PATERNAL EFFECTS ON PRE-IMPLANTATION EMBRYO DEVELOPMENT IN CATTLE

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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And hereby certify that in our opinion it is worthy of acceptance

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
APC	Anaphase Promoting Complex
ART	Assisted Reproductive Technology
ATP	Adenosine Triphosphate
BCL2	BCL2 Apoptosis Regulator
BL	Blastocyst Rate
BAD	BCL2 Associated Agonist of Cell Death
BRCA1	BRCA1 DNA Repair Associated
BSA	Bovine Serum Albumin
Cdk5	Cyclin Dependent Kinase 5
CDK2AP2	Cyclin Dependent Kinase 2 Associated Protein 2
cDNA	Complementary DNA
CEP162	centrosomal protein 162
CL	Cleavage Rate
CNOT6L	CCR4-NOT
COC	Cumulus Oocyte Complex
CREBBP	CREB Binding Protein
DAG	1,2-diacylglycerol
DDIT3	DNA damage Inducible Transcript 3
DNA	Deoxyribonucleic Acid
DPI	Days Post Insemination
EGA	Embryonic Genome Activation

EP300	E1A Binding Protein p300
FDR	False Discovery Rate
FSH	Follicle Stimulating Hormone
GADD45G	Growth Arrest and DNA Damage Gamma
GnRH	Gonadotrophin-Releasing Hormone
GO	Gene Ontology
HP	High Performing
HPF	Hours Post Fertilization
HPF1	Histone PARylation Factor
HPI	Hours Post Insemination
HSP	Heat Shock Protein
HSPB1	Heat Shock Protein Family B (Small) Member 1
ICM	Inner Cell Mass
IP3	Inositol 1,4,5-triphosphate
IVF	In Vitro Fertilization
IVP	In Vitro Production
KIF11	Kinesin Family Member 11
LC3	Microtubule-associated Protein 1 Light Chain 3
LP	Low Performing
MEG	Maternal Effect Genes
MFI	Mean Fluorescent Intensity
MKI-67	Marker of Proliferation Ki-67
miRNA	Micro RNA

MmRNA	Maternal Messenger Ribonucleic Acid
MPF	Maturation Promoting Factor
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NDUFA1	NADH Dehydrogenase Ubiquinone 1 Alpha Subunit 1
PABPC	Poly(A) Binding Protein Cytoplasmic 1
PDCD5	Programmed Cell Death 5
PG	Prostaglandin F2α
PHB	Prohibitin
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidyl 4,5-bisphophate
PLCζ	Phospholipase C-zeta
PUM	Pumillio
RBP	RNA-binding Protein
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSE	Regulatory Sequence Element
SCR	Sire Conception Rate
SIVA1	SIVA1 Apoptosis Inducing Factor
SMG	Smaug
SNP	Single Nucleotide Polymorphism
SYCP2	Synaptonemal Complex Protein 2

TE	Trophectoderm
TEX15	Testis Expressed 15
UBE3A	Ubiquitin E3 A Ligase
UFO	Unfertilized Oocyte
UQCR10	Cytochrome b-c1 Complex Subunit 9
UTR	Untranslated Region
ZP	Zona Pellucida
ZPA	Zona Pellucida A
ZPB	Zona Pellucida B
ZPC	Zona Pellucida C

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Abstract

Currently, sire fertility is measured using sire conception rate (SCR), which is not always indicative of embryo development. Since the majority of pregnancy loss in dairy cattle occurs during the early embryonic period, it is important to determine the effect of sire during this time period. Therefore, the goal of this research is to identify sires with high and low capacities to produce embryos and elucidate the effect of sire on early embryo development. To investigate this, 65 Holstein sires with SCRs ranging from -14.2 to 5.3 were run through an *in vitro* embryo production system and embryo development was monitored. Based on their in vitro development performance, eight high performing (HP) and 9 low performing (LP) sires were identified. The average blastocyst rate (BL) was 48% for HP and 14% for LP sires, respectively. In this dataset, there was no correlation between SCR and BL. However, there was an increase in embryos arrested at the 5-6 cell stage in LP sires compared to HP sires. Next, embryos were produced from HP and LP to determine autophagy levels, and blastocyst cell number. LP sires had a higher rate of autophagy than high performing sires, with no effect of SCR. However, the ratio of trophectoderm to inner cell mass cells in blastocysts did not differ between sire performance groups. RNA-Seq on 4-cell embryos identified 687, and 1411 genes with increased expression in HP and LP sires, respectively. Genes with increased expression in HP sires were involved in mRNA and cell cycle regulation, chromosome segregation, and sperm mitochondria clearance. Genes with increased expression in embryos from LP sires were indicative of sperm mitochondria retention, and an increase in

DNA damage and apoptosis. Lastly, embryos were produced in vivo from HP and LP sires. Interestingly, LP sires generated twice the number of degenerated embryos as HP sires.

In conclusion, this research demonstrates a clear effect of sire on pre-implantation embryonic development, where LP sires produced a higher proportion of embryos with developmental delays, increased autophagy and expression of DNA damage and pro-apoptotic genes, resulting in embryonic arrest at the 5-6 cell stage. Interestingly, SCR was not indicative of pre-implantation development in this study. The in vitro model used in this study to identify sires with negative effects in embryo development represents a useful tool to build a robust predictor of sire fertility that accounts for the sire's influence on the early stages of pregnancy.

CHAPTER 1: REVIEW OF THE LITERATURE

1.1 Introduction

Approximately 40-50% of pregnancy losses in dairy cattle are attributed to embryonic mortality, making pre-implantation embryonic development a crucial area of study in order to improve pregnancy outcomes (Wiltbank *et al.* 2016). This large incidence of pregnancy loss is in part due to selective breeding of dairy cattle for increased milk production, which is negatively associated with fertility (Olds *et al.* 1979; Beam and Butler 1999; Snijders *et al.* 2000; Berry *et al.* 2003; Dillon *et al.* 2006). Most of the research has been focused on elucidating the effects of maternal environment (Wise *et al.* 1988; García-Ispierto *et al.* 2006; Gernand *et al.* 2019), female fertility phenotype (Shore *et al.* 1998; Inskeep 2004; López-Gatius *et al.* 2004), and genetics (Pryce *et al.* 2004; Bamber *et al.* 2009; Cummins *et al.* 2012) on pregnancy loss. Recently, efforts have been made to understand the role of sire on embryo quality and viability (Ledoux *et al.* 2015; Ortega *et al.* 2018; Gross *et al.* 2019; Wu *et al.* 2020). These studies demonstrate an effect of sire on early pregnancy loss, however, how sires' influence the processes of pregnancy establishment and maintenance is still unknown. Given the reduced number of sires used in dairy herds, understanding sire influences on embryo development has the potential to make a substantial impact on pregnancy rates and overall fertility outcomes.

The current way to measure sire fertility is using sire conception rate (SCR), which is the probability a unit of semen from a given sire will produce a pregnancy compared to the average bull (Norman *et al.* 2011). This fertility

indicator uses data from at least 300 services within the last four years to measure conception rate (Norman *et al.* 2008). When calculating SCR, fixed effects of herd, year, state, month, registry status, parity, service number, milk yield, dam and sire age group, and length of the breeding interval are included (Kuhn *et al.* 2008). Additionally, random effects of AI organization of the bull, mating year, service bull, dam, and inbreeding coefficients of the bull and the potential resulting embryo are included in the model (Kuhn *et al.* 2008). However, variables such as quality of heat, health of the female, and human errors cannot be included in the model (DeJarnette *et al.* 2007). This leads to variation in SCR value and observed conception rates because it is not a direct measure of sire fertility. For example, if a herd with a 30% conception rate was serviced with a bull of average SCR, the observed conception rate could between 26-34%, which could mean a difference of 80 pregnant cows in a 1000 cow herd (DeJarnette *et al.* 2007).

Given that sire conception rate is a phenotype, efforts have been made to dissect the genetic component to create a more accurate measure of sire fertility. Previous studies have identified 14 single nucleotide polymorphisms (SNP) associated with SCR, all of which are in genes involved in gamete maturation, spermatogenesis, motility, and sperm-oocyte interactions. (Peñagaricano *et al.* 2012; Han and Peñagaricano 2016). Furthermore, inclusion of non-additive effects, and functional information show promising statistical methods to predict sire fertility (Abdollahi-Arpanahi *et al.* 2017; Nani *et al.* 2019; Schober *et al.* 2018) (Kropp *et al.* 2017a; Gross *et al.* 2019).

However, since SCR is determined by day 70 pregnancy rates, it is hard to determine where pregnancies from low SCR sires fail. For example, low SCR sires that are producing less pregnancies than average by day 70, may have an issue in embryo production, elongation, or other pregnancy processes that occur before day 70 (Ortega *et al.* 2018). Therefore, it is necessary to clearly characterize the phenotypes influenced by sire to elucidate the genetic component of sire fertility and create a consistent, reliable predictor for this trait.

1.2 Early Embryonic Pregnancy Loss

Pregnancy loss is a major economic concern in the dairy cattle industry, with early term losses costing around $500 per incidence (De Vries 2006; Lee and Kim 2007). One of the most researched areas in terms of pregnancy loss is the role of the maternal environment. For example, breeding during the warmer seasons, and increased milk production both result in lowered pregnancy rates (Wise *et al.* 1988; Ullah *et al.* 1996; Vasconcelos *et al.* 2006; García-Ispierto *et al.* 2006; Gernand *et al.* 2019). Additionally, there is evidence on how the uterine conditions affects embryo development, with excessive estradiol and insufficient progesterone resulting in pregnancy loss (Shore *et al.* 1998; Inskeep 2004; López-Gatius *et al.* 2004).

Although the maternal environment plays a large role in supporting pregnancy, an effect of sire can also be detected. Sire has effects on fertilization rates, blastocyst production, and pregnancy rates (Chaveiro *et al.* 2010a; Ledoux *et al.* 2015; Franco *et al.* 2018; Szelényi *et al.* 2018; Ortega *et al.* 2018). However, the specific causes of the sire's effect on pregnancy are not well

elucidated. For example, sperm characteristics such as motility, concentration, and morphology are well studied, but what makes a sire more or less fertile and how his fertility affects pregnancy is a lacking area of research.

Though, one studied effect of sire on the process of pregnancy is paternal age. Semen characteristics such as total volume, and sperm count increase while motility and acrosome integrity post cryopreservation decrease with age (Bhave *et al.* 2020; Llamas-Luceño *et al.* 2020). Additionally, Wu *et al.* (2020) analyzed sperm miRNAs and concluded that bulls aged 10-12 months have differing miRNA expression, which negatively affects embryo metabolism compared to embryos produced from 16 month old bulls. In summary, literature suggests younger bulls have improved sperm motility, but older sires have a positive effect on embryo development and possibly pregnancy rates. However, even though literature demonstrates a clear difference in pregnancy outcomes with different sires there is lacking evidence for how sires specifically influence pregnancy (Ledoux *et al.* 2015; Ortega *et al.* 2018).

The timing of pregnancy loss is also important when tackling the goal of reducing pregnancy loss. To better understand early pregnancy loss, it is important to understand all the processes involved from fertilization until the formation of a blastocyst.

1.3 Fertilization and Early Embryonic Development

1.3.1 Sperm Transport

During natural service in bovine and humans, semen is deposited in the anterior vagina. Once in the vagina, sperm utilizes its whip-like tail movement,

termed flagellation, to progress forward to reach the cervix. During estrus the cervix produces two types of mucus: sialomucin and sulfomucin (Heydon and Adams 1979). Sialomucin has a low viscosity, allowing forward moving motile sperm to swim through it to continue traveling towards the uterus (Mullins and Saacke 1989). Sulfomucin has a high viscosity, which will flush seminal plasma and abnormal non-motile sperm, downwards, away from the uterus (Mullins and Saacke 1989). After sperm penetrates the sialomucin, they move towards the wall of the cervix where they continue travel to the uterus. Sperm can bind to folds on the cervical walls to create temporary storage reservoirs, which allows for temporal release of sperm to increase the likelihood of sperm reaching the oocyte at the time of ovulation.

The dairy cattle industry widely uses artificial insemination, opposed to natural mating, in which case the sperm is deposited directly into the uterus (Rickard *et al.* 2019). Additionally, in natural mating in murine, sperm is deposited directly into the uterus. Once sperm reaches the uterus, it begins the process of capacitation, which is a process where sperm undergo morphological and molecular changes to gain the capacity to fertilize an oocyte. Uterine contractions caused by high levels of estrogen in the female allow the sperm to rapidly move from the uterine body into the oviduct (Hawk 1987). Once in the oviduct, sperm bind to oviductal epithelium, which delays advancement in the capacitation process and reduces motility to increase the lifespan of the sperm (Suarez 2016). While bound to the oviductal epithelium, sperm also undergo additional changes including: an increase in membrane fluidity, intracellular calcium, and tyrosine

phosphorylation of proteins (Naz and Rajesh 2004). After these changes, sperm can no longer bind to oviductal epithelium cells and they are released into the lumen of the oviduct (Suarez 2016). Sperm then become hyperactive as they are attracted to the site of fertilization by increased oviductal temperature and progesterone produced from the oocyte's cumulus cells (Hunter and Nichol 1986; Jeon *et al.* 2001; Bahat *et al.* 2005). Once the sperm reaches the oocyte, it undergoes the acrosome reaction and releases enzymes such as hyaluronidase and acrosin, which help penetrate cumulus cells, and bind to the zona pellucida, respectively (Baba *et al.* 1994).

1.3.2 Fertilization

For fertilization to occur, the sperm must first pass through the inner layer of cumulus cells termed the corona radiata, in order to bind the oocyte's outer membrane, the zona pellucida. In bovine, the zona pellucida is made of three glycoproteins: zona pellucida A (ZPA), zona pellucida B (ZPB), and zona pellucida C (ZPC), which are termed ZP1, ZP2, and ZP3, respectively in human and murine (Rankin and Dean 1996; Sutovsky 2018). All three zona pellucida proteins are important for the sperm to bind the zona pellucida. Specifically, ZPA maintains the sustained sperm-zona binding while ZPB and ZPC create a heterocomplex to allow the complete binding of the acrosome intact sperm to the zona pellucida (Yonezawa 2014). Once bound, sperm begins the process of exocytosis (Gadella 2010). After acrosomal exocytosis, oolemma-binding proteins on the sperm's head are exposed which facilitates its binding to the plasma membrane of the oocyte, termed the oolemma (Cuasnicú *et al.* 2016).

Once bound, the membrane of the sperm head fuses with the oolemma to insert its genetic material into the cytoplasm of the oocyte, termed the ooplasm. This is accomplished through a major influx of external calcium, known as the calcium oscillation, which also signals the oocyte to resume meiosis II (Belmonte *et al.* 2016). Once inside the oocyte, the sperm head grows in size to form the male pronucleus. Then, the pronuclear membrane will dissolve, and the paternal chromosomes will condense and participate in mitosis to form a 2-cell embryo, with each cell containing 60 chromosomes in bovine (Sutovsky 2018).

1.3.3 Preimplantation Cleavage

By 30 hours post fertilization the embryo has undergone its first mitotic cell division (cleavage), creating two blastomeres, each with half the cytoplasmic volume of the original zygote. The embryo will continue to divide from a 2-cell embryo to an 8-cell embryo, halving the size of the totipotent blastomeres each time which maintains the diameter of the embryo. These cleavages are not synchronized, meaning embryos can go from 2-cell to 3-cell, or 4-cell to 6-cell, instead of the traditional 2-cell, 4-cell, 8-cell divisions (Meseguer *et al.* 2011). During the early cleavages, the embryo's own genome has not been activated yet, therefore the embryo is reliant on maternal mRNAs and proteins accumulated in the oocyte before fertilization for development. These first cleavages are rapid, occurring every 8-12 hours in bovine, and every 12-14 hours in human and murine (Marlow 2010; Hlinka *et al.* 2012; Milewski and Ajduk 2017). This rapid cleavage is possible due to the lack of gap phases between synthesis and division phases while the embryo is still dependent on maternal

mRNAs and proteins (Marlow 2010). However, after the embryonic genome activation (EGA), termed zygotic genome activation (ZGA) in murine, the cell cycle will consist of four phases: synthesis, gap phase 1, division, and gap phase 2, which increases time between cleavages (Marlow 2010).

The embryo will continue to cleave and once the embryo is composed of greater than 16 cells it is termed a morula, which is defined as an embryo where individual blastomeres can no longer be distinguished, and will undergo compaction.

1.3.4 Compaction

In the bovine, compaction occurs between the 16-32 cell stage, which is approximately 4-5 days post insemination (dpi) (Soom *et al.* 1997). In murine and humans, compaction occurs at the 8-16 cell stage, which is day 3 and 4 post fertilization, respectively (Iwata *et al.* 2014; White *et al.* 2016). During compaction there is an increase in cell-to-cell adhesion, formation of tight junctions between blastomeres, and blastomere polarization (Nikas *et al.* 1996). Cell-to-cell adhesion is mediated by associations of E-cadherin with the actin cytoskeleton, which allows blastomeres to increase the area of contact with each other, thereby reducing the volume of the embryo (Johnson *et al. 1986*).

