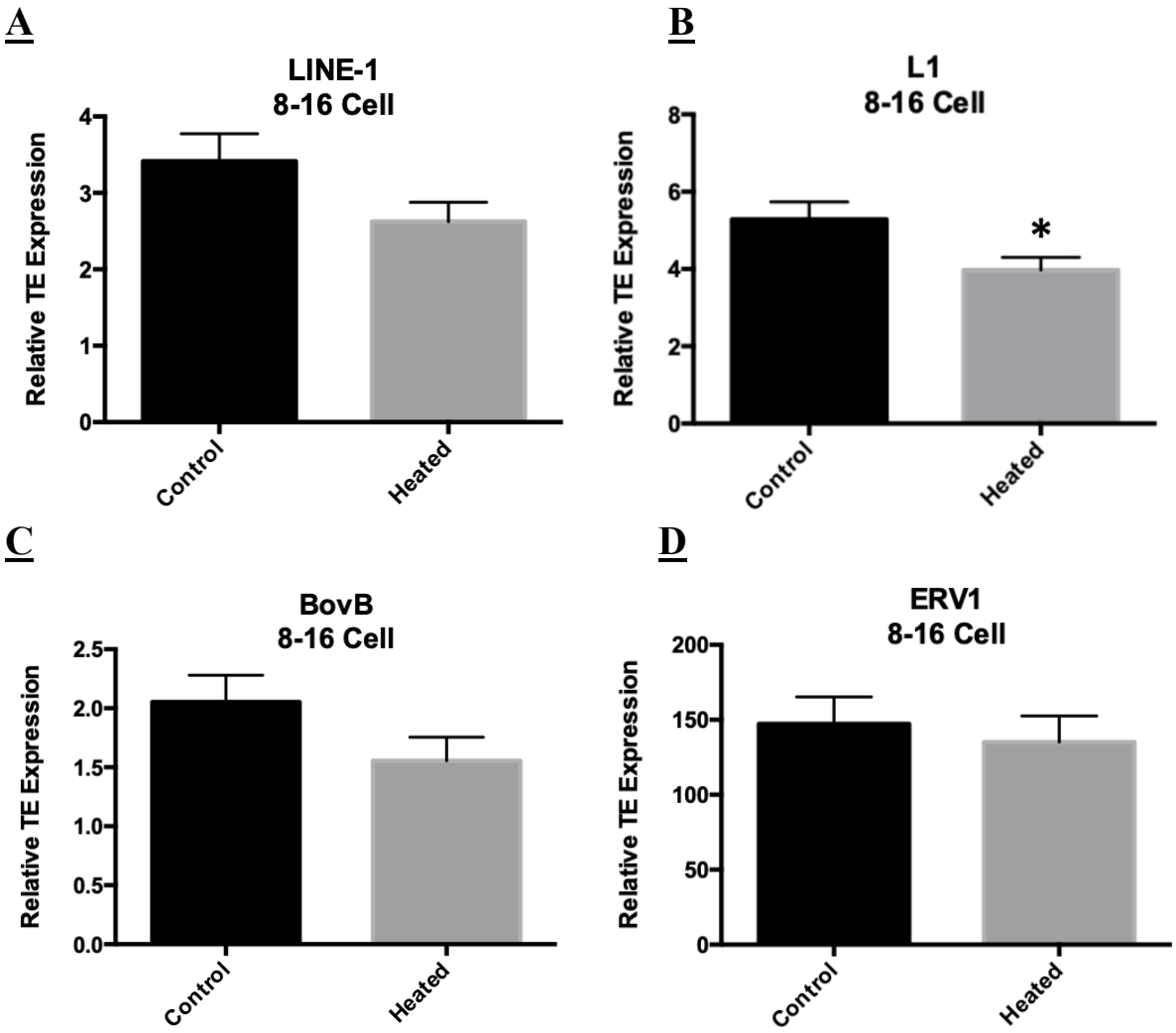

Figure 17 – Relative TE expression levels in 8-16 cell embryos. A) LINE-1 **B)** L1 **C)** BovB **D)** ERV1 expression levels from embryos exposed to heat (1 hr at 41^{O}C) immediately after aspiration, and control. Error bars represent +/- SEM and P<0.05. *P<0.05. n=5.