During this stage, blastomeres gain polarity. This is necessary to dictate which cells migrate to the outer and inner regions of the morula, which is associated with the developmental fate of blastomeres to the trophectoderm or the inner cell mass, respectively. To establish polarization, members of the protein kinase C family phosphorylate Lgl, Numb, and Mir to inhibit their

localization to the plasma membrane or the cell cortex which allows for the creation of a polar cell (Bailey and Prehoda 2015; Dong *et al.* 2015; Hong 2018). Polarization leads to localized expression of microvilli on the apical region of the cell, which is detected at the 16-cell stage in bovine, the 8-cell stage in murine, and the 10-cell stage in human embryos. (Reeve and Ziomek, 1981; Koyama *et al.* 1994; Nikas *et al.* 1996). Additionally, tight junctions form at this time to help blastomeres maintain polarity through regulation of paracellular transport (Watson *et al.* 1999). To accomplish this, tight junctions proteins: occludin, claudin and junctional adhesion molecules span the paracellular space, and interact with tight junction proteins ZO-1 and ZO-2 that are inside the cells to interact with actin (Citi, 1993). Tight junctions then regulate diffusion of lipids, integral membrane proteins, and polar substances, which allows for maintenance of blastomere polarity during compaction (Cereijido *et al.* 1998). After compaction, blastomeres have a developmental fate and the embryo will begin to form a blastocyst.

1.3.5 Blastocyst Formation

Once the cell fate of blastomeres is determined, the embryo begins to segregate the two cell populations: the inner cell mass, and the trophectoderm. To accomplish this, a fluid filled cavity begins to form between these two distinct cell populations. Through sodium potassium ATPase pumps and aquaporins, cells of the trophectoderm increase sodium concentrations in the center of the embryo, leading to osmosis of water inside the embryo, thus forming a large fluid filled cavity termed blastocoel (Bell et al. 2008). Once the blastocoel is more than

half the volume of the embryo, the embryo is now termed a blastocyst. During the formation of the blastocyst, the embryo undergoes the first lineage specification, with the trophectoderm (TE) cells lining the inside of the zona pellucida, and an inner cell mass cluster at one pole. This occurs on days 7-8 post insemination in bovine, 4-5 dpi in humans, and 3-4 dpi in murine (Cockburn and Rossant 2010; Putri Lubis and Halim 2019). After the blastocyst is formed, the inner cell mass undergoes a second lineage specification and segregates into two distinct populations: the hypoblast which gives rise to the yolk sac and the epiblast which gives rise to the embryo proper (Wei *et al.* 2017).

1.4 Maternal and Paternal Contributions to Embryonic Development

1.4.1 Maternal Contributions

A major maternal contribution to embryonic development is the quality of the ovulated oocyte. Since sperm does not contribute cytoplasm or mitochondria to the developing embryo, the quality of the oocyte's cytoplasmic content is critical to embryo development. Decreased oocyte quality can be indicated by morphological parameters such as increased oocyte diameter, granulated cytoplasm, in addition to abnormal polar bodies and zona pellucidas (Ebner 2000; Kahraman 2000; Rosenbusch 2002; Shi *et al.* 2014). These morphological qualities can indicate physiological abnormalities within the oocyte. For example, increased oocyte diameter and cytoplasm granulation is correlated with increased rates of aneuploidy (Kahraman 2000; Rosenbusch 2002). Aneuploidy is when a cell has the incorrect number of chromosomes and it decreases the ability of an oocyte to successfully produce an embryo capable of establishing

pregnancy (Hassold and Hunt 2009). Additionally, dark zona pellucidas are correlated with mitochondrial defects in differentiation, morphology, and redistribution which negatively affects embryo development (Shi *et al.* 2014). Correlations between numerous morphological and physiological abnormalities led to oocyte quality selection based off morphology for *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection procedures to increase fertilization and implantation rates. However, not every physiological abnormality manifests itself in a morphological manner. For example, increased lipid accumulation is correlated with decreased developmental competence of the embryo, but lipid droplets are not visible without immunofluorescence (Prates *et al.* 2014). Therefore, oocyte selection based off morphology alone cannot ensure the selection of only high-quality oocytes.

During oocyte growth and development, transcripts and proteins are produced and stored in the oocyte, and these products can affect early embryonic development. A population of genes, termed maternal effect genes (MEGs), are essential to embryonic development, and cannot be compensated for by the paternal genome (Kim and Lee 2014). Many MEGs have been knocked out in female mice, to demonstrate the maternal genotype determines the phenotype (Tong *et al.* 2000; Narducci *et al.* 2002 p. 1; Ramos *et al.* 2004; Roest *et al.* 2004). For example, knockouts of Padi6 and Ago2, which are genes involved in cellular metabolism, leads to embryonic death at the 2-cell stage (Esposito *et al.* 2007; Lykke-Andersen *et al.* 2008; Kaneda *et al.* 2009). Another protein, Atg5, which is involved in autophagy, leads to arrest at the 4- to 8-cell

stage (Tsukamoto *et al.* 2008a). This work demonstrates that in addition to the quality of the oocyte, the maternal genome influences early embryonic development before the activation of the embryonic genome.

1.4.2 Paternal Contributions

The sperm, contributed by the sire, contributes more than just its DNA to the developing embryo. At the time of fertilization sperm contributes two important pieces to embryo development: the oocyte activating factor, and the centriole. The oocyte activating factor signals the calcium oscillation within the oocyte which is responsible for the resumption of meiosis II and the formation of the female pronucleus. The sperm-specific protein responsible is believed to be phospholipase C-zeta (PLCζ) (Malcuit *et al.* 2006). After the sperm binds the oocyte, PLCζ activates the phosphoinositide pathway (Turner *et al.* 1984; Stith *et al.* 1994). In this pathway, PLCζ cleaves phosphatidyl 4,5-bisphophate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Rice *et al.* 2000). Then, IP_3 binds the type I IP_3 receptor on the endoplasmic reticulum, which stores calcium (Miyazaki 1988; Berridge 2002). This binding leads to the release of the calcium from the endoplasmic reticulum into the cytoplasm. Calcium then activates calmodulin-dependent protein kinase, which activates the anaphase promoting complex (APC) (Yamamoto *et al.* 2005). After activation, APC tags cyclin B, which regulates maturation promoting factor (MPF), to be degraded leading to the completion of metaphase II and entrance into anaphase II to finish meiosis (Gautier *et al.* 1990; Nixon *et al.* 2002).

The sperm also contributes its centriole during fertilization. Centrioles are organelles in the cytoplasm that help form and organize spindle fibers (Azimzadeh and Marshall 2010). Two centrioles together form a centrosome which provides structure to the cell and pulls apart chromatids during mitosis. Oocytes lack functional centrioles but retain a store of centrosomal proteins (Sutovsky *et al.* 1999). It is believed that oocytes and sperm degenerate their centrosomes in a reciprocal manner so upon fertilization, contributions from both gametes leads to a functional centrosome (Schatten 1994; Manandhar *et al.* 2005).

In addition to DNA, centrioles, and proteins, the sperm contain mRNAs and microRNAs (miRNAs) that are also delivered to the oocyte upon fertilization. It is important to note that although the major wave of the embryonic genome activation occurs at the 8-16 cell stage in bovine, some paternal genes are transcribed as early as the 2-4 cell stage (Gross *et al.* 2019). Differentially expressed miRNAs have been identified in sperm and 2-4 cell embryos from high and low fertility sires, indicating sperm RNAs have a direct effect on early embryo development (Gross *et al.* 2019). For example, sperm-borne microRNA-34c inhibits expression of BCL-2, which has an antiproliferative function, and thereby its absence has a negative effect on the first zygotic cleavage (Liu *et al.* 2012). Literature also demonstrates that sperm-borne miRNA-216b affects cell proliferation, with lower levels in high fertility sperm leading to higher levels of its target gene *K-RAS* in embryos, which promotes cell proliferation (Alves *et al.* 2019). Therefore, the sperm contributes many proteins and RNAs to the embryo

which have the potential to be consequential or favorable towards early embryo development.

1.5 Genetic and Physiological Mechanisms During Pre-Implantation Development

1.5.1 Maternal Transcript Recruitment

After an oocyte is fertilized, the embryo undergoes rapid mitotic divisions until the embryonic genome is activated. Genome activation occurs in two waves: the minor and the major. In bovine, the minor wave occurs at the 2-cell stage and major at the 8-cell stage (Graf, Krebs, Heininen-Brown *et al.* 2014). Whereas the minor and major genome activations occur at the 2- and 4-cell stage in humans, and the 1- and 2-cell stage in murine, respectively (Wang and Dey 2006; Sozen 2014). Until the EGA, the embryo primarily relies on maternal mRNAs (MmRNAs) that are stored in the oocyte during development and maturation. These MmRNAs are recruited to produce machinery to aid in development of the embryo before its genome is activated. Mechanisms behind MmRNA recruitment are not well studied in large domestic species, but studies in murine have identified several regulatory sequence elements involved.

Regulatory sequence elements (RSEs) regulate transcription of MmRNA by elongating the poly(A) tail. Elongation of the poly(A) tail is essential for MmRNA recruitment because lack of, or a short poly(A) tail leads to degradation of the MmRNA via exonucleases (Eichhorn *et al.* 2016). RSEs accomplish this by binding MmRNAs directly, or mediating modifications of mRNA binding proteins (Potireddy *et al.* 2006). The most studied RSEs involved in recruitment of

MmRNAs are cytoplasmic polyadenylation element, polyadenylation response element, and the hexanucleotide polyadenylation signal, which all recruit MmRNA by elongating the poly(A) tail so MmRNAs of interest are not degraded (Simon *et al.* 1992; Charlesworth *et al.* 2004). By utilizing MmRNAs the embryo is able to go from the 1-cell to the 8-cell stage within 3-4 days.

1.5.2 Degradation of Maternal RNAs

During the 8- to 16-cell stage, in bovine, the embryo transitions from use of maternal to embryonic transcripts. This process is termed the maternal-to-embryonic transition (MET) which involves degradation of maternal transcripts and simultaneous production of embryonic transcripts. In the beginning, maternal transcripts are degraded using maternally encoded products, but as development continues, they are also degraded through zygote produced products (Tadros and Lipshitz 2009). To begin the process of degradation, maternal transcripts must first be labeled for destabilization by microRNAs (miRNA). MiRNAs are single stranded and coded for by nuclear DNA. After their production they exit the nucleus and bind a RNA-induced silencing complex (RISC) (Kim *et al.* 2009). This complex will facilitate the binding of the miRNA to a 3’ untranslated region (UTR) of a maternal target mRNA. The 3’ UTR contains various sequences such as miRNA response elements, as well as the poly(A) tail and thus plays a crucial role in regulating gene expression. The binding of RISC to the MmRNA effectively tags it for destabilization by promoting its decapping and deadenylation of the poly(A) tail (Despic and Neugebauer 2018). Deadenylation of the poly(A) tail of MmRNAs is the main method of maternal transcript

clearance. The purpose of the poly(A) tail, which is made of adenosines, is to protect the mRNA from enzymes within the cell that can digest it. When the tail becomes too short through deadenylation, the mRNA is no longer protected, and the transcript body is vulnerable to exonucleolytic digestion.

RNA-binding proteins (RBPs) also play an important role in post-transcriptional processes and can either lengthen the poly(A) tail to stabilize mRNA, or promote shortening of the poly(A) tail, thus degrading the mRNA (Wigington *et al.* 2014; Sha *et al.* 2018). There are two well elucidated RBPs that are involved in maternal transcript degradation: Smaug (SMG), and Pumillio (PUM) (Hamm and Harrison 2018). In Drosophila, SMG binds to the CCR4/POP2/NOT-deadenylase complex, which then binds the MmRNA (Yartseva and Giraldez 2015). This interaction promotes deadenylation of the poly(A) tail, leading to enzymatic digestion of the mRNA (Guhaniyogi and Brewer 2001; Semotok *et al.* 2005). In mammals, the role of SMG in MmRNA degradation is unknown, but its' target, the CCR4/NOT complex is important for early embryo development in mice, suggesting a level of conservation between MET mechanisms in Drosophila and mammals (Liu *et al.* 2016).

The second well studied RBP, PUM, is known for its diverse role in translational repression in the murine, where it interacts with over 900 mRNAs (Gerber *et al.* 2006; Lin *et al.* 2018). It binds to the Pumilio-binding element sequence, which is enriched in destabilized MmRNAs (De Renzis *et al.* 2007). PUM binds to POP2 of the CCR4/POP2/NOT-deadenylase complex to promote

deadenylation of the poly(A) tail while also antagonizing polyadenylation of the tail via poly(A)-binding protein (Weidmann *et al.* 2014).

In addition to RBPs, there are other elements that have been shown to promote deadenylation of the poly(A) tail, thereby leading to MmRNA degradation. For example, embryonic deadenylation element (EDEN) is a recognition site close to the poly(A) tail and is comprised of U(A/G) repeats (Audic *et al.* 1998; Paillard *et al.* 1998). In Xenopus, EDEN binding protein is dephosphorylated after fertilization, which leads to an increase in target deadenylation, further demonstrating EDEN plays a role in MmRNA degradation (Detivaud *et al.* 2003). In summary, are various products within the cell that target MmRNA, most of which recruit deadenylases to shorten the poly(A) tail to degrade the MmRNA and pave the way for expression of embryonic genes.

1.5.3 Activation of the Embryonic Genome

During the process of clearance of maternal mRNAs, the embryo is simultaneously starting to produce its own transcripts, which starts with the EGA. In the bovine minor EGA, 390 genes are activated, and their functions relate to cell proliferation and the cell cycle, which are necessary for major genome activation to occur. During the bovine major EGA, 3,965 genes are activated, and they are involved in processes such as protein biosynthesis, cell adhesion, and maintenance of pluripotency (Graf, Krebs, Zakhartchenko *et al.* 2014). In bovine, the mechanisms behind EGA have not been elucidated, but mouse models can provide some insight. One studied mechanism is the recruitment of maternal cyclin A2 mRNA, and its effect on regulating transcription of the embryonic

genome. Cyclin A2 is involved in G2/M transition in germ cells, as well as activation of cyclin-dependent kinases (Cdk) (Ohashi *et al.* 2003). It is believed to be maternally derived due to the accumulation of its mRNA 6 to 12 hours post fertilization in murine, when EGA is initiated (Kaňka *et al.* 2009). To regulate transcription in embryos, Cyclin A2 binds to Cdk2, and the resulting complex binds to the Sp1 binding site, which is an abundant transcription factor with binding sites in numerous promoters and regulatory sequences in order to regulate transcription (Kaňka *et al.* 2009).

Another mechanism elucidated in murine is chromatin remodeling, which unwinds DNA, allowing RNA polymerase to bind and transcribe genes. A proposed mechanism behind chromatin remodeling in the embryonic genome activation involves the maternal SW1/SNF related chromatin remodeling complex that has a catalytic subunit, BRG1 (Kaňka *et al.* 2009). When recruited BRG1 exhibits DNA-dependent ATPase activity (Bultman *et al.* 2006). The energy from ATP hydrolysis breaks DNA-histone contacts, which allows for RNA polymerase to access DNA within a few hundred base pairs up or downstream (Bultman *et al.* 2006). This hypothesis was validated when mouse oocytes with depleted BRG1, showed reduced transcriptional activity, and a null mutation is embryonic lethal (Bultman *et al.* 2000). At day 4-5 post fertilization, the 16-cell embryo has completed the major EGA and the embryo is now dependent on its own transcripts (Lindner and Wright 1983).

1.5.4 Autophagy

Autophagy is a cellular pathway where damaged or unused proteins and organelles are degraded in a lysosome to recycle amino acids (Cecconi and Levine 2008). There are three types of autophagy: microautopahy, macroautophagy, and chaperone-mediated autophagy (Klionsky 2005; Cecconi and Levine 2008). The difference in each type of autophagy is the pathway in which components tagged for degradation enter a lysosome. In the main autophagy pathway, macroautophagy, cellular components marked for degradation are engulfed by a forming autophagosome which then fuses with a lysosome.

Formation of an autophagosome involves autophagy related proteins (Atg7, Atg16, Atg5, and Atg12) which form complexes to initiate the formation of the double membrane autophagosome. The microtubule-associated protein 1 light chain 3 (LC3) which is present in the cytoplasm as LC3-I, is recruited to the forming autophagosome complex, where it binds and turns into the membrane bound form, LC3-II. Once the autophagosome is completely formed, the result is a double membrane structure surrounding the components that will be degraded. The lysosome, which contains enzymes capable of degrading such components, will fuse with the autophagosome, and is then termed autolysosome. In this structure, the components of the autophagosome, including LC3-II will be degraded by lysosomal hydrolases, thus freeing the amino acids to be reused to generate new proteins and organelles (Deretic 2008). Due to the routine conversion of LC3-I to LC3-II, the turnover of LC3-II is commonly used to measure autophagy as its presence correlates to the amount of autophagosomes

formed, and its degradation correlates with the rate of autophagy (Tsukamoto 2015).

The way in which autophagy is activated is dependent upon the signals the cell receives. When sperm binds the oocyte, the phosphatidylinositol 3-kinase (PI3K) signaling pathway is activated, which promotes formation of the (Yamamoto *et al.* 2014) PI3K complex. This complex is responsible for the fusion of autophagosomes with lysosomes to create an autolysosome (Kang *et al.* 2011). In addition to the PI3K pathway, literature suggests transcription factor EB and forkhead box O transcription factors are capable of inducing autophagy at the time of fertilization, but the mechanisms are not well understood (Roczniak-Ferguson *et al.* 2012; van der Vos *et al.* 2012).

After its initiation at fertilization, autophagy plays an important role in early embryo development to help degrade maternal components in the maternal to embryonic transition (Tsukamoto 2015). Murine knockouts show that embryos null for autophagy related proteins such as: Atg9, Atg13, Ambra1, and Becn1 and PIK3c3/Vps34, all lead to embryonic lethality (Qu *et al.* 2003; Kuma *et al.* 2004; Fimia *et al.* 2007; Zhou *et al.* 2011; Kojima *et al.* 2015). Interestingly, when oocytes null for Atg5 (no autophagic activity) were fertilized with Atg5-null sperm, the resulting embryos died at the 4-8 cell stage, due to decreased protein synthesis (Tsukamoto *et al.* 2008). However, when using wild type sperm, these Atg5-null oocytes could be fertilized and develop normally (Tsukamoto *et al.* 2008). This study demonstrates the importance of autophagy in producing free

amino acids in early embryo development, as well as the important paternal role in this pathway (Tsukamoto *et al.* 2008).

Since autophagy is measurable within the embryo, and correlated with early embryo development, it is possible to use autophagy as an indicator of embryo quality. It is believed the higher rate of autophagy the better, as the embryo is more capable of degrading maternal components in addition to damaged organelles and proteins within the cytoplasm. A study by Tsukamoto *et al.* (2014) found that murine embryos with higher rates of autophagy are more likely to become blastocysts, and when transferred, these embryos result in larger litter sizes than embryos with low autophagic levels. It is hypothesized that embryos with lower rates of autophagy have decreased intracellular nutrients in addition to presence of factors that should be degraded within the embryo such as sperm-derived mitochondria, and maternal cytoplasmic factors, which can negatively affect embryo development (Tsukamoto 2015).

Lastly, there is a possible relationship between autophagy and chromosomal abnormalities in embryos. There is a correlation between low autophagic levels and the number of micronuclei in a cell (Tsukamoto 2015). Micronuclei are extra-nuclear bodies that form when a chromosome or chromosome fragment is not incorporated into one of the daughter nuclei after cell division (Luzhna *et al.* 2013). Therefore, micronuclei are associated with chromosomal instability and abnormalities within the embryo, thus decreasing embryo viability (Chester *et al.* 1998). Altogether, autophagy is an essential

mechanism in the early embryo, and its dysregulation can lead to lower embryo quality, and decreased developmental and birth rates.

1.5.5 Aneuploidy

To form an embryo, a haploid female pronuclei within the oocyte fuses with a haploid sperm pronuclei to form a diploid zygote. When the correct number of chromosomes are present in a diploid cell, being 40, 46, and 60 for murine, humans, and cows respectively, it is termed euploid. However, errors during meiosis and mitosis can lead to an embryo having the incorrect number of chromosomes for their species and is therefore termed aneuploid. If an error occurs during meiosis, meaning the embryo is formed from an aneuploid gamete, all resulting cells in the embryo will be aneuploid, which negatively affects embryo viability (Jones and Lane 2013).

In females, oocytes are arrested in prophase of meiosis I prior to birth. At the time of puberty, meiosis continues, and the oocyte will arrest in metaphase of meiosis II (Marangos and Carroll 2012). When sperm binds the oocyte after ovulation, it will trigger resumption of meiosis II which will result in a fertilized embryo (Marangos and Carroll 2012). Literature suggests a majority of aneuploidy in embryos arises from maternal meiotic divisions, making the elucidation and recognition of aneuploid oocytes key to successful embryo development (Hassold and Hunt 2009; Nagaoka *et al.* 2012).

Oocyte aneuploidy can arise from lack of segregation or pre-divisions. The improper segregation of chromosomes is termed non-disjunction. When non-disjunction occurs in meiosis I, homologous chromosomes are not equally

divided between the oocyte and the first polar body leading to an aneuploid oocyte (Stolakis and Bertero 2019). If non-disjunction occurs during meiosis II, the sister chromatids are not equally shared between the second polar body and the oocyte (Stolakis and Bertero 2019). Causes of non-disjunction are not known, but it is hypothesized that structural abnormalities of chromosomes lead to abnormal chromosome pairing, thus promoting non-disjunction (Sparkes and Crandall 1972; Pellestor 2002; Oliver *et al.* 2008). However, there is a clear effect of increased maternal age on the rate of non-disjunction, and aneuploidy in oocytes (Pellestor 2002; Gilliland and Hawley 2005; Hassold and Hunt 2009). In addition to chromosome segregation errors, aneuploid oocytes can arise from pre-divisions. In the case of pre-divisions, cohesion between homologous chromosomes or sister chromatids within a chromosome are lost resulting in their premature separation (Lister *et al.* 2010). This leads to independent segregation of chromosomes in meiosis I, or sister chromatids in meiosis II (Lister *et al.* 2010). Pre-division errors can occur due to irregular chromosome attachment, decreased sensitivity of spindle assembly checkpoints, and loss of cohesion that holds chromosomes together (Jones and Lane 2013). Both of these cases of non-disjunction and pre-division in the oocyte lead to aneuploidy which can affect embryonic development.

Males can also form aneuploid gametes, thereby creating aneuploid embryos (Uroz and Templado 2012). In male gamete formation, spermatogonia mitotically divide to produce primary spermatocytes which remain dormant until puberty (Chandra *et al.* 2010). Then they undergo meiosis I and II to produce 4

spermatozoa from one spermatogonia (Chandra *et al.* 2010). One major difference between male and female gametogenesis is that forming spermatozoa do not extrude polar bodies. Therefore, issues in segregation will not only result in aneuploidy for one gamete, but for two as each cell resulting from meiotic division results in a spermatozoa. Aneuploidy in male gametes can arise through non-disjunction and pre-division, just as in female gametes (Uroz *et al.* 2008; Uroz and Templado 2012).

After an oocyte is fertilized the male and female pronuclei fuse and the resulting zygote undergoes mitotic divisions to increase cell number in the embryo. If an error occurs during mitosis within embryo development, some blastomeres will be aneuploid, and some will be euploid, and the resulting embryo is termed mosaic (Ambartsumyan and Clark 2008). Mitotic errors can arise from anaphase lagging and non-disjunction. Anaphase lagging is a major cause of pre-implantation mosaicism and occurs when spindle do not attach to chromatids properly leading to some chromatids or chromosomes lagging behind and ultimately not being incorporated into one of the daughter cells (Coonen 2004). A consequence of anaphase lagging is the formation of micronuclei which results in defective DNA replication and ultimately, DNA damage (Chavez *et al.* 2012; Crasta *et al.* 2012). There is extensive literature demonstrating the decreased developmental capacity of aneuploid embryos. Aneuploid embryos are less likely to cleave, form blastocysts, and successfully undergo implantation (Rubio *et al.* 2003; Ljunger *et al.* 2005; Fragouli *et al.* 2013; Schaeffer *et al.* 2019; Middelkamp *et al.* 2020). However, the molecular mechanisms responsible for

aneuploidy and aberrant cleavages are still unknown because chromosome segregation in embryos is poorly described (Tšuiko *et al.* 2019).

1.5.6 Embryo Kinetics

The timing of development, termed kinetics, is an important area of research when determine embryo quality and competency. Advances in live-imaging technology has allowed for assessment of pronuclei presences, cleavage timing, and other developmental milestones such as blastulation, to identify superior embryos. Lemmen *et al.* (2008) found that human zygotes that reached at least the 4-cell stage by 24 hours post insemination (hpi) had earlier pronuclei disappearance and improved development compared to embryos that only reached the 2-3 cell stage by 24 hpi. Live imaging also allows for quantification of the time between embryonic cleavages. The duration of the first cytokinesis, defined as the appearance of the cleavage furrow to the separation of the two daughter cells, was indicative of blastocyst formation with decreased cytokinesis time being more desirable (Wong *et al.* 2010). In addition to cleavage timing, the timing of mitotic events can also be determined. A mitotic event is based on individual blastomeres, and not the same as a cleavage event. For example, an embryo dividing from a 2-cell embryo to a 4-cell embryo is termed its second cleavage event, and a 2-cell embryo becoming a 3-cell embryo is its second mitotic event. Cleavage and mitotic event timings are not the same because individual blastomeres do not cleave at the exact same time as other blastomeres in the embryo, but ideally there is a short window between these cleavages. However, too fast of cleavages can be indicative of errors in DNA

replication. Rubio *et al.* (2012) analyzed the pregnancy outcomes of human embryos that went from the 2-cell stage to the 3-cell stage in less than 5 hours, and only 1% implanted compared to the 20% implantation rate of embryos that took 12 hours for the same cleavage. Wong *et al.* (2010) concluded the time for a human 3-cell embryo to become a 4-cell embryo should be around 1 hour, and the longer this division takes, the less likely the embryo will go on to form a blastocyst. Additionally, time from fertilization to the 8-cell stage are lower in embryos that form blastocysts compared to those that do not (Desai *et al.* 2014). More specifically, embryos that reach the 8-cell stage by 61 hpi are more likely to develop to blastocysts than those that reach the 8-cell stage by 65 hpi, which is correlated with embryonic arrest (Dal Canto *et al.* 2012). Lastly, timing between all cleavages up to the 8-cell stage is correlated with the ability of a blastocyst to expand (Dal Canto *et al.* 2012). These data validates that the timing of the first few mitotic and cleavage events can be indicative of embryo development.

However, predicting the ability of an embryo to become a blastocyst does not always mean that embryo will successfully implant *in vivo*. Desai *et al.* (2014) demonstrated the timing from fertilization to the 2-, 3-, 5-, and 8-cell stage are lower in blastocysts that successfully implant compared to those that do not, but later developmental stages such as the 9-cell to expanded blastocyst is not different between embryos that successfully and unsuccessfully implant in humans. Altogether these data suggest early embryo kinetics is indicative of embryo quality, blastocyst rate, and implantation rates and there is a clear

developmental delay in embryos that ultimately degenerate, form poor-quality blastocysts, or fail to implant (Dal Canto *et al.* 2012; Desai *et al.* 2014).

1.5.7 Paternal Chromatin Remodeling

In somatic cells and the oocyte, chromosomes are composed of chromatin, which is DNA wrapped around four core histone proteins to form a nucleosome. Histones and nucleosomes allow the DNA to be compact in order to create the structure of a chromosome. However, sperm DNA has the need to be even more compact, and therefore undergoes a multi-step process to remove histones and replace them with protamines during spermatogenesis (Wouters-Tyrou *et al.* 1998). The first step in replacing histones with protamines is the relaxation of the nucleosome structure through acetylation of H4, and ubiquitination of H2B and H3 in murine and rats, respectively (Meistrich *et al.* 1992; Jason *et al.* 2002). Secondly, histones are replaced by transition proteins TP1-4, during spermatid elongation and nuclear condensation (Yelick *et al.* 1987). Lastly, transition proteins are replaced by protamines, although sperm chromatin may retain up to 15% of its original histones (Gatewood *et al.* 1987).

Sperm chromatin is transcriptionally inactive, possibly due to its unique chromatin structure. However, at the time of fertilization sperm chromatin must be remodeled by the oocyte to a transcriptionally active form (McLay and Clarke 2003). This remodeling occurs in three phases. First, the paternal chromatin disperses while the oocyte resumes anaphase of meiosis II (Wright and Longo 1988). Secondly, when the oocyte is completing telophase II the sperm chromatin recondenses to approximately one-half of its original size (Wright and

Longo 1988). Lastly, the chromatin decondenses inside the male pronucleus. This last step is concurrent with the oocyte chromatin decondensing in the female pronucleus to prepare for the first mitotic event (Adenot *et al.* 1991).

The remodeling of sperm chromatin coincides with replacement of protamines by histones. Sperm chromatin is void of protamines by the time the oocyte is completing anaphase II, and histones are present within the 1-cell embryo (Rodman *et al.* 1981; Adenot *et al.* 1997). It is theorized that reduction of inter-protamine sulfhydryl bonds within protamines is responsible for their removal, however this mechanism is not well elucidated (Perreault 1992). Once protamines are replaced by histones, histones organize into nucleosomes and the paternal chromatin decondenses in the male pronucleus. At this time, paternal chromatin is still lacking important proteins which will be imported from the ooplasm to the male pronucleus. For example, centrosome proteins CENP-A and CENP-B are not present in paternal chromatin inside the sperm, but are present on paternal chromatin inside the embryo (Schatten *et al.* 1988). Additionally, the male pronucleus lacks important proteins for DNA replication, which are also believed to be contributed by the oocyte (McLay and Clarke 2003). Two such proteins are Cdc6, which initiates DNA replication, and Mcm2, a DNA helicase (Tachibana *et al.* 2010). Both proteins are present in the female pronucleus, and not the male pronucleus immediately after fertilization, but later became detectable (Tachibana *et al.* 2010). Therefore, the oocyte is important for paternal chromatin remodeling within the embryo and provides the necessary proteins so it can begin transcription after fertilization.

1.5.8 Paternal Chromatin Integrity

Paternal chromatin integrity is known to affect embryo quality. The main reason for decreased chromatin integrity is increased DNA damage, which is a major contributor to male infertility in both bulls and humans (Sadeghi *et al.* 2009; Mukhopadhyay *et al.* 2011; Agarwal 2011; Rybak *et al.* 2012; Simões *et al.* 2013; Simon *et al.* 2014; Kumaresan *et al.* 2017; Castro *et al.* 2018; Boe-Hansen *et al.* 2018 p.; Colaco and Sakkas 2018). DNA damage is twofold higher in bulls of below average fertility compared to those of above average fertility (Kumaresan *et al.* 2017). The increased DNA damage in ejaculates from low fertility bulls could be due to the presence of immature spermatogonia, cytoplasmic droplets, sperm head malformations, in addition to aberrant protamine ratios and increased oxidative stress (González-Marín *et al.* 2012; Boe-Hansen *et al.* 2018). However, in the case of commercial sires who undergo routine morphological screening, a high incidence of spermatid maturation errors, characterized by round sperm heads, in semen straws sold for use is unlikely. Ratios of the two protamines, P1 and P2, that replace histones during chromatin remodeling in the sperm, can be indicative of DNA damage if they significantly differ from the normal 1:1 ratio (García-Peiró *et al.* 2011). Lastly, oxidative stress can occur within the male reproductive tract during epididymal travel. Epithelial cells in the epididymis are highly metabolically active, leading to increased reactive oxygen species (ROS) production. ROS increases DNA damage in the sperm which is detrimental to embryo development (Ochsendorf 1999; Moustafa 2004; Simões *et al.* 2013; Iommiello *et al.* 2015; de Castro *et al.* 2016). Antioxidants present in

semen usually protect sperm from ROS, but if the ROS generation is too high it can exceed the antioxidants capabilities to protect sperm. Overall, sperm chromatin integrity can be affected even before ejaculation due to increased DNA damage, which negatively affects embryo development.

1.5.9 Embryo and Semen Handling in Assisted Reproductive Technologies

Assisted reproductive technologies (ART) have largely benefited the cattle industry. However, ART has its own implications as gametes and embryos are being removed from their normal environment and subjected to different environmental stressors.

For males, ejaculates are collected and extended with a semen extender to normalize concentration and aid cryopreservation. Each aspect of sperm handling can alter sperm quality and resulting embryo development. The method of semen collection, being either artificial vagina, electro-ejaculation, or manual massage, can affect semen characteristics. For example, bovine semen collected via transrectal massage have increased progressive motility, velocity, and acrosomal integrity compared to those collected from electro-ejaculation (Sarsaifi *et al.* 2013). After collection, semen is extended using a liquid diluent before being packaged into semen straws. Semen extenders usually contain a pH buffer, a source of nutrients, antibiotics, and cryoprotectants. Different substrates added to the extenders can affect sperm quality. For example, ethylene glycol opposed to glycerol as a cryoprotectant decreases sperm motility (Büyükleblebici *et al.* 2014). In addition, an egg yolk based extender contains high-density lipoproteins which reduce sperm respiration and motility (Moussa *et*

al. 2002). After the ejaculate is extended, it will most likely undergo cryopreservation to allow for long term storage and travel. The process of cryopreservation is stressful for sperm, with up to 50% of sperm dying during the process (Nijs *et al.* 2009; Oberoi *et al.* 2014). In addition, sperm that has been cryopreserved have increased head morphology abnormalities (Gravance *et al.* 1998).

In ART, it is common to collect oocytes via ultrasound-guided transvaginal follicular aspiration, termed ovum pick up (OPU). During OPU a vacuum pump is used to aspirate the oocytes. The pressure of the vacuum has been shown to affect oocyte quality, which can affect results of *in vitro production* (IVP) of embryos (Horne 1996). In addition to obtaining oocytes, the handling during IVP can influence developmental outcomes. Studies show the experience and quality of the technician can influence cleavage and blastocyst rates during embryo culture *in vitro* (Yang *et al.* 1995; Dumoulin 2001; Wale and Gardner 2016). Lastly, the media used for *in vitro* maturation, IVF, and IVP are synthetic, and not truly representative of the fluid an embryo would be exposed to *in vivo*. Therefore, the static environment and artificial media also affect oocyte and embryo development, which increases metabolic stress, alters gene expression, decreases blastocyst quality and cryopreservation survival compared to their *in vivo* counterparts (Farin and Farin 1995; Lechniak *et al.* 1996; Enright *et al.* 2000; Rizos *et al.* 2002a; Lopes *et al.* 2007; Tesfaye *et al.* 2009; Driver *et al.* 2012; Noguchi *et al.* 2020). Therefore, during ART there are many different critical

points that can influence the sperm and or the oocyte, which can affect embryo development.

Given the complexity of pregnancy, there are countless ways for embryo development to be influenced. Maternal effects on embryo development are well characterized as the dam has been in the forefront of pregnancy loss research for decades. The recent shift of focus to sires has allowed for detailed characterization of the effect of sire on fertilization, blastocyst, and pregnancy rates. Although efforts have been made to elucidate how a sire affects pregnancy, specific mechanisms within the embryo that are influenced by sire are still unknown. It is crucial to first determine what processes are influenced by sire, to then identify relevant genes to study as candidates for a genetic predictor of sire fertility that is not only consistent and reliable but is also reflective of a sire's ability to produce embryos capable of establishing pregnancy.