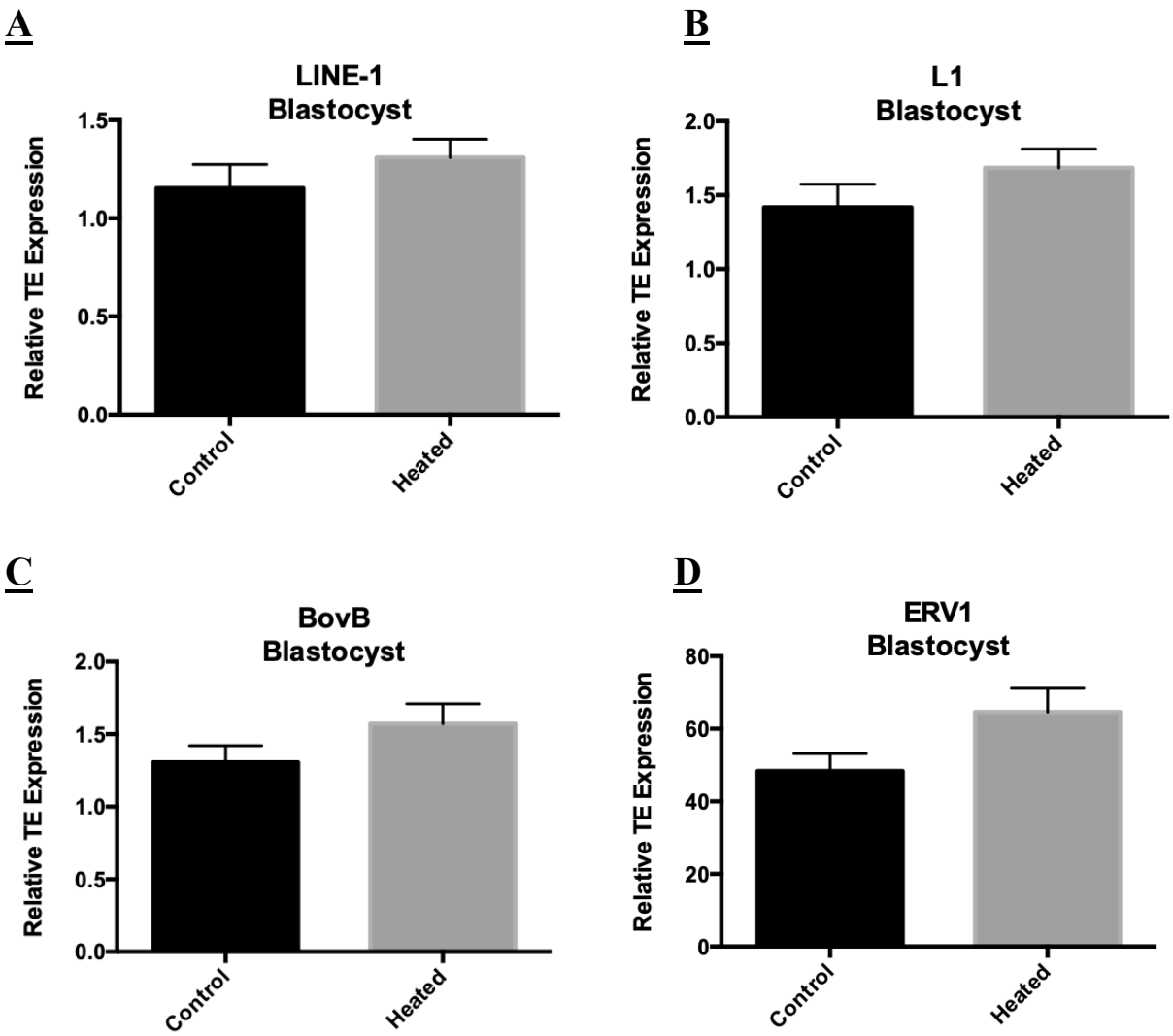

Figure 18 – Relative TE expression levels in blastocysts. A) LINE-1 **B)** L1 **C)** BovB **D)** ERV1 expression levels from embryos exposed to heat (1 hr at 41^{O}C) immediately after aspiration, and control. Error bars represent +/- SEM and P<0.05. *P<0.05. n=4.

Cleavage Rates

For the third objective of this thesis, heat-treated and control oocytes were fertilized after maturation for 24 hours. A significant difference ($P<0.005$) was observed in the cleavage rates of the heat treatment group compared to the control group with an average of 64% (Figure 19).

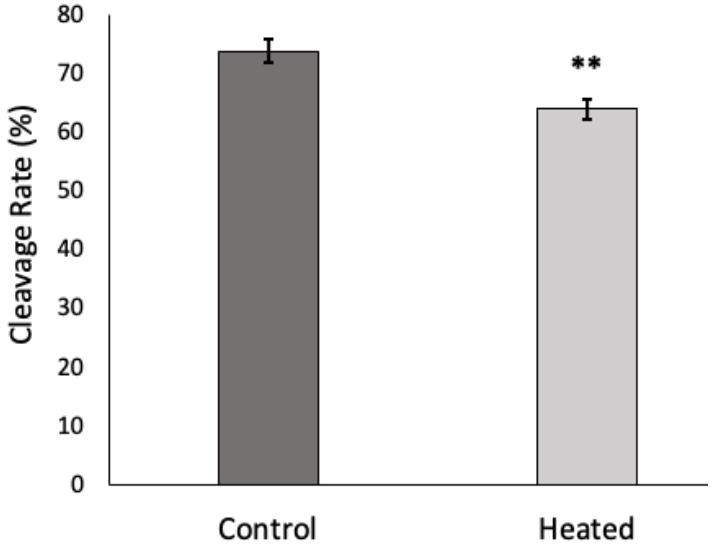

Figure 19 - Cleavage rates after heat treatment (1 hr at 41^{O}C). The total amount (%) of cleaved zygotes at 45-48 hrs post-fertilization (n=6). Error bars represent +/- SEM and $P<0.05$. **$P<0.005$

1.5 Discussion

The current study highlights dynamic trends in the expression of several developmentally important TEs after exposure to heat (1 hr at 41^{O}C) at the oocyte maturation phase of bovine *in vitro* development. Previous studies have illustrated the importance of TEs in oocyte maturation and embryo development, however, little is known concerning the specific expression levels throughout development and how these TEs may change in response to specific stressors (Georgiou *et al.*, 2009). The current study is the first to our knowledge to assess the effects of heat on TE expression levels when bovine oocytes are exposed during maturation, then fertilized and allowed to develop to the blastocyst stage. It is important to note that oocytes were only treated once directly following oocyte aspiration in the GV stage of development. Hence, all results presented here reflect the impacts of exposure only during oocyte maturation. Additionally, low developmental rates and/or changes in heat-treatment groups suggest that environmental stressors on the oocyte may have lasting consequences, and a measurable impact later in embryo development.

The overall success of embryo development relies on the early stages of oocyte maturation. Environmental factors have the possibility to reduce oocyte quality and potential to become fertilized effectively during this stage of development. Oocyte exposure to heat, in the current study, impaired oocyte competence, as cleavage rates were significantly reduced in the heat treatment group compared to the control (Figure 19). This highlights the significance of optimal environmental conditions during the early stages of development. These low rates may be explained by heat stress altering the cellular environment, leading to altered culture conditions, incomplete oocyte maturation, impaired genome activation or DNA fragmentation (Ferré *et al.*, 2020). Environmental stressors have the potential to change DNA methylation

patterns, leading to changes in TE expression which may also cause these impairments and lower cleavage rates (Li *et al.*, 2016b; Barrera *et al.*, 2017). Studies that have exposed developing embryos to longer periods of heat shock (>12 hrs.) have noted no noticeable changes in the cleavage rates (Edwards and Hansen, 1997; Lawrence *et al.*, 2004). This may demonstrate the differences between short-term and long-term exposure to heat stress. Short-term heat stress may have early developmental consequences (lower cleavage rates) but long-term heat stress may have less noticeable developmental consequences early during development (normal cleavage rates). Stressors that occur before fertilization, may have the potential to impact fertility or growth after fertilization, as seen in the current experiment's decreased cleavage rates.