CHAPTER 2: PATERNAL EFFECTS ON PRE-IMPLANTATION EMBRYO DEVELOPMENT IN CATTLE

2.1 Introduction

Approximately 40-50% of pregnancy losses in dairy cattle are attributed to early embryonic mortality, which occurs during the first week of gestation (Cerri, Juchem *et al.* 2009; Cerri, Rutigliano *et al.* 2009; Hackbart *et al.* 2010; Wiltbank *et al.* 2016). During this time, the embryo undergoes important events such as the embryonic genome activation, compaction, cell differentiation, and formation of a blastocoel to from a blastocyst by day 7 of development (Soom *et al.* 1997; Holm *et al.* 1998; Graf, Krebs, Zakhartchenko *et al.* 2014). The effect of the dam on embryo development is well studied, and literature suggests the most important maternal contribution to embryo development is the intrinsic quality of the oocyte (Rizos *et al.* 2002b; Boni *et al.* 2002; Rahman *et al.* 2012). For example, oocytes that are euploid with increased mitochondrial potential, decreased lipid accumulation, and adequate essential cytoplasmic factors are more likely to develop to the blastocyst stage (Renard *et al.* 1994; De Sousa *et al.* 1998; Watson *et al.* 1999; Guerin 2001; Hassold and Hunt 2001; Wakefield *et al.* 2008; Prates *et al.* 2014; Shi *et al.* 2014). However, specific sperm attributes have yet to be tied to early embryonic development.

Several parameters have been used to determine the developmental capacity of an embryo to develop to the blastocyst stage, and its likelihood to establish pregnancy (Wydooghe *et al.* 2014; Li *et al.* 2016; Heras *et al.* 2016). Among these we find morphological grading (Van Soom *et al.* 2003), timing to

first cleavage (Desai *et al.* 2014), cell number (Ebner *et al.* 2016), chromosome number (Turner *et al.* 2019), stress indicators such as reactive oxygen species accumulation (Guerin 2001), and more recently, autophagy levels (Song *et al.* 2012; Kuma *et al.* 2017). However, the effect of sire on these measurements remains unknown. Previous studies have investigated the effect of sire fertility on cleavage and blastocyst rates in vitro (Anchamparuthy *et al.* 2009; Chaveiro *et al.* 2010b; Kropp *et al.* 2017a; Gross *et al.* 2019; Keles *et al.* 2021), with mixed results. Likely because these studies, much like the industry, relied on sire conception rate to determine high and low fertility sires.

Sire conception rate (SCR), is a phenotypic trait determined by day 70 pregnancy rates, and is defined as the probability a unit of semen from a given sire will produce a pregnancy compared to the average of all bulls tested (Norman *et al.* 2011). Holstein sires must have at least 300 services in the last four years for a SCR value can be determined, which makes SCR an indicator rather than a predictor of male fertility (Norman *et al.* 2011).

During this time, many biological processes including fertilization, embryonic development, elongation, and placentation occur (Hashizume 2007; Blomberg *et al.* 2008), making it difficult to underline which processes, specifically, are affected by sire. This complex phenotype is also reflected in several studies indicating the effect of sire on embryo development and pregnancy establishment is individually driven, rather than attributable to SCR alone (Williams *et al.* 1988; Ward *et al.* 2001; Anchamparuthy *et al.* 2009; Chaveiro *et al.* 2010a; Kropp *et al.* 2017b; Ortega *et al.* 2018). Likely, this is also

one of the reasons why, to date, the genetic component of SCR has not been fully elucidated (Peñagaricano *et al.* 2012; Abdollahi-Arpanahi *et al.* 2017; Rezende *et al.* 2019; Pacheco *et al.* 2020).

The aim of this study is to identify sires with high and low capacity to produce embryos and provide insights on the phenotypic and physiological characteristics of embryos produced from these sires. We hypothesized that pre-implantation development is sire dependent, and that embryos produced from high and low sires have physiological differences apparent in the early stages of embryo development. The long-term goal of this work to elucidate the phenotypic and genetic components attributable to sire in each process from fertilization to pregnancy establishment to build a predictor of sire fertility.

2.2 Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All statistical analysis was performed using Statistical Analysis System (SAS) version 9.4, and significance was defined as $P < 0.05$ unless otherwise stated. Semen straws used in all experiments was gifted by Select Sires Inc. (Great Plains, OH) and were processed in the same commercial house, using the same semen extender and quality testing for all sires. The average age of sires at the time of collection was 28 months old, and up to two different batches of semen were used per sire. All experimental designs are depicted in Figure 2.1.

<u>Experiment 1: Sire phenotype in vitro</u>

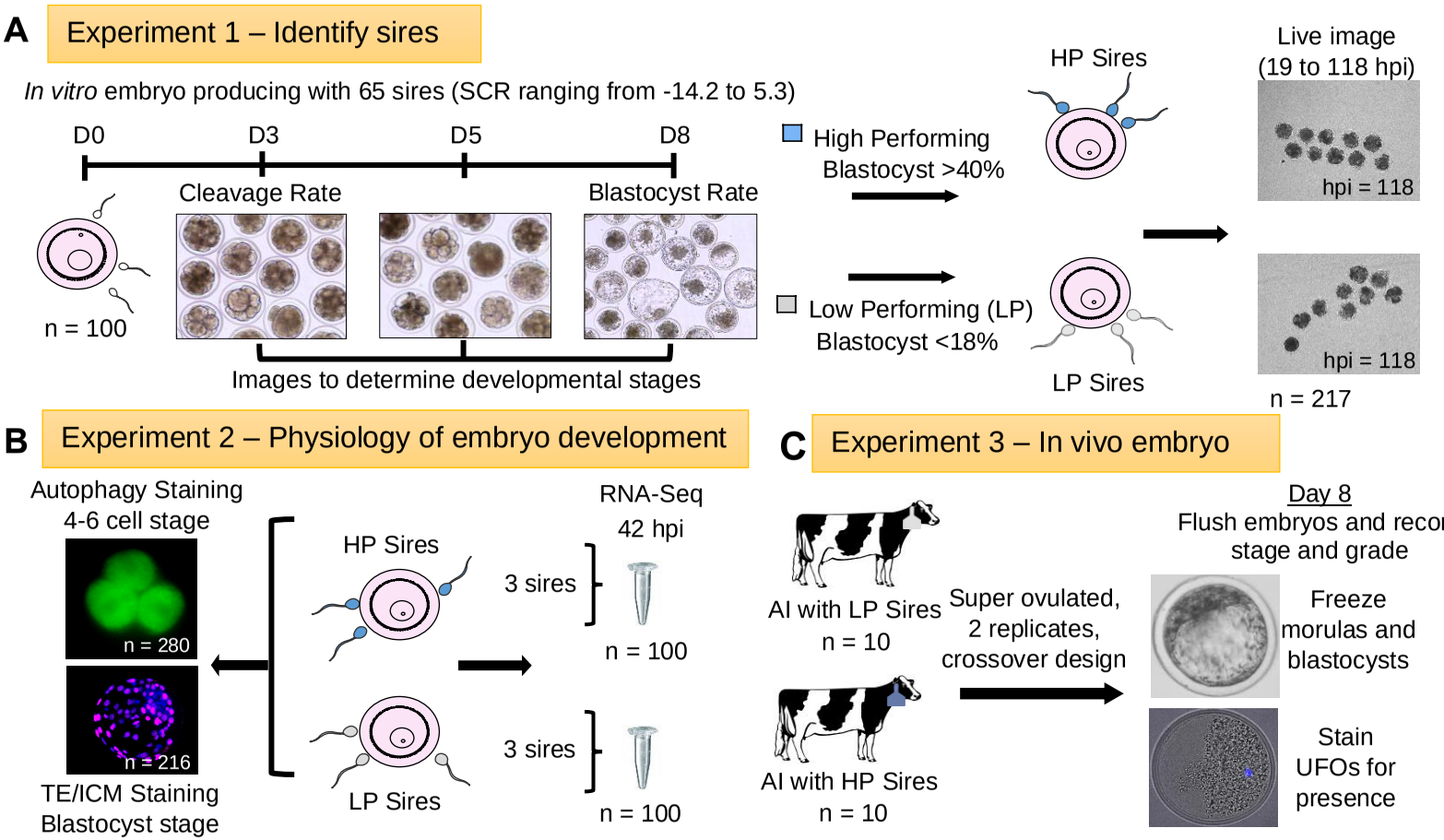

Figure 2.1. Study Design. A) Sires were run through in vitro embryo production (IVP) to identify sires with high and low blastocyst (BL) rates, and termed HP and LP sires, respectively. Embryo development was tracked using images taken days 3, 5, and 8 of culture, as well as live imaging to determine developmental arrest stages. B) Embryos were produced from HP and LP sires to determine autophagy levels, blastocyst cell number, and differentially expressed genes of 4-cell embryos. C) Heifers were super ovulated, artificially inseminated, and resulting embryos were flushed on day 8. Using a crossover design, 10 heifers were artificially inseminated with a sire of high or low performance each replicate.

A total of 65 Holstein sires with SCRs ranging from -14.2 to 5.3 were tested using an in vitro embryo production (IVP) system, and all media for IVP was prepared as previously described (Ortega *et al.* 2017). Briefly, cumulus-oocyte complexes (COCs) were collected at a commercial abattoir (DeSoto Biosciences, Seymour, TN, USA), and COCs with at least three layers of compact cumulus cells and homogeneous cytoplasm were placed in oocyte maturation medium equilibrated with air containing 5% (v/v) CO_2. Tubes with COCs were shipped overnight in a portable incubator (Minitube USA Inc., Verona, WI, USA) at 38.5˚C to the University of Missouri. A total of 100 COCs were used per sire and sperm was prepared for fertilization as previously described (Ortega *et al.* 2020). For all sires, the final concentration of sperm in the fertilization plate was $1x10^6$/ml, and fertilization time was 18h. At the end of fertilization cumulus cells were removed, and putative zygotes were placed in SOF-BEII culture medium. Cleavage rates (CL) were determined on day 3 post insemination by dividing the number of embryos with 2+ cells by the number of putative zygotes in the well. Blastocyst rates (BL) were determined on day 8 by dividing the number of blastocysts by the number of putative zygotes in the well. All sires were tested across 14 IVP replicates. Each IVP run contained sires considered high and low fertility based on their SCR value, and a control sire with known IVP performance.

To determine the effects of sire and SCR class on embryonic development each embryo was considered an observation, and CL and BL data were analyzed with a binomial logistic regression using the GLIMMIX procedure. An effect of sire, IVP run, and a random sire x IVP interaction were included. To

determine performance in vitro, sires were then assigned into quartiles using PROC MEANS based off BL and BL/CL, and the differences in means of quartiles was identified using the pdiff option of LSMEANS with the Scheffe adjustment. Individual comparisons between all sires were made to identify high performing (HP) and low performing (LP) sires with divergent BL and BL/CL using the pdiff option of LSMEANS. Lastly, the correlation between BL and SCR was determined using PROC CORR of SAS.

Developmental arrest

To determine at which stage embryos that did not make it to the blastocyst stage stop developing, pictures of each well were taken on days 3, 5, and 8 at 72, 120, and 192 hours post insemination (hpi), respectively. Brightfield images were taken using a Nikon Eclipse Ti-S at 40X magnification (Nikon, Melville, NY, USA). The number of embryos at the 1-cell, 2-3 cell, 4-6 cell, 7-8 cell, 9-16+ cell, morula, blastocyst or degenerated were recorded from each picture. Degenerated embryos were defined as shrunken 1-cell embryos, or lack of blastomere membranes due to apoptosis. After accounting for embryos becoming blastocyst, developmental arrest was estimated as the most frequent cell stage at which embryos stopped development. Differences in development between HP and LP sires were determined by one-way ANOVA using the PROC GLIMMIX procedure of SAS, and differences in means was determined using the pdiff option of LSMEANS with the Scheffe adjustment.

Live Imaging

To validate developmental arrest stages, putative zygotes from 3 HP and 3 LP

sires were live imaged to follow embryo development. Oocytes were obtained from a different commercial abattoir (Simplot, Kuna, ID), to determine if embryo development was consistent, regardless of oocyte source. Sires were run in pairs, one HP and one LP sire per replicate, across 3 replicates. All sires tested had previously exhibited cleavage rates above 70%, which is considered normal in IVP (Lee *et al.* 2012).

A total of 40 putative zygotes per sire were produced as described above. Putative zygotes were then placed in 16 µl drops of SOF-BEII (10 zygotes/drop) on a glass bottom dish (ThermoFisher Scientific, Waltham, MA, USA), overlaid with 2 ml of mineral oil. At 19 hpi, zygotes were placed inside a chamber fitted to the heated stage of a Lecia DMi8 scope (Leica, Wetzlar, Germany). The chamber was held at 38.5°C and contained a humidified atmosphere of 5% CO_2 (v/v), 5% O_2 (v/v), and 90% N_2 (v/v). Brightfield images were taken of each droplet, every 3 hours, for 4 days, at a magnification of 100X. The intensity was set between 10-20 and z-stack images were taken using a z-step size of 10 µm. Video analysis was done in LASX (Version 3.7.4.23463), and the stage of each embryo was recorded at each time point with stages being: 1-cell, 2-cell, 3-4 cell, 5-6 cell, 7-8 cell, 9-16+ cell, morula, and degenerated embryos. A degenerated embryo was defined by a lack of cell membranes due to apoptosis. Unfertilized oocytes were removed from further analysis. The percentage of embryos that reached the at least the 5- or 7- cell stage at each timepoint was determined using a binomial logistic regression with an effect of performance across hpi in PROC GLIMMIX. The difference in means between performance groups was

determined using the pdiff option of LSMEANS, and significance was defined as $P \leq 0.10$.

Experiment 2: Physiological characteristics of embryos derived from HP and LP sires

Autophagy

To determine autophagic activity, embryos were produced in vitro using 6 HP and 8 LP sires across 5 replicates, as previously described. Of these sires, six had a negative SCR (<0), and eight had a positive SCR (≥0). On day 2 post insemination, 4-6 cell embryos (n = 20/sire) with evenly granulated, and homogenous cytoplasm were collected. Autophagosomes were stained using the CYTO-ID Autophagy Detection Kit 2.0 (Enzo Life Sciences, Farmingdale, NY, USA), as previously described (Balboula *et al.* 2020). Counterstaining was performed by incubating the embryos in SOF-BEII with 1 mg/ml polyvinylpyrrolidone and Hoechst 33342 (Invitrogen Molecular Probes, Waltham, MA, USA) at 1 µg/ml. The mean fluorescent intensity (MFI) of each embryo was determined using ImageJ Software 1.46r (National Institutes of Health). For statistical analysis, a one-way ANOVA was performed using a fixed effect of sire performance or SCR on the MFI in PROC GLIMMIX. Both total MFI per embryo, and MFI per cell were tested. Differences in means between sire performance groups or SCR classifications were determined using the pdiff option of LSMEANS with the Scheffe adjustment.

Cell number

To investigate the number of trophectoderm (TE) and inner cell mass (ICM) cells

in blastocysts produced from HP (n = 120 blastocysts) and LP (n = 96 blastocysts) sires, differential staining was performed across 3 replicates. On the morning of day 7 post insemination, blastocysts were collected and TE cells were identified by immunolocalization of nuclear CDX2 [anti-CDX2 (mouse monoclonal antibody) BioGenex Fremont, CA, USA], and nuclei were stained with Hoechst 33342 as previously described (Stoecklein *et al.* 2021). The number of ICM cells were determined by subtracting the number of TE from the total number of nuclei. The stage of each blastocyst at the time of collection was also noted. To account for differences in blastocyst stages, the number of TE was divided by the number of ICM to create a ratio. The effect of sire performance on the ratio of TE/ICM cells, and the blastocyst stage were determined by one-way ANOVA in PROC GLIMMIX and the pdiff option of LSMEANS.

RNA-Sequencing

To elucidate the differences in embryonic development between embryos from HP and LP sires, RNA from 4-cell embryos, the embryonic stage just prior to the observed embryonic arrest, were sequenced. Embryos were produced as previously described, using 100 COCs for each HP (n = 3) and LP (n = 3) sire. At 42 hpi, all 4-cell embryos present were washed in DPBS-PVP, then incubated in 0.1% pronase for 60 seconds to remove any sperm and thin the zona pellucida. Then, embryos were washed 3x in DPBS-PVP and collected in ~5-10 µl of SOF-BEII. Embryos were then snap frozen and placed at -80°C until RNA extraction. This experiment was repeated three times. Embryos were pooled according to sire performance for each replicate. The total number of embryos collected from

HP sires was 113, 104, and 111 and 122, 102, and 92 for LP sires for replicates 1, 2, and 3, respectively.

RNA was isolated using the PicoPure Kit (ThermoFisher Scientific), following manufacturer instructions, however, Nano spin columns (Luna Nanotech, Toronto, Ontario, CA) were used instead of the minicolumns provided with the kit. Due to the low amount of RNA, less than 10 ng of RNA per sample, the Takara SMART-Seq v4 Ultra Low input RNA Kit (Takara Bio, Mountain View, CA, USA) was used to generate full length cDNA from mRNA (Usa). Samples were sequenced to an average read count of 50 million per sample using 75 bp paired-end sequencing carried out on the Illumina NovaSeq 6000 by the University of Missouri DNA Core Facility. FASTQ files were 3' trimmed to remove Illumina adapters and ambiguous nucleotides. Reads with fewer than 10 nucleotides were discarded. FASTQ reads were aligned to the bovine genome assembly (Bos_taurus.ARS-UCD1.2) using Hisat2 (Kim *et al.* 2019). Then, feature counts was used to determine the number of read counts mapping to each gene (Liao *et al.* 2014). Analysis of differentially expressed genes (DEGs) was performed using the package Robust from R (Robinson *et al.* 2010). To identify significant DEGs, a false discovery rate of 5% was used. After identification, DEGs were run through the ToppGene platform to determine relevant biological processes and pathways enriched in embryos from HP and LP sires (Chen *et al.* 2007).

Experiment 3: Sire performance in vivo

To validate in vitro results, 7 HP and 9 LP sires were tested for their ability to produce embryos in vivo. All animal procedures were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee at the University of Missouri.

Estrus synchronization and super ovulation

A total of 20 heifers, with an average of 18 months old, and 384 kilograms, were first pre-synchronized using the Ovsynch-56 protocol (Pursley and Wiltbank 1995). After pre-synchronization, a 7-day CO-Synch + CIDR protocol was implemented, with day 0 being the day of CIDR insertion (Eazi-Breed CIDR; 1.38 g P4; Zoetis Animal Health, Parsippany, NJ, USA) (Stevenson *et al.* 2008). To achieve superovulation, FSH (Folltropin Vetoquinol, Quebec, CA) shots were administered intra muscularly in the morning and evening, in decreasing dosages beginning on the morning of day 4 and finishing on the afternoon of day 7, totaling 300 mg per heifer. Two adjustments were made to the typical 7-day CO-Synch + CIDR protocol. First, an additional PG shot (25 µg) (Lutalyse, dinoprost tromethamine; Zoetis Animal Health) was administered on day 8 to ensure lysis of all corpora lutea, and artificial insemination (AI) was performed on the morning and evening of day 9, using 2 straws per insemination. The timeline of estrous synchronization, super ovulation, and AI protocol is shown in Figure S1.

To minimize the effect of heifer on embryo production, two replicates were performed using a crossover design, where heifers were inseminated with a HP or LP sire in replicate 1, and with a sire of opposite performance in replicate 2.

Embryos were collected non-surgically 8 days after AI using a 23” 30 CC silicone catheter (Agtech, Manhattan, KS, USA), complete flush media (Agtech) and an embryo collection filter with 75 µm mesh (WTA, São Paulo, Brazil). The stage and grade of recovered embryos were recorded according to the International Embryo Technology Society (IETS) manual (Stringfellow and Givens 2010). Recovered oocytes were fixed for 20 minutes in 4% paraformaldehyde, and incubated with Hoechst 33342 (Invitrogen Molecular Probes, Waltham, MA, USA) at 1 µg/ml for 10 minutes to see if sperm were bound. For analysis, embryos were classified as non-transferable (2-12 cell degenerated embryos) and transferable (morula and blastocyst stage grade 1 or 2). Unfertilized oocytes (UFOs) made up 11% of the structures recovered and were evenly distributed across sires and heifers and were removed from analysis. Data were analyzed using the PROC GLIMMIX with a fixed effect of sire or sire performance. Differences between transferable and non-transferable embryos were determined using the pdiff option of LSMEANS with the Tukey adjustment.

2.3 Results

Experiment 1: Sire phenotype in vitro

The purpose of this experiment was to identify sires with high a low capacity to produce embryos in vitro. The CL for all sires ranged from to 44.38% to 92.59% and BL ranged from 8.15% to 61.57%. Sires were divided into quartiles based on BL rates to identify sires on the extreme ends of the range, and quartile 1 had BL rates ranging from 8.15% to 21.42%, while quartile 4 BL rates ranged 32.64% to 61.72%. The mean blastocyst rate of each quartile was different ($P < 0.05$), and

there was no correlation between SCR value and BL rate ($P = 0.90$) shown in Figure S2. The mean cleavage rate of quartile 1 was significantly lower than quartiles 2 – 4 ($P < 0.05$). Given the lowered cleavage rate in quartile 1, BL/CL rates were used moving forward to focus on embryo development rather than fertilization capacity (Figure 2.2A). Eight sires within quartile 4 for both BL and BL/CL were considered HP, and 9 sires in quartile 1 for both BL and BL/CL were considered LP (Figure 2.2B). Additionally, BL/CL rates for sires with multiple IVP runs is shown in Figure S3 to demonstrate the consistency of sire performance. Regardless of sire performance, 75% of sires had most of their non-blastocyst embryos stopping development at the 2-6 cell stage (Figure 2.3A). The 2-6 cell stage category was further investigated, and more specifically, the 4-6 cell stage was termed the most common arrest stage with the percentage of arrested embryos in this category being different between HP and LP sires ($P < 0.05$) shown in Figure 2.3B. Live imaging showed a developmental lag in embryos produced from LP sires, with embryos from HP sires reaching later developmental stages sooner than those from LP sires, shown in Videos S1 and S2, and summarized in Figure 2.4. There were no differences in the percentage of embryos capable of reaching the 5-6 cell stage based off sire performance (Figure 2.5A), however fewer embryos produced from LP sires reached the 7-8 cell stage (Figure 2.5B).

<u>Experiment 2: Physiological characteristics of embryos derived from HP and LP sire</u>

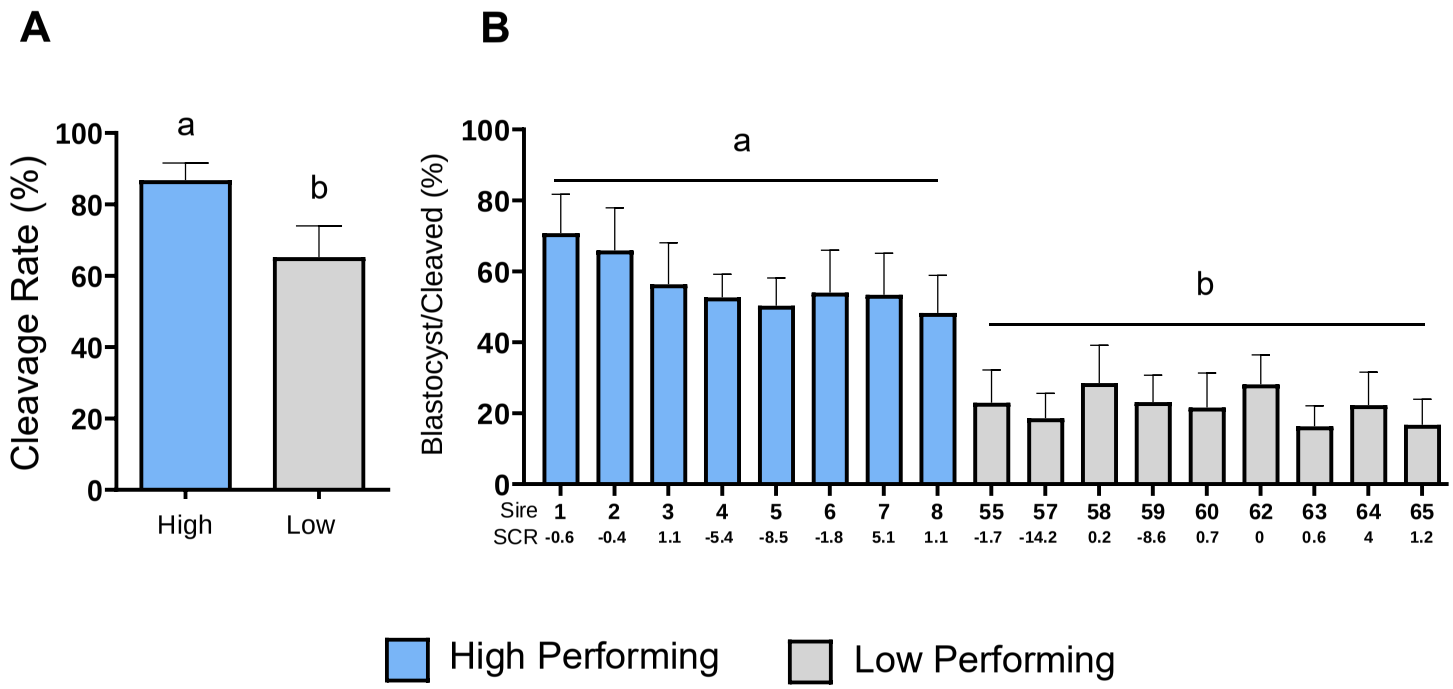

Figure 2.2. Cleavage and Blastocyst/Cleaved Rates of HP and LP Sires. Sires were ranked according to blastocyst/cleavage rates, and sires with the highest and lowest rates were termed high and low performing sires, respectively. A) Cleavage rates of high and low performing sires, and differing letters indicate significance. B) Blastocyst/cleavage rates of high and low performing sires. Bars with different letters are statistically different ($P < 0.05$). SCR = sire conception rate

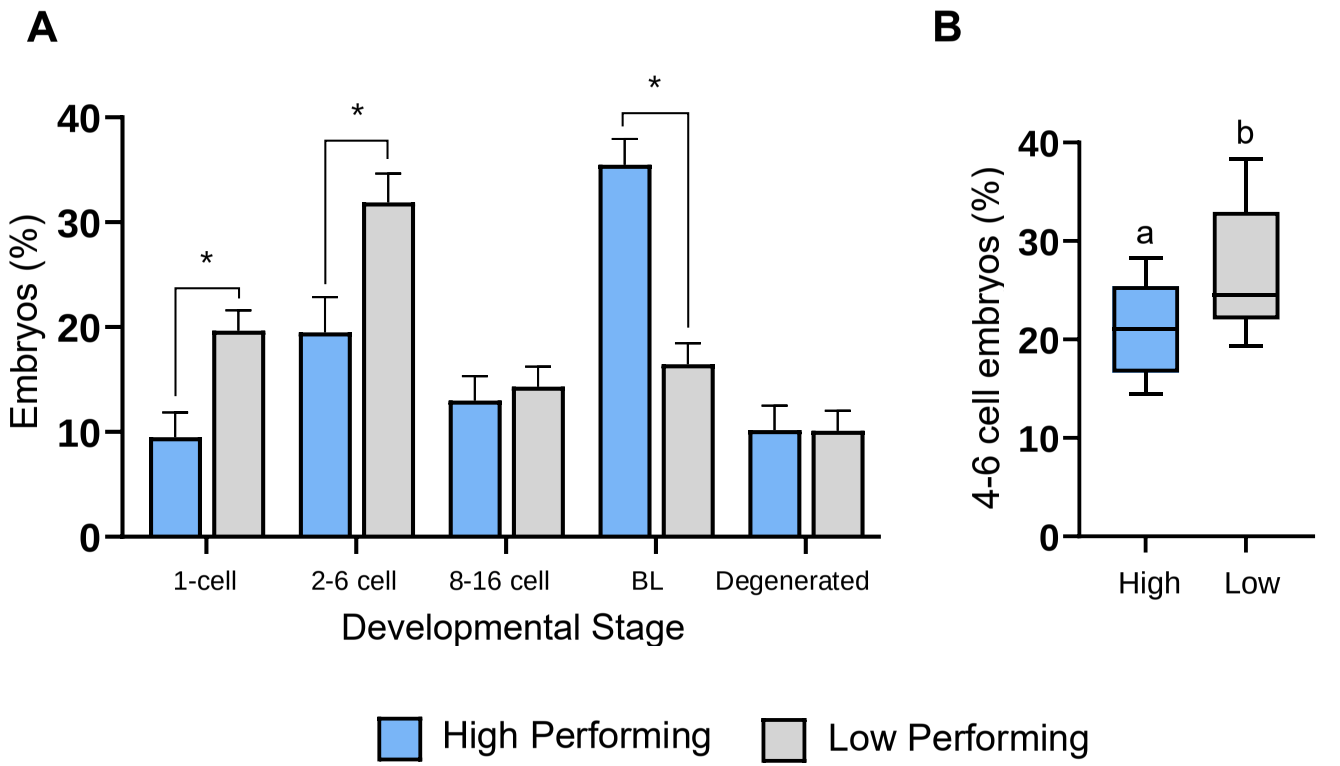

Figure 2.3. Developmental Stages of Embryos from HP and LP Sires. A) Developmental stages of embryos produced from high and low performing sires. Significance difference between groups is noted by asterisks. B) The percentage of embryos arrested in this most common arrest stage, the 4-6 cell stage, of high and low performing sires. Bars with different letters are statistically different (*P < 0.05)*.

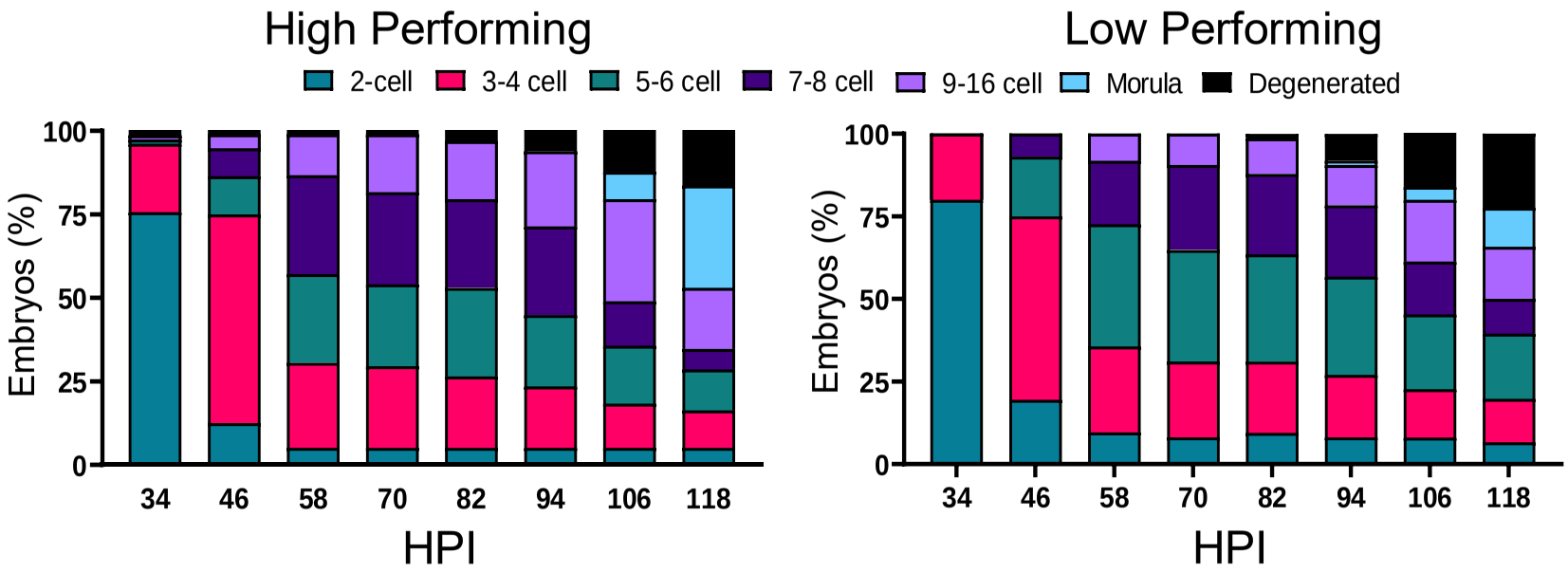

Figure 2.4. Developmental Delay in LP Sires. Fertilized embryos were live imaged, and stages were recorded every 3 hours. Stacked bars are shown to represent the percentage of embryos at each developmental stage at 12-hour increments beginning at 34 hours post insemination (hpi) and ending at 118 hpi.

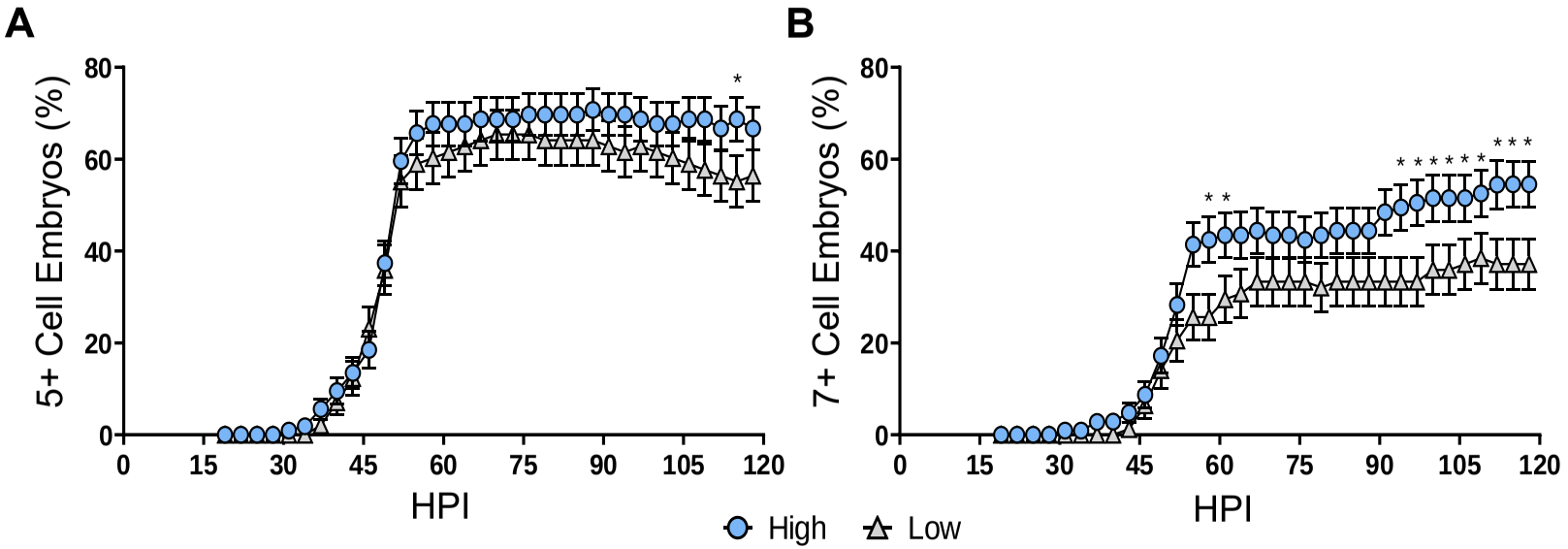

Figure 2.5. Developmental Arrest of 5-6 Cell Embryos. A) The percentage of embryos from high and low performing sires that reached at least the 5-cell stage at each timepoint. B) The percentage of embryos from high and low performing sires that reached at least the 7-cell stage. Points with asterisks are statistically different (*P < 0.10*).