Trends towards changes in the TE expression levels were seen in almost all stages of development between treatment groups, however, a significant decrease in L1 expression was documented at the 8-16 cell stage of the heated group compared to the controls (Figure 17B). It has been previously documented that the bovine embryonic genome becomes fully activated at the 8-16 cell stage (Memili and First, 2000), so it may be possible that profound genetic or TE effects in response to heat are not seen during the early developmental stages. LINE-1 is the most common TE found in the mammalian genome, therefore, it was not surprising to see a significant change in this element during development. However, it was perhaps surprising to see a significant decrease in expression in the heated group compared to the control. Studies have documented the ability of many TEs to become more active and expressed during periods of stress during development (Horváth *et al.*, 2017; Lanciano and Mirouze, 2018), therefore, our observation of lower expression in this context requires further examination. One potential explanation for this contradictory finding regarding not only L1 at this stage of development, but also the other TEs throughout development, could be due to various protective pathways

activated in the genome leading to the degradation of this transcript in the stressed group. There may be an immediate effect associated with the stressor, such as increased expression, but a compensating increase of silencing genes and pathways may also be present (Lapp and Hunter, 2019). The specific timing of this initial expression and any compensating silencing pathway, is unknown, but is worth exploring in the future. In addition to the PIWI pathway discussed already in the literature review, there are other specific TE silencing mechanisms and pathways. One of these is histone modifications. H3K9 is a histone modification that has been seen to silence transcription and lead to heterochromatin formation (Kouzarides, 2007; Hyun *et al.*, 2017). Specifically during times of acute stress, levels of H3K9 are increased (Lapp and Hunter, 2019). Interestingly, this histone marker is highly abundant in the maternal pronucleus and studies have identified high levels of H3K9 in regions of high LINE/LTR density (Pauler *et al.*, 2009; Canovas and Ross, 2016). We suspect that, in the current study, increased H3K9 may be present due to the acute heat stress, silencing LINE for the majority of development up to the blastocyst stage. Future studies should measure global H3K9 levels throughout development, with particular emphasis on the 8-16 cell stage where significant decrease in TE levels are seen.

During oocyte maturation, all LINE-1 primers (LINE-1, L1 and BovB) identified similar trends in expression (Figure 14). A trend towards decreased expression levels in heated GV and MII oocytes were seen compared to the controls, and higher control MII expression compared to GV controls (Figure 14B and C). These trends were slight, and were not statistically significant. Because these trends were so slight, heat stress under the current experimental conditions may not change the measured TE expression levels during this stage of development. Additionally, one-hour of heat stress may have not been a long enough period to measure quantifiable changes of these TEs during maturation.

BovB expression diverged from LINE-1 and L1 at the 2-4 cell stage of development where there was a trend towards increased expression with the heated group compared to the controls (Figure 16C). Although some trends were notable, no statistically significant changes were observed, perhaps due to the limited sample size. Some LINE-1 groups approached statistical significance, specifically during the 2-4 cell stage of development (Figure 16 B & C, P=0.09), so we speculate that with additional replicates, statistical significance may be achieved. Any identified changes in LINE-1 TE expression levels may ultimately impact downstream genes that affect fertility outcomes and embryo development, and may be a factor contributing to the low developmental rates in the current study. One report examining human embryos and LINE-1 expression levels established that, during the GV stage, a cluster of hypermethylated LINE-1 elements upstream of an androgen receptor (AR) gene caused the AR gene to be expressed during the GV stage of development (Yu *et al.*, 2019). Importantly, AR genes are responsible for follicle maturation and growth, reinforcing the importance of appropriate TE expression levels during development for overall embryo success (Gleicher *et al.*, 2011) and suggesting one possible mechanism by which they impact development. AR genes have also been seen to contribute to additional early fertility events including the activation of the germinal vesicle breakdown (Li *et al.*, 2011). These studies illustrate the cascade effects that might ensue if LINE-1 expression levels become altered by environmental stressors during early development, and the consequences this might have on the overall embryo fertility.

Information regarding BovB elements is limited and it is unknown whether this TE directly impacts nearby genes related to embryo development and fertility success. It is, therefore, difficult to suggest any mechanism that may underlie the trends towards altered expression levels. It is known that BovB resides in the same family (LINEs) as LINE-1, and the

overall expression patterns seem to be similar during oocyte maturation, 8-6 cell, and blastocyst stage of embryo development (Figure 14, 17 and 18), suggesting that it may act in a similar manner to the LINE-1 elements in this regard (Ivancevic *et al.*, 2018). Additionally, information regarding BovB's ability to transfer between animal species via a vector species (eg. tick or virus) has been emerging in the past few years, so it may be more prevalent in the genome and in multiple species than previously recognized (Ivancevic *et al.*, 2018). The current results illustrate the element's unusual stage specific changes as it demonstrated a trend towards altered expression at the 2-4 cell stage compared to the other LINE primers. It may be interesting to look into this differentiation further and determine whether low levels of targeting piRNAs or other factors may be contributing to this increase in expression during this specific stage of development.

There are a number of important defense mechanisms that have evolved to help protect the genome against damage caused by TEs. One of these defense mechanisms is the PIWI pathway. The bovine PIWI pathway is essential to early embryo development (Roovers *et al.*, 2015). PIWI protein expression varies throughout bovine development, peaking at the 2-4 cell stage where it significantly decreases at it develops to the blastocyst stage (Russell *et al.*, 2016). Interestingly, at the blastocyst stage, ERV1 (Figure 18D, P=0.09) displayed an upwards trend in expression in the heated group. Once again, this was not a statistically significant finding, however results were trending towards significance, so we speculate with additional replicates this may be achieved. One possible explanation for this may be the PIWI pathway. This pathway may be facilitating the control of TE expression and over-expression that may be induced by heat stress throughout oocyte maturation and the 2-4 cell and 8-16 cell stage of development, however, at the blastocyst stage as the levels of PIWIL1 significantly decline, the measured TEs

display an increased expression in the heated group. Previously, PIWI proteins were only identified in male reproductive organs and sperm, but as we learn more about the role of the PIWI complex in female reproduction, the more we are understanding their important cellular role in mediating appropriate development. Very recent studies have confirmed the presence of additional PIWI proteins (PIWIL3) in bovine and human oocytes (Roovers *et al.*, 2015; Tan *et al.*, 2020). Between other TE groups at the blastocyst stage, there was a lack of significant differences which may suggest that the ERV class of TEs respond differently to stress than LINE-1 elements during maturation and development. During the GV and MII stage of maturation, ERV1 was the only element to show a trend towards increased expression compared to the controls (Figure 14D). Also unique to this element is the observation that the denuded oocytes and cumulus cells responded differently to heat stress, implying that there may be defense mechanisms present in the cumulus cells controlling TE activation and expression that may not be present in the oocytes. One study examining ERV expression levels in early mouse development found that the expression levels remained relatively low throughout oocyte maturation and only peaked after fertilization at the 4-cell stage of embryo development (Gifford *et al.*, 2013). This is opposing the results of the current study as the MII stage trended toward higher expression level than the GV stage of oocyte maturation (Figure 14D). The repression of this ERV element during oocyte maturation in Gifford *et al*. (2013) study can be partially attributed to a zinc finger protein 42 (ZFP42), which is consistent with the results and discussion of other work (Guallar *et al.*, 2012). This may be the result of lower ZFP42 levels in bovine than in mice or other species. ERV elements have been shown to play a role in the restriction of infection from other viruses such as murine leukemia viruses (MuLVs) in rodents (Gifford *et al.*, 2013). If they play a similar role in cattle, increased ERV expression during maturation in the