Autophagy

Autophagy levels in embryos produced from HP and LP sires were measured as an indicator of cellular stress. The total MFI per embryo and per cell yielded similar results, therefore only total MFI per embryo is presented. Interestingly, the LP sires had a higher ($P < 0.0001$) rate of autophagy compared to HP sires (Figure 2.6A). However, when autophagy levels were analyzed by SCR, opposed to sire performance, there was no difference ($P > 0.05$) between groups (Figure 2.6B).

Cell number

To determine if blastocysts from HP and LP sires have differing developmental potential, the number of TE and ICM cells was determined by immunofluorescence (Figure 2.7A). Four embryo stages were observed at the time of collection: early blastocysts, blastocysts, expanded blastocysts, and hatched blastocysts (Stringfellow and Givens 2010). The TE/ICM ratio was analyzed to normalize for differences in embryo stage. There was no effect ($P > 0.05$) of performance on the TE/ICM ratio (Figure 2.7B).

RNA-Sequencing

Alignment rates ranged from 86-92%, and mapping rates ranged from 40-61%. One sample, LP3, from LP sires IVP run 3, did not pass quality control for the number of mapped reads and was discarded from analysis. There were 1411 genes with increased expression in embryos from LP sires, and 687 genes with increased expression in HP sires (Figure 2.8, $FDR < 0.05$). Based on edge R and Toppgene biological processes analysis, embryos from HP sires had increased

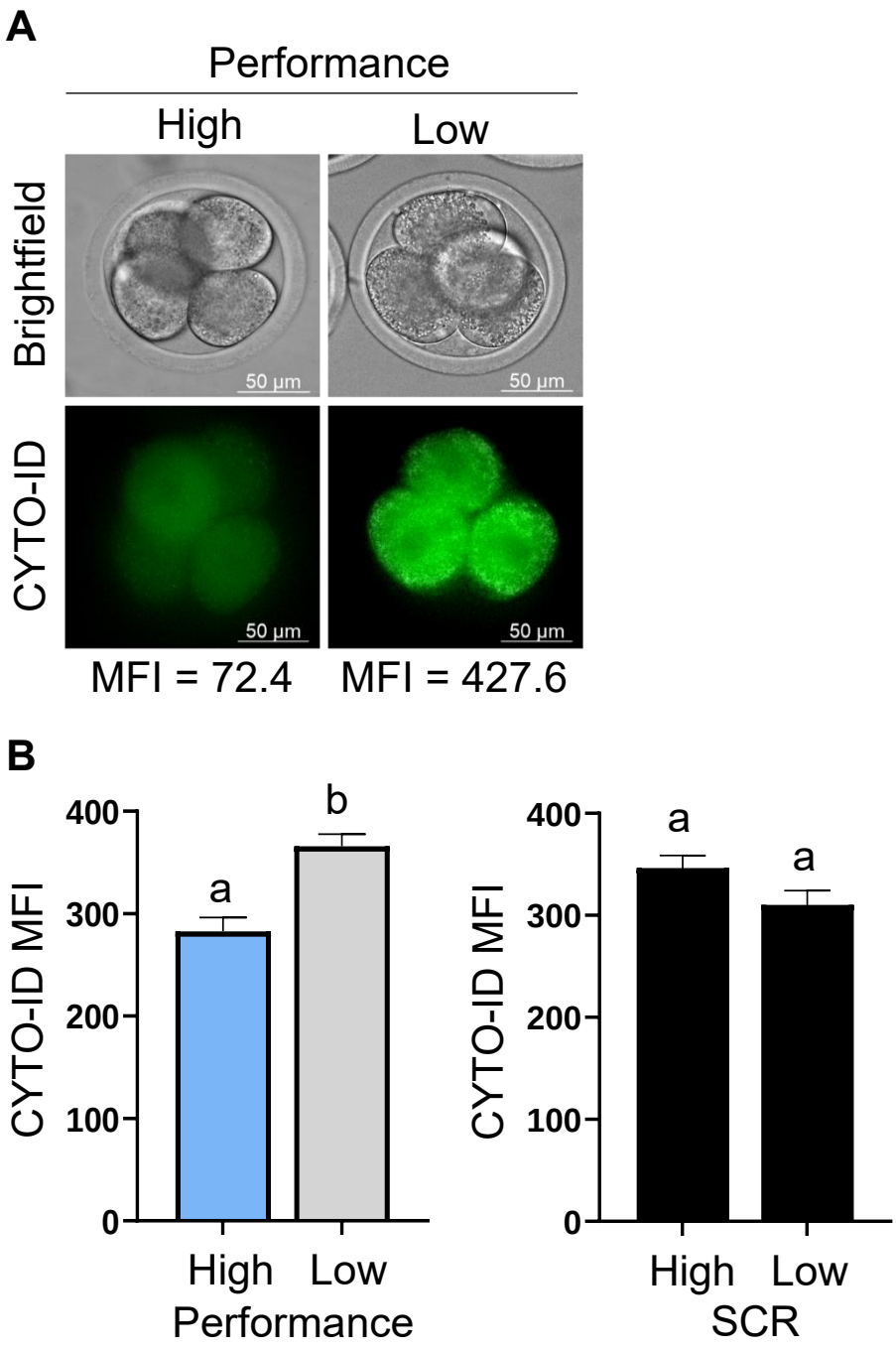

Figure 2.6. Autophagy of 4-6 Cell Embryos. A) Embryos from high and low performing sires were stained with CYTO-ID dye to tag autophagosomes and measure rates of autophagy. The mean fluorescent intensity of each embryo is shown. B) On the left, mean fluorescent intensity of embryos from high and low performing sires. On the right, data was analyzed using SCR opposed to sire performance. Bars with different letters are statistically different ($P < 0.05$). MFI = mean fluorescent intensity.

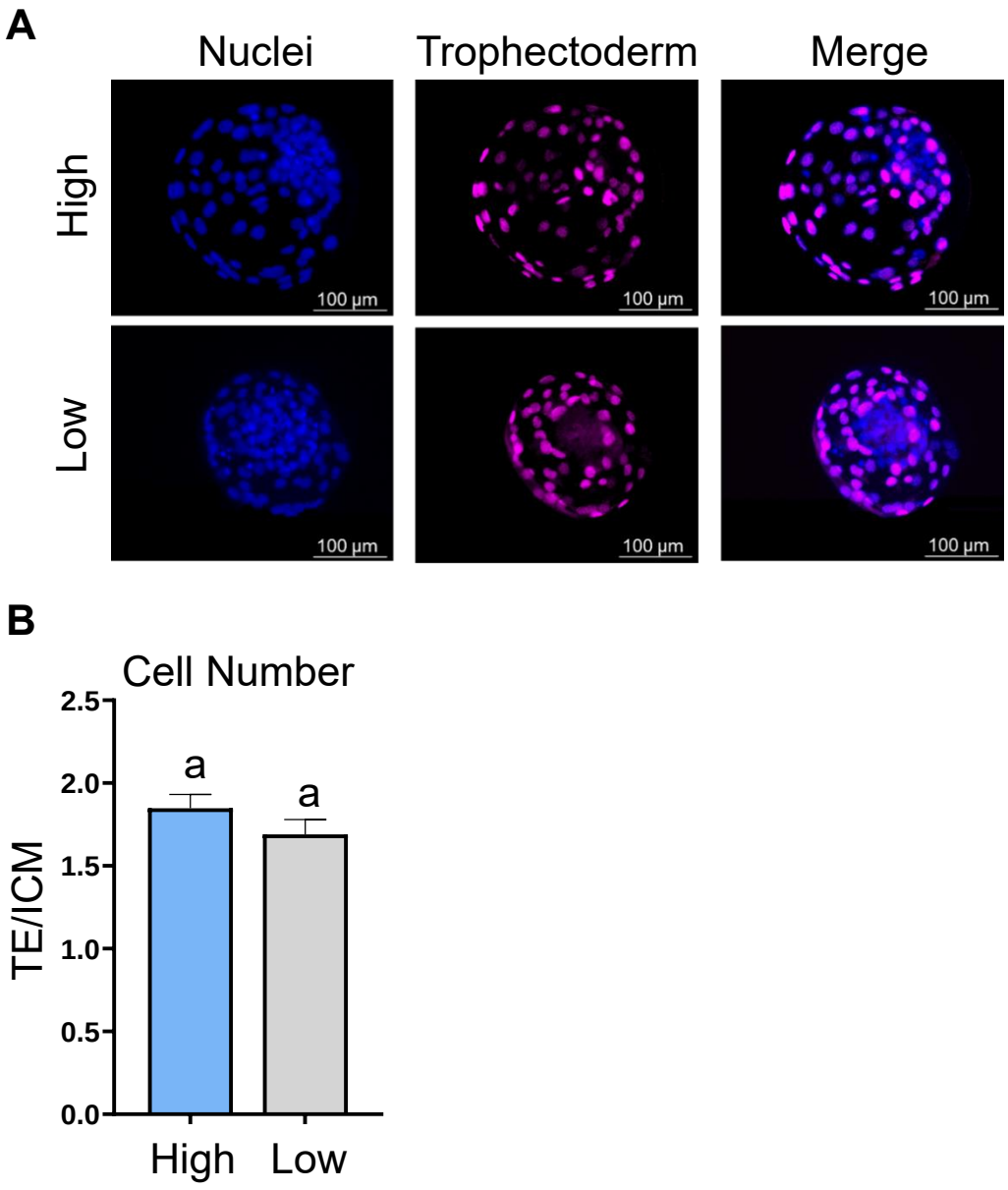

Figure 2.7. Blastocyst Cell Number. Blastocysts from high and low performing sires were differentially stained to quantify the number of trophectoderm and inner cell mass cells. A) Expanded blastocysts from high and low performing sires, nuclear stain is shown in blue, and trophectoderm cells which are CDX2 positive are noted in magenta. B) The ratio of trophectoderm to inner cell mass cells is shown to account for different blastocyst stages at the time of collection. Bars with different letters are statistically different ($P < 0.05$). TE = trophectoderm, ICM = inner cell mass

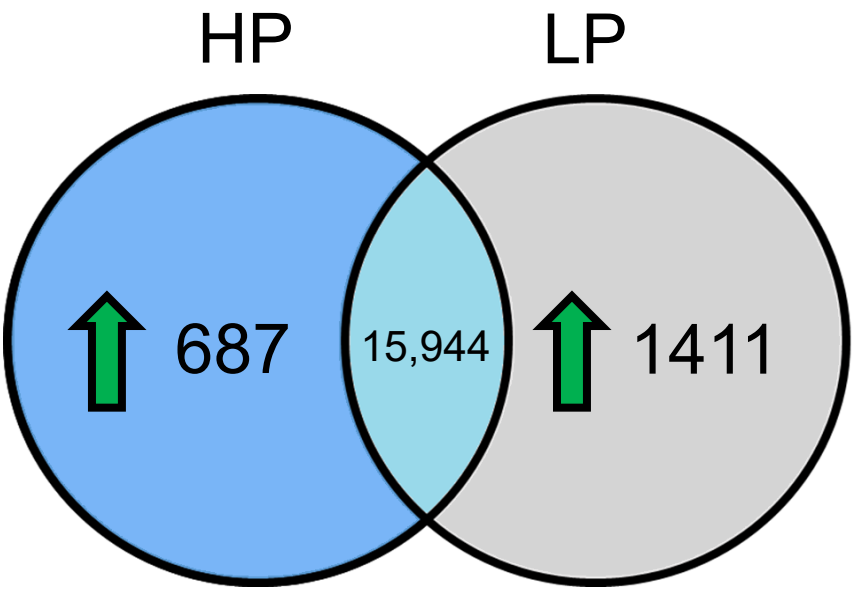

Figure 2.8. Differentially Expressed Genes of 4-cell Embryos Produced from HP and LP Sires. HP = high performing, LP = low performing. False discovery rate was < 0.05.

expression in genes related to sperm mitochondrial clearance, regulation of mRNA, and the cell cycle, shown in Figure 2.9. Embryos from LP sires had increased expression in genes related to sperm mitochondria, DNA damage, apoptosis, and cellular stress, shown in Figure 2.10. Gene ontology of biological processes and pathways with increased expression in HP and LP sires are shown in Tables S1 and S2, and Tables S3 and S4, respectively.

Experiment 3: Sire performance in vivo

Embryos were produced in vivo with HP and LP sires to determine if sires had similar performance in vivo and in vitro. Grade and stages of embryos collected are shown in Table S5, and all recovered oocytes had no sperm bound. The percentage of transferable and nontransferable embryos was not affected ($P > 0.05$) by sire performance (Figure 2.11). However, LP sires had 18 degenerated embryos when compared to 8 produced from HP sires. Interestingly, even with the low sample numbers, the same trend in the performance in vitro and in vivo was still identified for half of the sires.

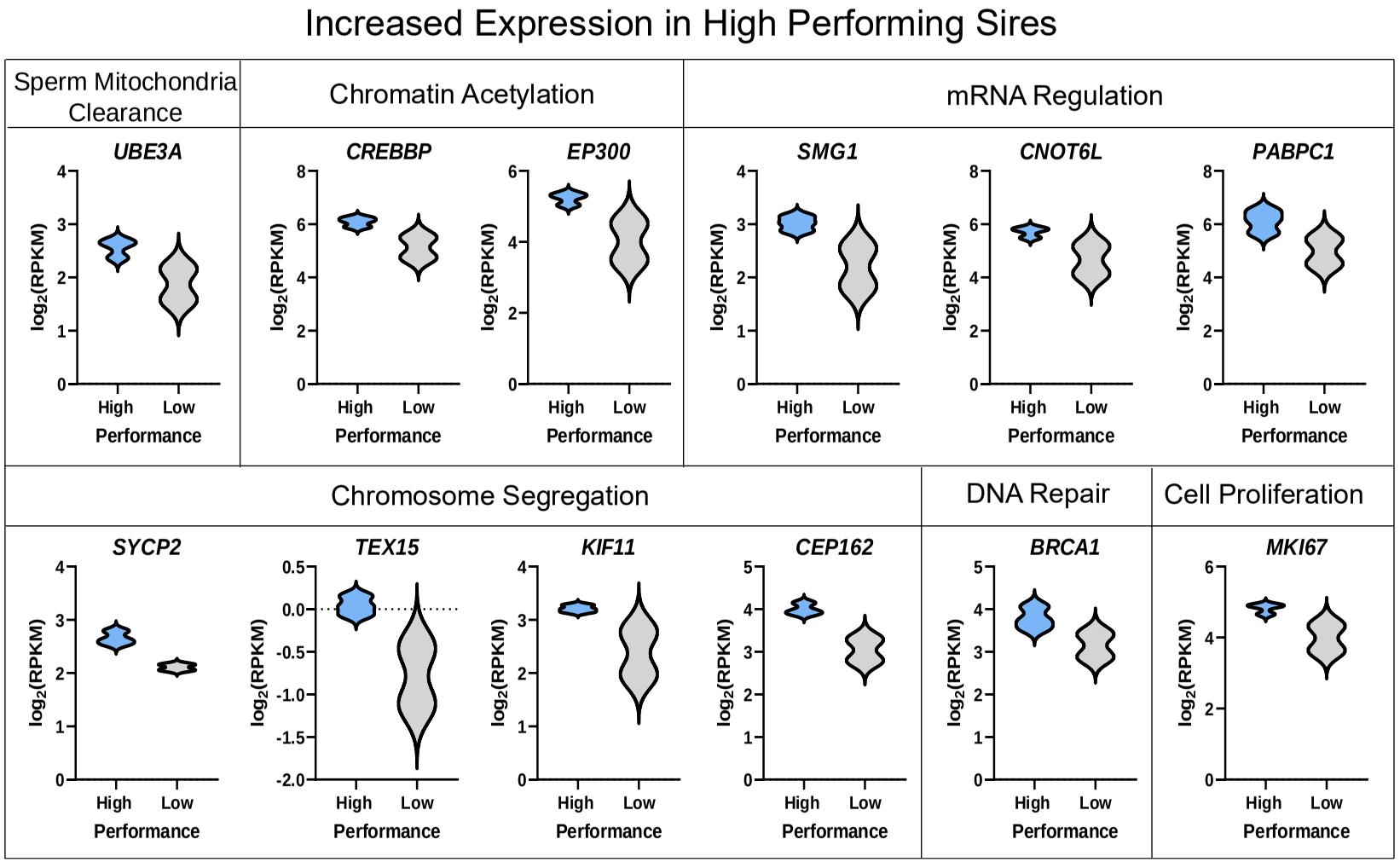

Figure 2.9. Genes with Increased Expression in 4-cell Embryos Produced from HP Sires. Violin plots of differentially expressed genes with increased expression in high performing sires. The respective biological importance is noted above each section of genes.

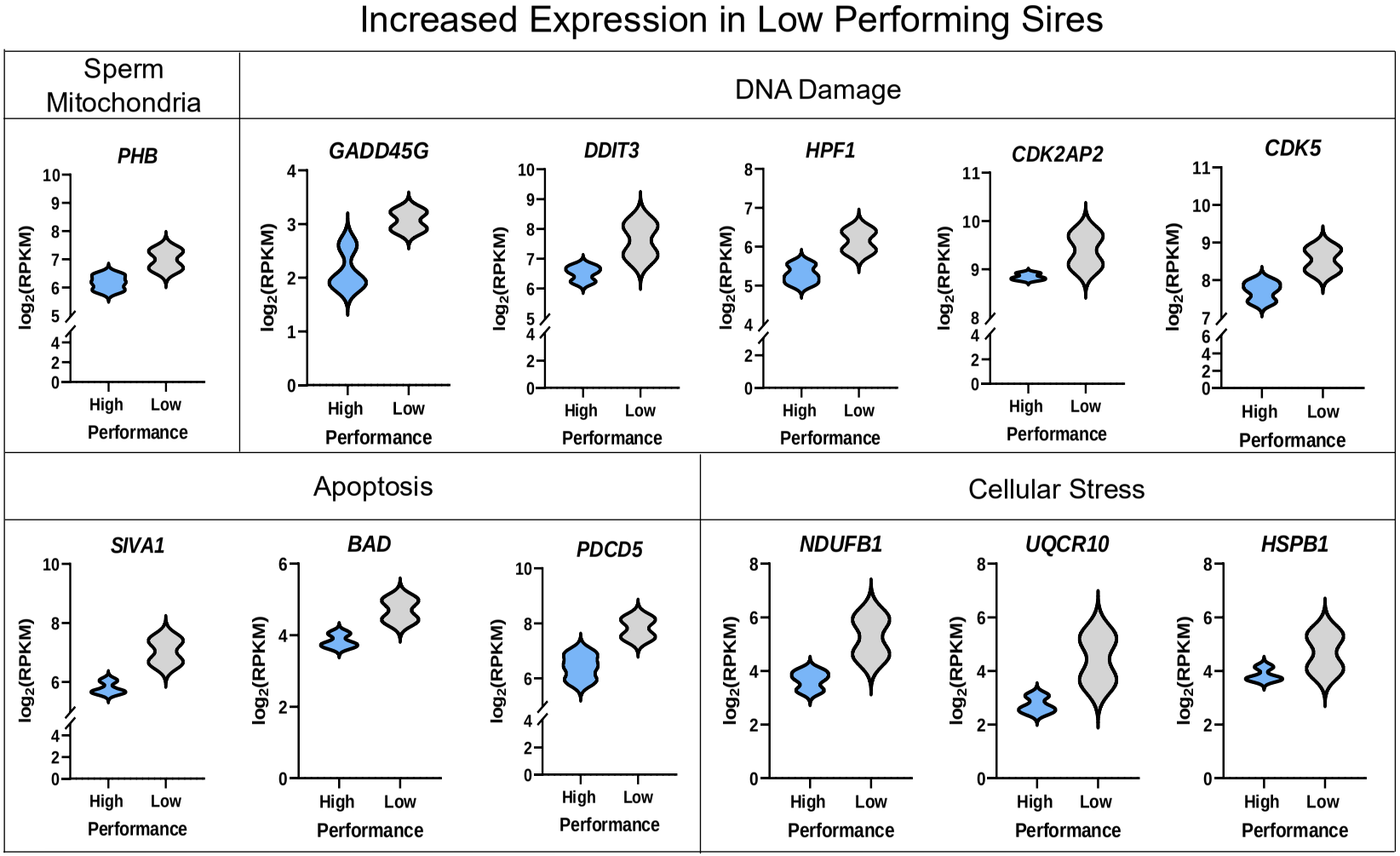

Figure 2.10. Genes with Increased Expression in 4-cell Embryos Produced from LP Sires. Violin plots of differentially expressed genes with increased expression in low performing sires. The respective biological importance is noted above each section of genes.

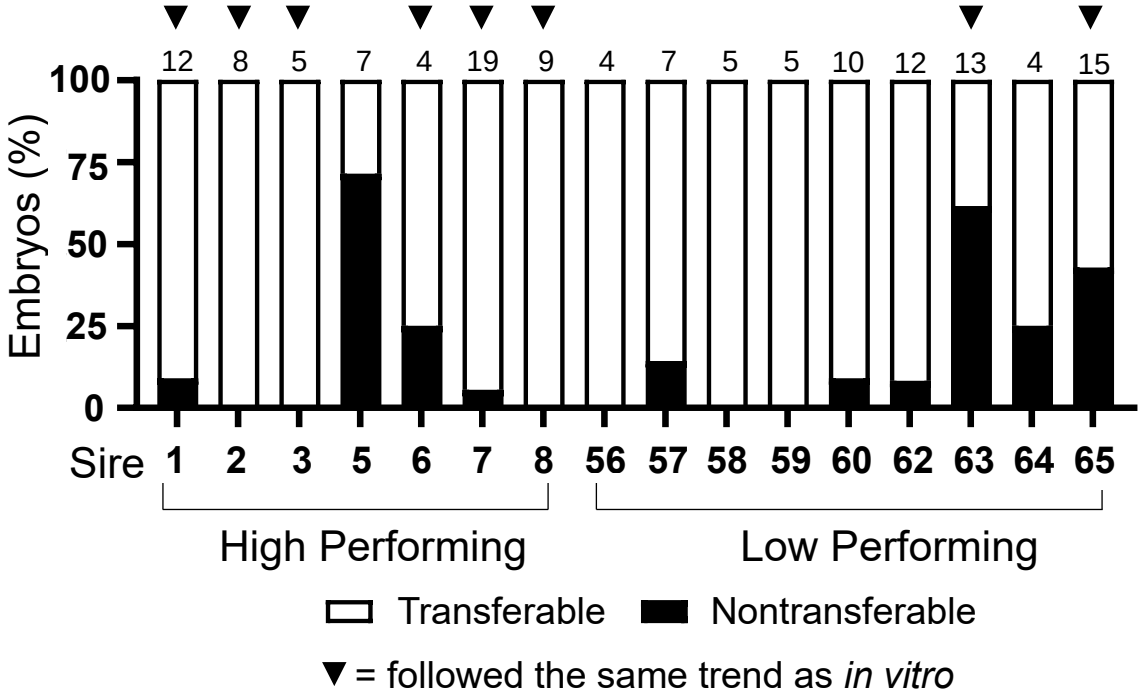

Figure 2.11. Embryos Recovered from Super Ovulated Heifers. Heifers were super ovulated, artificially inseminated, and embryos were flushed on day 8. The percentage of embryos recovered from each sire that was either transferable, or nontransferable is shown. Transferable is defined as morula or blastocyst stage, and nontransferable embryos are defined as degenerated 2-12 cell embryos. The number of embryos recovered for each sire is located above bars. Sires that maintained the same trend as *in vitro* are indicated using black triangles.

Table 2.1. Stage and grades of recovered *in vivo* produced embryos

Embryo Stage	Low Performing Sires		High Performing Sires	
	Grade 1	**Grade 2**	**Grade 1**	**Grade 2**
Morula	28	2	30	4
Early BL	9	0	9	1
BL	12	0	4	1
Expanded BL	6	0	7	0
Degenerated [a]	18		8	
UFO [b]	5		12	
Total	80		76	

The number of embryos collected at various developmental stages. BL = blastocyst, UFO = unfertilized oocytes. Stages and grades were assigned according to IETS standards.
[a] Degenerated 2-12 cell embryos
[b] Unfertilized Oocytes

Discussion

The majority of pregnancy loss in dairy cattle occurs during the first week of gestation. Maternal influences on embryo development and pregnancy loss are well studied, but the paternal effects remain unclear. One explanation for the lack of clarity on the paternal effect of embryo development could be the use of the current sire fertility measurement, SCR, as fertility classifications for a majority of studies. Due to the variation, in embryo development, between sires within the same SCR classification, these studies yield limited results. For example, Ortega *et al.* (2018) identified a sire with a low SCR that had embryo development similar to sires with high SCRs, in addition to low SCR sires that had decreased embryo development compared to high SCR sires. Therefore, to study how sires affect embryo development it is important to first identify sires with different capacities to produce embryos, rather than using SCR classification which can create noise as not all low SCR sires with have an issue producing embryos.

The purpose of this research was to identify sires with high and low capacities to produce embryos, to then provide insights on how they affect embryo development. The sperm is responsible for supplying the oocyte activating factor to resume meiosis, the centrioles necessary for zygotic division, RNAs that regulate transcription and embryonic development, in addition to the paternal genomic material (Schatten 1994; Malcuit *et al.* 2006; Alves *et al.* 2019; Gross *et al.* 2019). Therefore, we hypothesized a sire specific effect on pre-implantation embryo development, that cannot be predicted using SCR alone.

A clear separation of sire performance in IVP supports the idea of a paternal effect on embryonic development, through either a genetic or non-genetic contributions to the embryo. The striking developmental delay, determined by kinetics, in embryos from LP sires suggest these embryos fall behind in development soon after fertilization. In addition to the developmental delay, a large portion of embryos from LP sires fail to pass the 5-6 cell stage. These data suggest the sire plays an important role during the first few cleavage events, possibly through the contribution or lack thereof of important genetic components, RNAs, or organelles, involved in early embryo development. For example, Liu *et al.* (2012) identified a miRNA present in murine sperm important for the first zygotic cleavage. Therefore, the developmental delay and increased embryonic arrest in embryos produced from LP sires may be due to an influence of sire on early embryonic cleavages. Another reason for the developmental delay could be that embryos from LP sires inherit organelles or molecules that are consequential to embryo development, that are not present, at least to the same magnitude, in embryos from HP sires. For example, retention of sperm mitochondria in LP sires could explain the increased rates of autophagy observed in this experiment, possibly contributing to the developmental delay observed. In conclusion, the delay in embryo development from LP sires suggests these embryos have an additional challenge to overcome during early development compared to those produced from HP sires.

The timing of the observed arrest in embryos from LP sires is concurrent with the embryo preparing to undergo the major wave of the EGA at the 8-16 cell

stage. It is believed the embryo begins transcription of select genes at the 2-4 cell stage to produce the large amount of transcriptional machinery needed once the embryo reaches the 8-cell stage (Kaňka *et al.* 2009; Abe *et al.* 2018). Therefore, arrest after the minor wave of the EGA, but before the major wave, suggests these embryos are not able to activate their genome, which results in cell death. Additionally, the increased autophagy in 4-6 cell embryos from LP sires indicates increased embryonic stress at the end of the minor wave, leading up to the initiation of the major wave. Altogether this data indicates a sire specific contribution, either genetic or non-genetic, to embryo development that results in an embryo not being able to successfully complete the minor EGA and initiate the major EGA, which are essential for early embryo development.

However, it is still unknown what paternal contributions are crucial to embryo development. It is important to identify specific genes or genomic signatures in sperm that are reflective of embryo development to build a predictor of sire fertility. Previous studies have identified SNPs located in genes essential for spermatogenesis that are present at different levels among high and low fertility sires. For example, Peñagaricano *et al.* (2012) identified eight SNPs in genes involved in process such as sperm maturation, and chromatin remodeling during spermatogenesis, supporting the idea that a sire's ability to produce embryos can be influenced from the beginning of gamete production. Additionally, work by Han and Peñagaricano (2016) found genes important in motility and sperm-oocyte interactions were strongly associated with sire fertility, suggesting importance of post gametogenesis processes in the effect of sire on

embryo development. However, sire specific genomic signatures, RNAs, organelles, or molecules that negatively influence embryo development are not entirely known. This research suggests the importance of increased sperm mitochondria retention, DNA damage, and ROS in delaying and arresting development in embryos from LP sires due to a genetic or non-genetic contribution of the sire.

Currently, the commercial semen industry routinely measures semen concentration, sperm motility, and sperm morphology, to determine a sire's fertility alongside pregnancy rate data. However, contributions of the sperm to the embryo that do not manifest in a phenotype (i.e. decreased motility) cannot be controlled for under the current screening method. Therefore, in addition to elucidating a genetic component of sire on embryo development, there is also a possibility of discovering an intrinsic quality within the sperm that negatively affects embryo development. Therefore, further investigation of sperm from HP and LP sires can elucidate differences such as reactive oxygen species accumulation, and double stranded DNA breaks in the sperm. This could be useful in the commercial industry as an additional quality parameter to gauge the fertility of a sire based on their effect on embryonic development and create a basis for mechanisms to look into for identifying a genetic component of sire fertility.

This study demonstrates an *in vitro* and *in vivo* model for determining sire fertility, specifically in regards to pre-implantation embryo development, where a majority of pregnancy loss occurs in dairy cattle. Embryos from sires were

phenotyped both *in vitro* and *in vivo* and LP sires consistently produced degenerated, developmentally incompetent embryos suggesting a relationship between IVP and *in vivo* artificial insemination outcomes using the same sire. Given the consistent developmental delay using two sources of abattoir ovaries, and different heifers *in vivo,* it is clear that the harmful contributions from LP sires can be detected regardless of oocyte source. Therefore, investigating sires using the *in vitro* model may be a more powerful tool than *in vivo* super ovulation because it allows for production of hundreds of embryos from oocytes of different environmental and genomic backgrounds to get predict the *in vivo* fertility of a sire in a diverse population of females.

The investigation of mechanisms within the early embryo in this study creates opportunities to building a phenotype of an embryo that is more likely to form a blastocyst, which is very important within the ART industry. Embryo kinetics are well studied in human IVF clinics, but there is less data available for bovine embryo development. The generation of a dataset for developmental stages, specific to bovine, could be an asset to the industry as a method of identifying embryos that are more likely to reach the blastocyst stage. Additionally, the increased autophagy in embryos from LP sires creates a foundation for a measurement of autophagy in early embryos as a marker for developmental competence in bovine. However, baseline levels of autophagy from embryos produced using average fertility sires is still needed to fully understand when and how much autophagy is expected to change in different stages of development for embryos that go on to form blastocysts. Then,

autophagy levels measured from different sires could be a useful quality parameter to not only gauge the quality of an embryo, but also the impact of a sire on embryo development, whether positive or negative. Altogether, by identifying the effect of sire fertility on autophagy and embryo kinetics, this research demonstrates a foundation for these two measurements being used as possible quality indicators in bovine embryo development.

In conclusion, sire-specific contributions affect physiological mechanisms within the embryo, shown in Figure 2.12, that can either promote or halt development to the 8-cell and blastocyst stage. Furthermore, SCR in this experiment was not indicative of pre-implantation embryo development, indicating the need of a more robust model of sire fertility to predict a sires influence on pregnancy before his first mating. This research also provides a foundation for the potential use of additional quality indicators such as DNA breaks and ROS accumulation, and measurement of embryo kinetics and autophagy levels to assess developmental competence of bovine embryos.

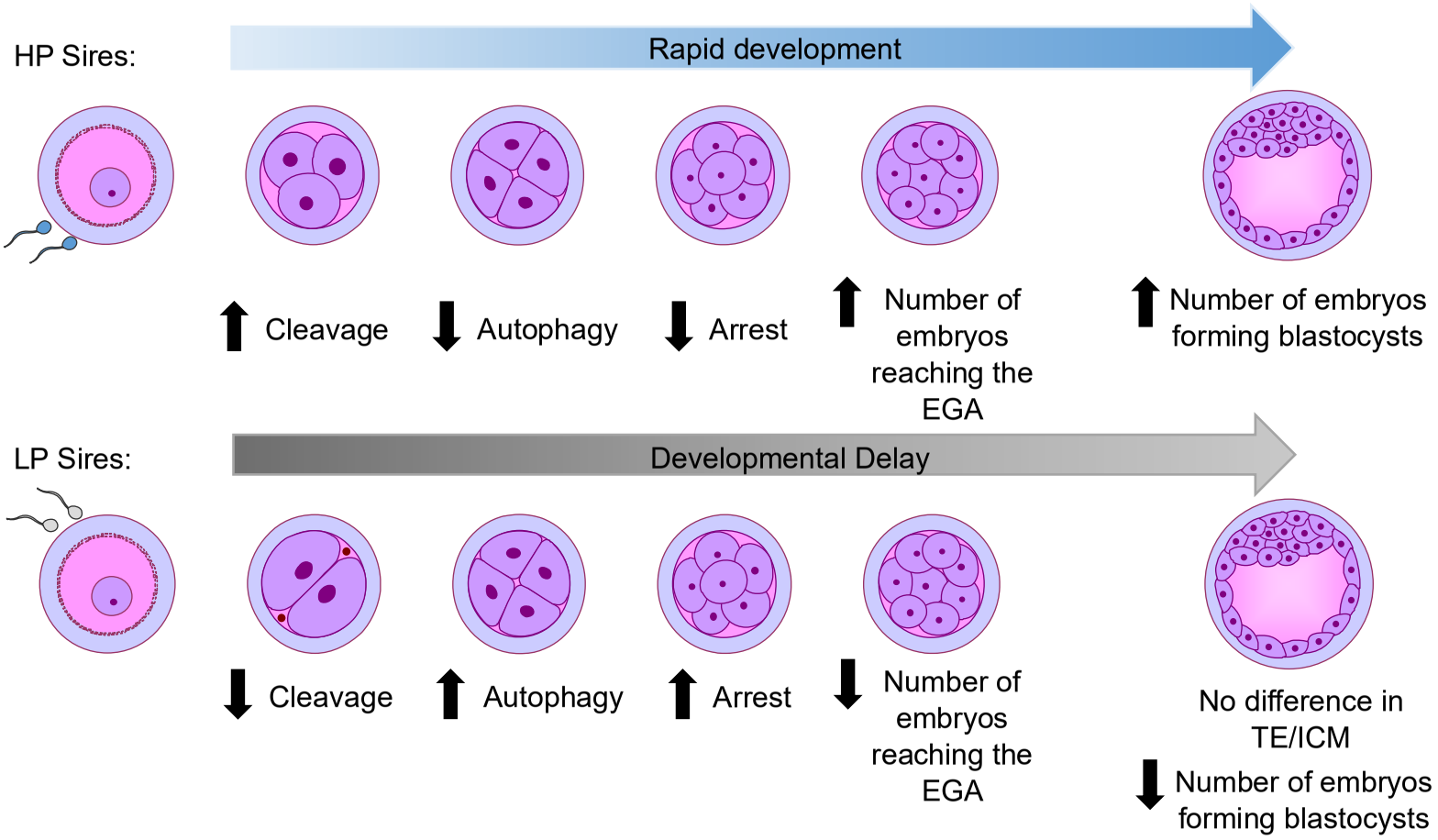

Figure 2.12 Proposed Model of Pre-Implantation Embryo Development from HP and LP Sires. Low performing sires (LP) produce developmentally delayed embryos with decreased cleavage rates, increased autophagy, and increased 5-6 cell arrest compared to high performing (HP) sires. HP sires produce embryos that develop faster and have an increased number of embryos reaching the embryonic genome activation and forming blastocysts. There is no observed observed difference in the ratio of trophectoderm (TE) to inner cell mass cells (ICM) in blastocysts from HP and LP sires. EGA = embryonic genome activation

Supplemental Video 1. Embryos from high performing sires were live imaged beginning at 19 hpi and ending at 118 hpi.