presence of a stressor may contribute to greater developmental success of the embryo (Hare et al., 1985). Interestingly, in studies utilizing high oxygen tension as an environmental stressor of bovine embryos, increased expression at the blastocyst stage was also seen, expanding the relevance of the current ERV1 results (Li *et al.*, 2016b). These studies may indicate ERVs particular developmental significance or importance in the cellular stress response. There has been some suggestion that ERV elements can act as regulatory elements in human preimplantation embryos, meaning they help control various gene expression patterns (Pontis *et al.*, 2019). ERV regulatory gene expression has been reported later on in embryo development at the 8-16 cell stage, therefore, it is interesting that we have noted trends toward expression level changes before fertilization. Changes to these regulatory elements may have the potential to alter phenotypes which can lead to diseases and problems such as those involving fertility (Sultana *et al.*, 2017). Overall, there is potential for the involvement of multiple pathways and genes in suppressing ERV expression during early development that may not be present or present at different levels during oocyte maturation and this topic remains to be further explored.

Interestingly, the expression levels of LINE-1 as detected by both primer sets ("LINE-1" and "L1") had slight variations during development, specifically at the 2-4 cell and 8-16 cell stages (Figure 16 and 17A, B). Both primer sets were designed to amplify the LINE-1 transcript, but target different regions of that transcript. Slight differences in the relative expression levels (specifically at the 8-16 cell stage with L1 having a significant decrease in expression vs. LINE-1 not exhibiting a significant decrease) highlight one misconception regarding TE structure- that all copies present in the genome are present as full-length elements. Large numbers of full and partial sequences for LINE-1 are present in the genome (Luo *et al.*, 2016) and the trend towards differences in LINE-1 expression in response to heat stress is likely due to transcription of some

partial sequences that are covered by only one set of primers. This is why the modern genome of eukaryotic organisms has been described as a heterogenous soup of modern and ancient, full-length and shortened, active and broken TEs (Gifford *et al.*, 2013). A graphical representation describing the genesis and expression of LINE-1 elements of different lengths is presented below and described elsewhere (Figure 20) (Lowe *et al.*, 2007).

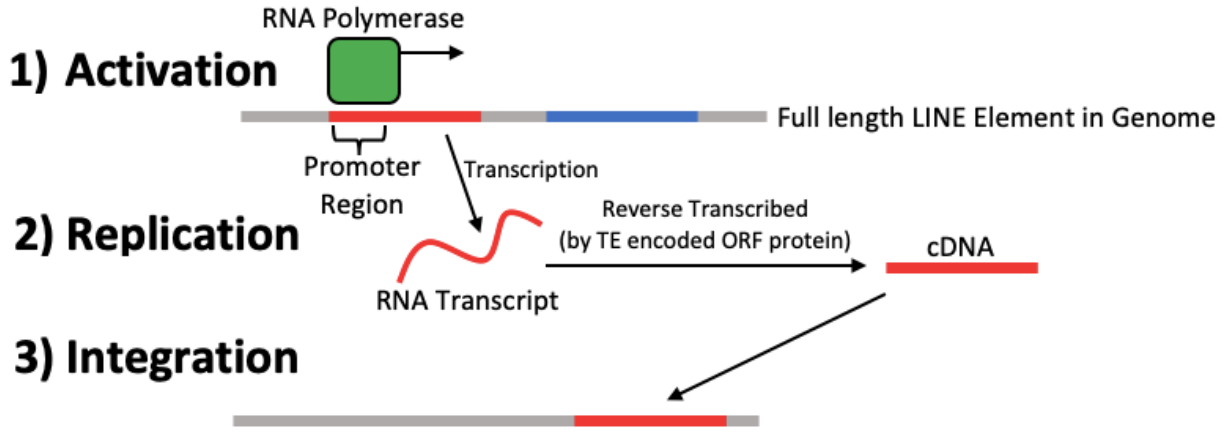

Figure 20 – Integration of truncated LINE elements into the genome. Some TEs within the genome are represented by a full and complete gray LINE element in the genome, or others may only be the red or blue segments. This means that there may be transcripts of longer length LINEs or shorter lengths LINEs. **1)** The first step is activation where smaller/truncated parts of the LINE element can be transcribed as long as they contain a promoter region. **2)** Once transcribed into an RNA transcript, they are reverse transcribed by its own RT encoded in the ORF protein. **3)** Once replicated to cDNA, the element can be integrated into another section of DNA.

This was the first study, to our knowledge, to measure TE expression in denuded oocytes and associated cumulus cells separately. This was done to identify cell-specific expression patterns of TEs and determining if TEs were only expressed in oocytes. Surprisingly, all four measured TEs were expressed in both cumulus cells and denuded oocytes (Figure 14 and 15). Cumulus cells do not undergo reprogramming in the same manner as oocytes. However, these cells are connected to the oocyte through transzonal projections (TZPs) and gap junctions that form a network mediating cross-talk between the two structures (Figure 21) (Nuttinck, 2018;

Baena and Terasaki, 2019). Previous studies have illustrated the importance of this cumulus-oocyte dialogue in development and how the environment may alter this dialogue impairing the developmental competence of the oocyte (Nuttinck *et al.*, 2011; Richani and Gilchrist, 2018). This dialogue is essential for proper oocyte maturation as shown in a previous study where inhibition of cumulus prostaglandin E2 was shown to impact oocyte fertility by decreasing meiosis progression (Nuttinck *et al.*, 2011). Through these connections, there has been evidence that small molecules such as metabolites and even larger RNA can be shared between cumulus and oocyte (Macaulay *et al.*, 2014; Biase and Kimble, 2018). It may be interesting to hypothesize that this transfer of resources could include TEs as well, because both cumulus and oocyte had expression of all four TEs measured in the current experiments. This may also explain the surprising observation in the current study that there was a difference between the denuded oocytes trending towards higher expression in the heated group of ERV1 (Figure 14D) but trending towards lower expression in the cumulus cells of the heated group (Figure 15D). As mentioned in the introduction, one previous study has reported LINE-1 expression in cumulus cells from women suffering from PCOS (Pruksananonda *et al.*, 2016). The results showed higher LINE-1 levels in the cumulus cells of mature (MII) oocytes in PCOS patients with no change in the immature (GV) stage oocytes (Pruksananonda *et al.*, 2016). This is why we chose to examine cumulus cells from mature oocytes, however, now that we have identified some expression in these cells, it may be useful to measure GV stage cumulus cells and see how they differ from their counterparts surrounding mature oocytes. Collecting additional replicates may also statistically resolve changes that can only be reported as trends in the current results.

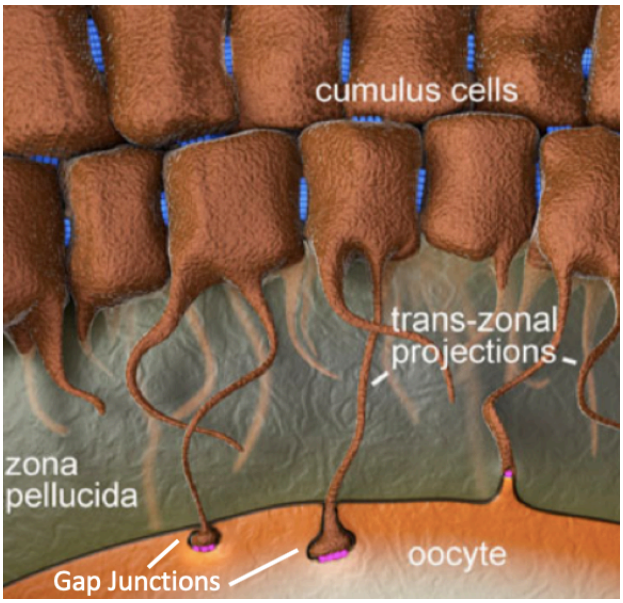

Figure 21 – Interaction between the cumulus cells and the oocyte. Transzonal projections reach from the cumulus cells towards the oocyte where they form gap junctions, allowing them to can pass small molecules back and forth and interact with one another. Adapted from Winterhager and Kidder, 2015.

It is surprising to some extent that GV stage oocytes display trends towards changes between heat and control groups in all measured TEs as GV oocytes were collected directly following the heat treatment, and this may not have allowed adequate time for TE expression levels to be altered in that stage. This was done to establish a baseline level of oocyte expression against which any rises in development could be measured. It is probable that TEs induced as a normal part of development take time to become transcribed and accumulate in the cell following environmental stress. This may be why over-expression started to emerge in the heated group at the blastocyst stage of development (Figure 18). Even though the general level of transcription in maturing oocytes is very low, it is particularly interesting to find TE expression through this period (Figure 14). Due to this early response, the factors and mechanisms that account for this are likely to be particularly important in development. In particular, a role for the transzonal projections described above warrant further study.

In addition to the timing of heat stress, a supplementary factor is the duration of exposure to that stress. The current study exposed all oocytes to one-hour of an elevated temperature, which is important for mechanistic dissection of the underlying timing, but does not fully reflect naturally occurring heat stress. A previous paper tracking the changes in the heat shock (HS) proteins in the maturing bovine oocyte and developing embryo examined four different time-intervals where oocytes were exposed to heat; 6-hours, 12-hours, 18-hours and 22-hours (Báez *et al.*, 2019). It was noted in the results of that study, that only in the 22-hour exposure groups were some specific developmental abnormalities observed. All groups did show decreased maturation of GV oocytes to the MII stage. Additional studies have increased the heat stress period during maturation up to 24 hours, where internal changes such as fewer total nuclei, abnormal chromosome organization and an amorphous zona pellucida were observed (Lawrence *et al.*, 2004; Báez *et al.*, 2019). These results suggest that increasing the duration of heat exposure may be more likely to induce developmentally relevant TE expression in this period of development.

In summary, the data presented in this chapter partially supports the hypothesis that heat as an environmental stressor may cause changes in the expression levels of some TEs in the maturing bovine oocyte *in vitro*, although changes during some of the stages of development are limited in their statistical significance. The changes observed in the LINE elements (LINE-1, L1 and BovB) and the developmental rates suggest possible downstream consequences in the overall embryo development and fertility. This is relevant as there is previous evidence that LINE elements are necessary for different aspects of developmental success. The variability in oocyte TE expression results may indicate that the effects of heat may impact each oocyte uniquely or that maturation may represent a time-point too early in development to be strongly affected in terms of TE expression changes. By continuing our studies of TE expression levels, and

incorporating different TE subtypes, our understanding of these unique sequences of DNA and their roles during oocyte maturation and embryo development should be enhanced.

GENERAL DISCUSSION

A decline in mammalian, and particularly bovine, fertility over the past decade has emerged, with infertility becoming a significant challenge for many producers to overcome. This trend has become pronounced in the dairy industry with a trend towards a decline in overall bovine fertility well-documented (Santos *et al.*, 2004; Diskin and Morris, 2008). Possible explanations could include an increase in selection for milk production traits, or environmental influences such as stress (Lucy, 2001). The goal of many producers is to improve cattle resistance to environmental stressors, while minimizing the adverse effects on productivity. It has been extensively documented that the reproductive performance and fertility of lactating cows is reduced during periods of heat stress (Thatcher, 1974; Collier *et al.*, 1982; Hansen and Aréchiga, 1999). Methods such as timed AI procedures, sprinklers and fans have been recommended and used to increase the pregnancy rates and cool down the herd during these periods (Collier *et al.*, 2006). Often these methods can be costly and time consuming to the farmers. Although the pregnancy rates have improved when these methods are employed, they are not perfect, and it is important to understand the underlying epigenetic regulatory pathways to help design and implement further strategies to improve the reproductive performance of these animals.

This thesis is one of the few studies to analyze the effects of environmental stressors on TE expression and overall fertility during the early stages of embryo development. This is also the first study of its kind to analyze TEs in bovine cumulus cells when exposed to an environmental stressor. The overall goal of this thesis was to investigate any association between environmental stressors, particularly heat, and changes in several developmentally important TEs throughout embryogenesis.

Collecting additional samples and identifying additional TE sequences would likely have given us a better understanding regarding the overall expression levels of TEs in the maturing oocyte, and how these expression levels change (or do not) in the presence of environmental stressors. Unfortunately, this was not possible in the current research environment due to pandemic related laboratory and sample restrictions. Quantification of small changes during these developmental stages may therefore require additional experiments utilizing a more sensitive method to detect minute shifts in expression levels such as digital droplet PCR (ddPCR). Using larger pools of initial oocytes to increase the measured amount of RNA may also eliminate the error of a low template concentration if ddPCR is not feasible. It would also be interesting to identify associations between any observed changes in TE expression and the expression levels of genes associated with LINE elements that are known to affect fertility, such as the AR gene, and determine whether expression levels change in line with the documented TE changes when exposed to environmental stressors. Additionally, measuring proteins implicated in defense pathways such as PIWIL1 or DNA methylation markers may be warranted to determine whether any correlations exist when certain TEs are suppressed or over-expressed during development. These steps may further explain fertility consequences that heat stress at the GV stage of development may have during oocyte maturation and embryo development.

Previous studies have identified significant decreases in blastocysts rates during longer periods of heat shock (>12 hrs.) (Edwards and Hansen, 1997; Lawrence *et al.*, 2004) and during periods of high oxygen tension as an environmental stressor (Li *et al.*, 2016b). These studies may demonstrate how long-term exposure to heat stress may have noticeable long-term consequences (lower blastocyst rates) later in development. Unfortunately, the current study did not allow sufficient documentation of blastocyst rates in the heat treated group due to time constraints and

inability to collect ovaries due to the COVID-19 pandemic. Future experiments may seek to assess additional stress-induced damage later in development to determine whether significant fertility responses such as decreased blastocyst rates are seen.

Since we documented the presence of TEs in both cumulus cells and denuded oocytes, it is possible that TEs may be involved in regulatory events. Emerging studies have identified specific regulatory pathways that are altered through heat stress, and the genes involved in these pathways, leading to negative developmental outcomes such as failed cumulus expansion in COCs (Latorraca *et al.*, 2020). With heat shock as low as 12-hours in duration, morphological abnormalities to important oocyte structures such as the zona pellucida are noted, appearing rough and spongy on the surface (Báez *et al.*, 2019). If structures such as these are damaged, it may negatively impact the cross-talk between the oocyte and its surrounding cumulus cells, possibly leading to failed shared products important for oocyte growth or fertilization. Overall, there have been limited studies documenting TEs in cumulus cells, therefore, little is known about the dialogue between these two structures and how TEs may or may not play a role in normal or hindered oocyte development. Previous studies have documented increased LINE-1 levels and DNA methylation marks in cumulus cells of lower fertility patients (suffering from PCOS), suggesting that these cells may play a role in the gene regulation affecting both the pathology and physiology of mature oocytes under specific environmental conditions (Pruksananonda *et al.*, 2016). Here, we provide evidence concerning LINE elements and ERV element expression that have the potential to participate in maturation, however, additional research and experimentation is needed to confirm these trends. Previous studies have utilized a variety of methods including staining RNA and immunofluorescence to visualize RNA movement between cumulus-oocyte gap junctions (Macaulay *et al.*, 2014). These methods may

be employed to stain TEs or various regulatory sequences to visualize additional movement between structures, and how heat may impact this movement.

Limitations to the current study also include the time of year, the season the oocytes were collected and the age of the cattle who provided the ovaries. Although we attempted to ensure consistent aspiration times throughout the data collection period, there were times when only afternoon collection was available versus the morning time period. Additionally, the heat shock performed in the present study may have been too subtle to induce more profound, significant differences in TE expression levels. The global pandemic (COVID-19) caused a laboratory closure for several months, precluding continued data collection and halting ovary collections. This inhibited continued collections of oocytes and additional replicates for the current study. These factors should be taken into consideration for the lack of significance and replicates for the presented data.

Mechanisms behind the heat stress induced TE changes that were observed have not yet been addressed, and subsequent outcomes from these changes have not yet been functionally linked with developmental outcomes. The results in the current study display changes across almost all measured TEs and developmental time points between the heated and control groups. These changes could be dependent on the class or TE sub-type measured, the developmental stage of the embryo or what cells were analyzed. Heat may alter multiple different pathways that may affect these variables differently, accounting for the mixed results seen here. Subsequent studies would be needed, focusing on pathways speculated to be involved in TE expression.

Specific genetic and other consequences of the over or under-expression of TEs during development are not easily identified and were beyond the scope of this study, although we have correlated some developmental parameters with TE expression. There were significant changes

noted in the developmental rates, however, to specifically implicate a specific TE type or level in these differences will be challenging. Additionally, with respect to the expression of TEs, we can note how they change from the control, but it is difficult to say by how many copies. It is important to note that subtle changes in TE expression may have profound impacts on the embryo, however, it is difficult to define the specific threshold level for these impacts. Subtle changes in TE expression throughout development may cause significant fertility problems later in development, but limitations in the laboratory with regard to culturing embryos beyond the blastocyst stage hampers further studies. The TE expression patterns studied here reveal a trend towards increased LINE-1 expression during the blastocyst stage. It would be advantageous to examine TE expression after implantation into a maternal host, which can only be achieved *in vivo*. Furthermore, to more fully reflect the situation *in vivo,* it is likely that TE mediated detrimental effects caused by environmental stressors may require more prolonged exposure above the 1-hour duration studied here. Consistent and prolonged exposure may lead to the accumulation of small changes in TE expression, leading to larger and noticeable phenotypic changes later in development.

By understanding the underlying mechanisms associated with oocyte maturation, embryo development and overall bovine fertility, we may be able to discover important cellular features such as TE expression levels that may help identify embryos that will develop successfully. Environmental stressors will continue to influence mammals, hence, the importance of understanding mechanisms that may inform the development of mitigative measures to improve fertility.

SUMMARY AND FUTURE DIRECTIONS

This research was the first in this field to follow and compare normal TE expression patterns throughout early bovine embryo development. Additionally, it is the first study to identify TE expression in the cumulus cells of bovine oocytes. This thesis completes objective 1 of analyzing the observed effect of TEs in maturing oocytes when exposed to normal and stressed environments, and objective 2 concerning how an environmental stressor may impact subsequent embryo development. By investigating more than one segment of the LINE-1 element through two primers ("LINE-1" and "L1") in addition to BovB and ERV1 primers, we were also able to view similarities and differences amongst different sub-types of TEs. This gave us a wider view of the true diversity of TE expression. These results, in part, support the initial hypothesis concerning the impact that heat, as an environmental stressor, has on TE expression levels in oocytes and embryos and the downstream effects on fertility. We conclude that there are trends towards changes in TE expression in heated oocyte groups compared to the controls across all stages of development, however, lack of statistical significance limits our interpretation of these results. Although heat impacts fertility parameters, there is no evidence in the current study for mechanistic pathways attributing this directly to the over or under-expression of TEs.

This study is important in laying the groundwork and protocols that future research in this field can be built on and results achieved at an accelerated pace. This research can be seen as a pilot study for future experimentation in embryo work for the improvement of assisted reproductive biotechnologies in livestock and humans. There are many directions to follow, and more to discover concerning TEs and how environmental stressors may be impacting organisms at an epigenetic level. The most promising area for future research may lie in establishing connections between epigenetic pathways to TEs and determining how these connections are

influenced by environmental stressors, so that clinical applications can be developed for both human and agricultural reproductive practices. In addition, the novel findings concerning TE expression in cumulus cells warrants further exploration. More replicates need to be analyzed to determine whether these trends reflect statistically significant changes and whether the expression levels change in GV cumulus cells versus mature cumulus cells. Visualizing the potential transfer of TE RNA between the cumulus cells and oocyte will also be interesting to study further. Emerging evidence from these interactions and exchange of genetic information are proving to be important aspects of developmental competence and overall embryo viability.

Although we have gained knowledge concerning how individual reproductive elements respond to heat stress, and how their TE levels change *in vitro*, it is important to continue these studies *in vivo* to truly understand the fertility consequences. In addition, there may be other pathways and mechanisms *in vivo* not present in the laboratory setting that contribute to repressing TE over-expression during these periods of heat stress. To understand how these other reproductive cells and tissues interact with one another and share genomic information (or TEs), it would be invaluable to determine what happens during periods of heat stress in a whole-organism.

To further the agriculture application of this research, experimentation on different breeds of cattle in warmer climates would be of interest. *Bos indicus* (South Asia) cattle are better adapted than *Bos Taurus* (European) breeds to warmer climates, and are less likely to suffer from hyperthermia (Mwai *et al.*, 2015). There was evidence from a study between these two breeds indicating that a greater proportion of *B. indicus* embryos reached higher developmental stages (morula and blastocyst) than *B.Taurus* when heat stressed (Krininger *et al.*, 2003). This evidence suggests environmental adaptive advantages may be in place as early on as embryo development.

It would need to be examined further to determine whether TEs may contribute to this difference between species, but comparing TE expression levels in these species will further illuminate potential differences.

To conclude, establishing significant observed effects of TEs in the oocyte and embryo during periods of environmental stress may enhance our understanding of environmental influences on female fertility. This work will allow for a better understanding of pathways associated with TE suppression such as DNA methylation, histone changes and the PIWI pathway, and under what conditions and where TEs are expressed. By enhancing our understanding, we may ultimately implement appropriate clinical and management to protect livestock and other species against potentially harmful outcomes of TEs.

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APPENDIX I

All reagents were prepared in the Reproductive Health and Biotechnologies Laboratory, the University of Guelph, Guelph, ON

IVF and IVC Reagents:

Table 1. HEPES/Sperm TALP Reagent Constituents

Chemical	**HEPES/SP Salts**	
Sodium Chloride (NaCl)	14.25 g	28.5 g
Potassium Chloride (KCl)	0.59 g	1.18 g
Sodium Phosphate Monobasic (MW=137.99)	0.12 g	0.24 g
Calcium Chloride ($CaCl_2$)	0.735 g	1.47 g
Magnesium Chloride ($MgCl_2$)	0.5 g	1.01 g
Water	500 mL	1000mL

Table 2. IVF Talp Reagent Constituents

Component	**nM**	**mg/L**
Sodium Chloride (NaCl)	114	6669
Potassium Chloride (KCl)	3.2	238.4
Sodium Phosphate Monobasic (MW=137.99)	0.4	55.2
Calcium Chloride ($CaCl_2$)	2	222.2
Magnesium Chloride ($MgCl_2$)	0.6	101.65
Sodium Bicarbonate	25	2100
Sodium Lactate	10	1.416
Volume		
Sodium Pyruvate	1 mL	
Gentamicin	100 µL	
Heparin	1mL	

Agarose Gel Reagents:

Table 1. 1% Agarose Gel Constituents

Component	**Volume/Mass**
Agarose Powder	1 g
TAE Buffer	100 mL
Ethidium Bromide (EtBr)	3.5 mL

Table 2. 50x TAE Buffer Stock Solution

Reagent	**Concentration**	**Volume/Mass**
Tris Base	2 M	242 g
Acetic Acid	1 M	57.1 g
EDTA Disodium Salt	0.5 M	100 mL
Deionized Water	-	800 mL

APPENDIX II

Post qPCR Agarose Gel Images:

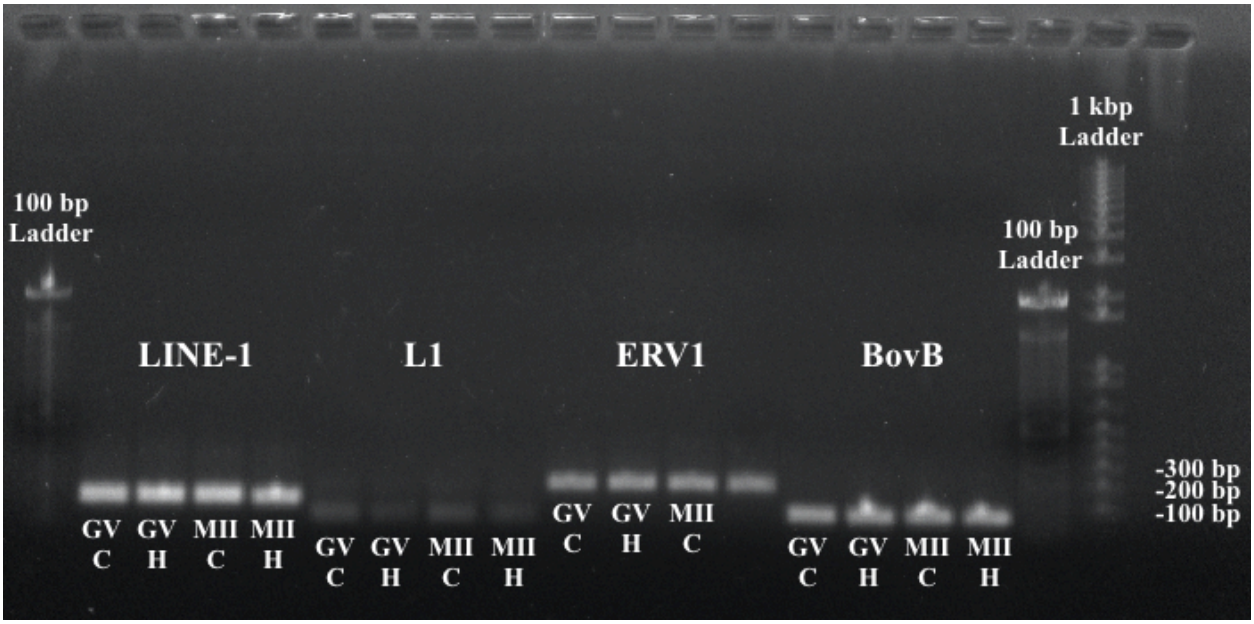

Figure 1- Oocyte Transposon Primers Replicate 1 on Agarose Gel. The product size (bp) of the LINE-1 primer was 200, L1 was 133, ERV1 was 277 and BovB was 147, which is in line with the results run here on the gel.

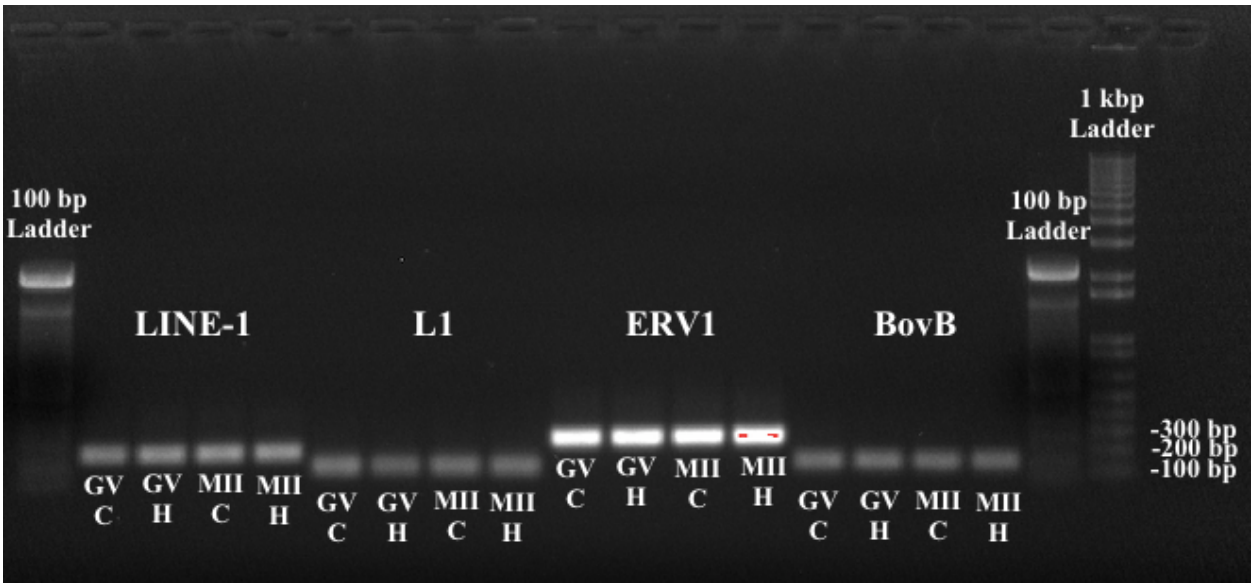

Figure 2 – Oocyte Transposon Primers Replicate 5 on Agarose Gel. The product size (bp) of the LINE-1 primer was 200, L1 was 133, ERV1 was 277 and BovB was 147, which is in line with the results run here on the gel.

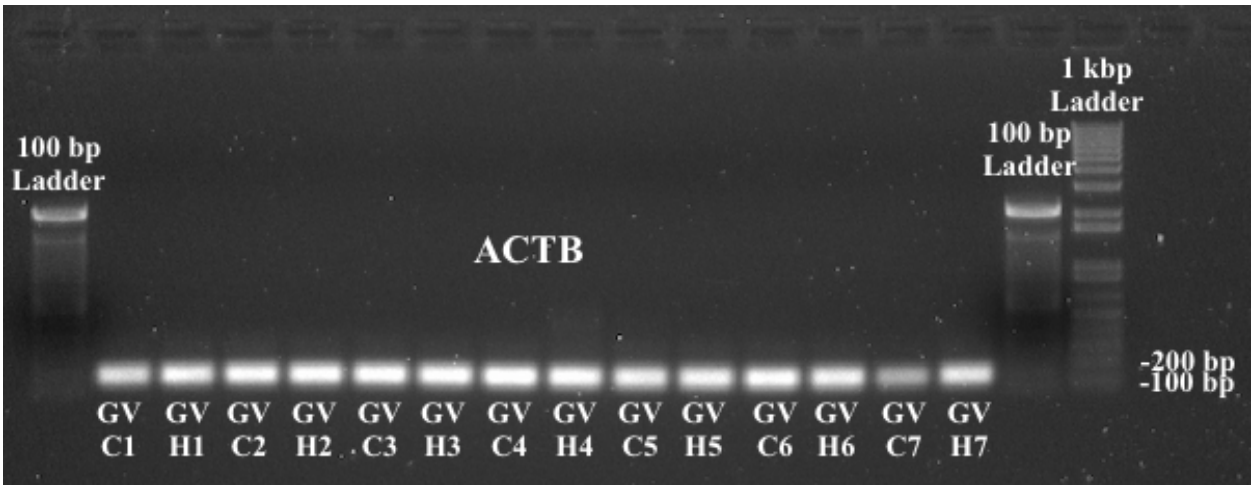

Figure 3 – GV stage Oocytes with one housekeeping gene ACTB on Agarose Gel. The product size (bp) of the ACTB primer was 186, which is in line with the results run here on the gel.