Supplemental Video 2. Embryos from low performing sires were live imaged beginning at 19 hpi and ending at 118 hpi.

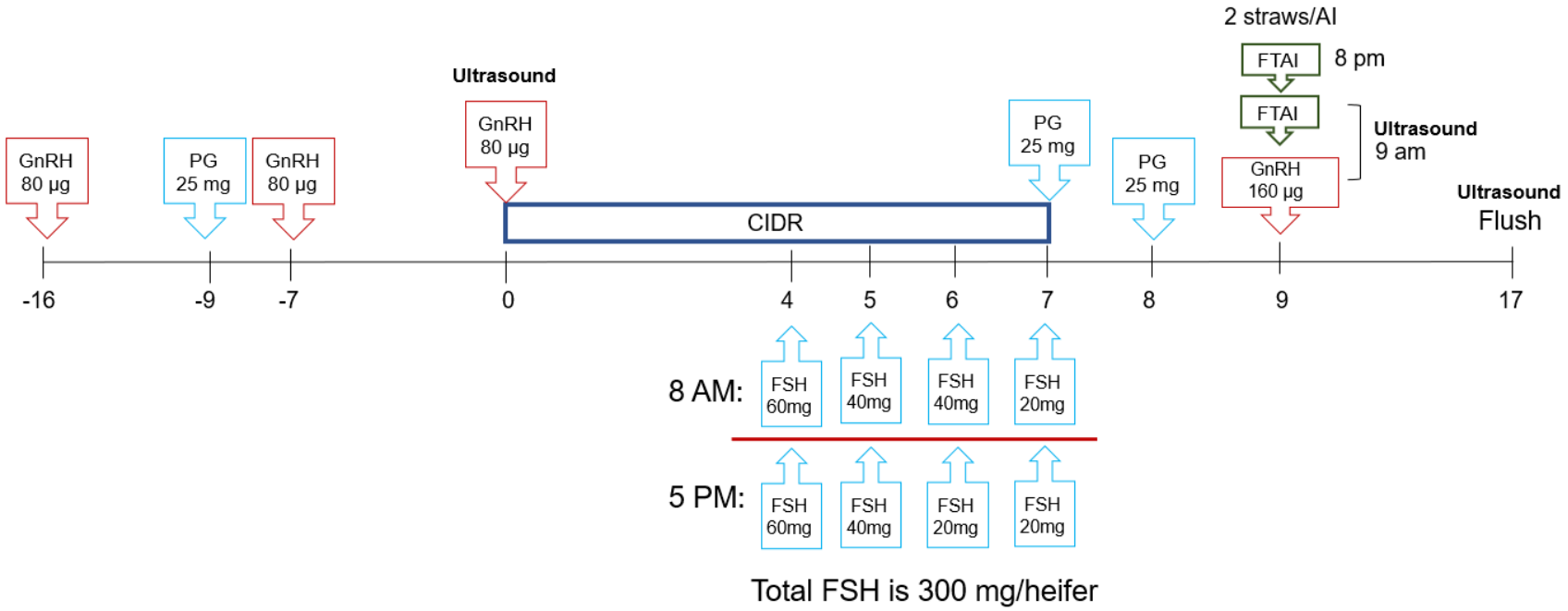

Figure S1. The estrous synchronization and super ovulation protocol used for in vivo validation of sires.

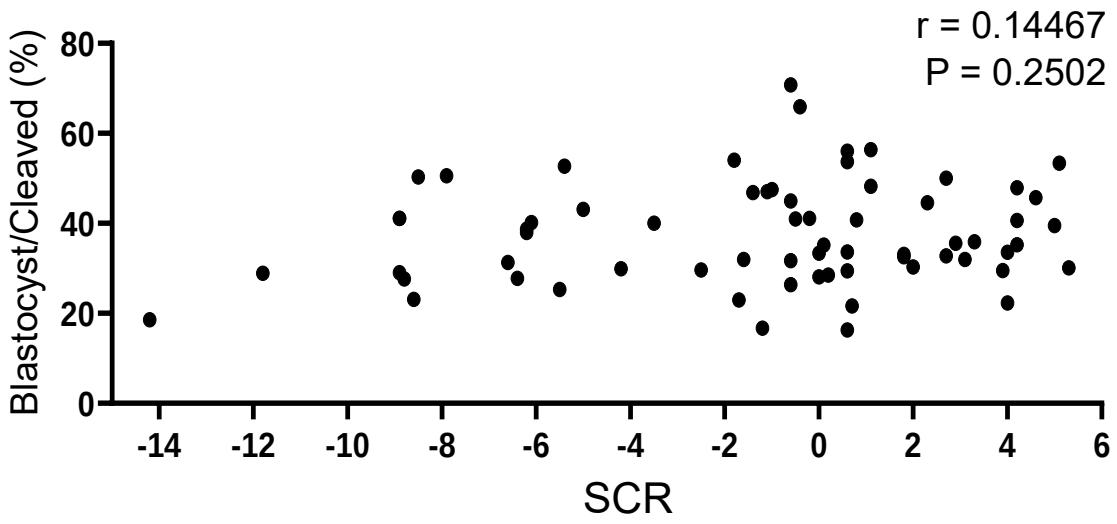

Figure S2. The Correlation of Blastocyst/Cleaved and SCR. The correlation between BL/CL and SCR for the 65 sires used in experiment 1.

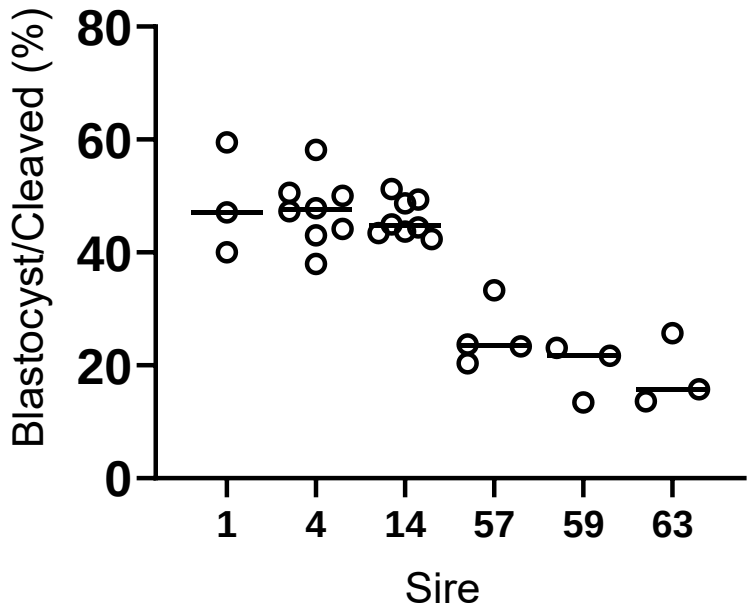

Figure S3. Variability of Blastocyst/Cleaved Rates in IVP. Sires were repeatedly run through IVP of embryos to demonstrate the lack of variability between runs, indicating one IVP run is a good representation of a sire's performance.

Table S1. ToppGene biological processes output for genes with increased expression levels in embryos from low performing sires.

ID	Name	P-value	FDR	Genes from Input
GO:0006613	cotranslational protein targeting to membrane	2.10E-47	1.47E-43	62
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	2.14E-46	7.50E-43	60
GO:0045047	protein targeting to ER	1.08E-44	2.52E-41	63
GO:0072599	establishment of protein localization to endoplasmic reticulum	1.40E-43	2.45E-40	63
GO:0006119	oxidative phosphorylation	7.22E-42	1.01E-38	68
GO:0015986	ATP synthesis coupled proton transport	8.47E-41	8.49E-38	68
GO:0015985	energy coupled proton transport, down electrochemical gradient	8.47E-41	8.49E-38	68
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	1.36E-40	1.19E-37	59
GO:0006414	translational elongation	5.73E-40	4.01E-37	149
GO:0006412	translation	5.73E-40	4.01E-37	149
GO:0009141	nucleoside triphosphate metabolic process	1.41E-38	8.96E-36	81
GO:0009144	purine nucleoside triphosphate metabolic process	1.88E-38	1.10E-35	77
GO:0006754	ATP biosynthetic process	2.21E-38	1.19E-35	71
GO:0006518	peptide metabolic process	2.53E-38	1.20E-35	168
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	2.63E-38	1.20E-35	73
GO:0009201	ribonucleoside triphosphate biosynthetic process	2.74E-38	1.20E-35	74
GO:0009145	purine nucleoside triphosphate biosynthetic process	3.86E-38	1.54E-35	73
GO:0070972	protein localization to endoplasmic reticulum	3.96E-38	1.54E-35	64
GO:0043043	peptide biosynthetic process	5.34E-38	1.97E-35	149
GO:0009205	purine ribonucleoside triphosphate metabolic process	5.80E-38	2.03E-35	75
GO:0016071	mRNA metabolic process	7.23E-38	2.41E-35	160
GO:0009199	ribonucleoside triphosphate metabolic process	8.11E-38	2.58E-35	76
GO:0009142	nucleoside triphosphate biosynthetic process	1.20E-37	3.65E-35	75
GO:0042773	ATP synthesis coupled electron transport	1.15E-36	3.37E-34	52

GO:1902600	proton transmembrane transport	4.21E-36	1.18E-33	81
GO:0000956	nuclear-transcribed mRNA catabolic process	4.69E-36	1.27E-33	72
GO:0070126	mitochondrial translational termination	1.02E-35	2.51E-33	48
GO:0070125	mitochondrial translational elongation	1.02E-35	2.51E-33	48
GO:0042775	mitochondrial ATP synthesis coupled electron transport	1.04E-35	2.51E-33	51
GO:0034622	cellular protein-containing complex assembly	1.17E-35	2.74E-33	189
GO:0022904	respiratory electron transport chain	3.79E-35	8.57E-33	55
GO:0006402	mRNA catabolic process	5.01E-34	1.10E-31	95
GO:0043604	amide biosynthetic process	2.15E-33	4.57E-31	156
GO:0006413	translational initiation	2.35E-33	4.84E-31	67
GO:0006612	protein targeting to membrane	1.33E-32	2.65E-30	70
GO:0006401	RNA catabolic process	1.36E-32	2.65E-30	99
GO:0006415	translational termination	1.55E-32	2.93E-30	49
GO:0032543	mitochondrial translation	3.70E-32	6.82E-30	55
GO:0022900	electron transport chain	6.51E-32	1.17E-29	63
GO:0033108	mitochondrial respiratory chain complex assembly	3.45E-31	6.04E-29	47
GO:0019080	viral gene expression	4.20E-31	7.18E-29	68
GO:0043603	cellular amide metabolic process	1.76E-30	2.94E-28	182
GO:0019083	viral transcription	5.05E-30	8.23E-28	64
GO:0009152	purine ribonucleotide biosynthetic process	1.56E-28	2.48E-26	78
GO:0034655	nucleobase-containing compound catabolic process	2.50E-28	3.89E-26	110
GO:0044270	cellular nitrogen compound catabolic process	2.79E-28	4.25E-26	115
GO:0007005	mitochondrion organization	4.43E-28	6.61E-26	111
GO:0009260	ribonucleotide biosynthetic process	5.16E-28	7.54E-26	79
GO:0046034	ATP metabolic process	7.00E-28	1.00E-25	81
GO:0140053	mitochondrial gene expression	1.64E-27	2.29E-25	56

Table S2. ToppGene biological processes output for genes with increased expression levels in embryos from high performing sires.

ID	Name	P-Value	FDR	Genes from Input
GO:0010558	negative regulation of macromolecule biosynthetic process	9.42E-22	2.96E-18	125
GO:0010557	positive regulation of macromolecule biosynthetic process	1.33E-21	2.96E-18	136
GO:2000113	negative regulation of cellular macromolecule biosynthetic process	1.36E-21	2.96E-18	124
GO:0051254	positive regulation of RNA metabolic process	5.89E-21	9.64E-18	128
GO:0009890	negative regulation of biosynthetic process	7.80E-21	9.65E-18	128
GO:0031327	negative regulation of cellular biosynthetic process	8.85E-21	9.65E-18	126
GO:0051276	chromosome organization	1.50E-19	1.40E-16	101
GO:0033043	regulation of organelle organization	1.96E-19	1.61E-16	101
GO:0006325	chromatin organization	9.98E-19	7.26E-16	77
GO:1903508	positive regulation of nucleic acid-templated transcription	2.19E-18	1.25E-15	118
GO:0045893	positive regulation of transcription, DNA-templated	2.19E-18	1.25E-15	118
GO:1902680	positive regulation of RNA biosynthetic process	2.29E-18	1.25E-15	118
GO:0016569	covalent chromatin modification	1.09E-17	5.51E-15	56
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	3.16E-17	1.48E-14	112
GO:0051253	negative regulation of RNA metabolic process	9.02E-17	3.94E-14	106
GO:0016570	histone modification	2.76E-16	1.13E-13	53
GO:0045892	negative regulation of transcription, DNA-templated	6.54E-16	2.52E-13	99
GO:1903507	negative regulation of nucleic acid-templated transcription	7.49E-16	2.72E-13	99
GO:1902679	negative regulation of RNA biosynthetic process	8.20E-16	2.82E-13	99
GO:0022402	cell cycle process	2.03E-15	6.63E-13	108
GO:0010564	regulation of cell cycle process	4.43E-15	1.38E-12	70
GO:0051726	regulation of cell cycle	7.67E-15	2.28E-12	91
GO:0045944	positive regulation of transcription by RNA polymerase II	9.84E-15	2.80E-12	92

GO:0031399	regulation of protein modification process	1.99E-14	5.43E-12	112
GO:0010638	positive regulation of organelle organization	3.87E-14	1.01E-11	58
GO:0051130	positive regulation of cellular component organization	1.15E-13	2.89E-11	88
GO:0051247	positive regulation of protein metabolic process	2.97E-13	7.20E-11	104
GO:0031401	positive regulation of protein modification process	5.06E-13	1.15E-10	82
GO:0009790	embryo development	5.09E-13	1.15E-10	87
GO:0051493	regulation of cytoskeleton organization	1.17E-12	2.56E-10	51
GO:0031329	regulation of cellular catabolic process	1.92E-12	4.06E-10	67
GO:0009894	regulation of catabolic process	2.40E-12	4.90E-10	75
GO:0032270	positive regulation of cellular protein metabolic process	2.69E-12	5.34E-10	98
GO:0007010	cytoskeleton organization	3.07E-12	5.91E-10	103
GO:0030030	cell projection organization	4.25E-12	7.95E-10	109
GO:0045787	positive regulation of cell cycle	6.00E-12	1.09E-09	43
GO:0040007	growth	6.95E-12	1.23E-09	78
GO:0000902	cell morphogenesis	7.59E-12	1.31E-09	79
GO:0030182	neuron differentiation	1.29E-11	2.13E-09	97
GO:0120036	plasma membrane bounded cell projection organization	1.30E-11	2.13E-09	106
GO:0048589	developmental growth	1.65E-11	2.64E-09	61
GO:0048666	neuron development	1.76E-11	2.74E-09	85
GO:0000278	mitotic cell cycle	2.18E-11	3.09E-09	74
GO:0140014	mitotic nuclear division	2.18E-11	3.09E-09	74
GO:1903047	mitotic cell cycle process	2.18E-11	3.09E-09	74
GO:0009057	macromolecule catabolic process	2.21E-11	3.09E-09	91
GO:0000122	negative regulation of transcription by RNA polymerase II	2.22E-11	3.09E-09	69
GO:0022008	neurogenesis	3.00E-11	4.10E-09	108
GO:0033044	regulation of chromosome organization	3.38E-11	4.51E-09	34
GO:0031175	neuron projection development	4.96E-11	6.49E-09	77

Table S3. ToppGene pathway output for genes with increased expression levels in embryos from high performing sires.

ID	Name	P-Value	FDR	Genes from Input
M19428	Wnt signaling pathway	2.92E-08	3.25E-05	21
M101	Signaling events mediated by HDAC Class I	2.23E-07	1.24E-04	13
M39682	Pathways Affected in Adenoid Cystic Carcinoma	1.62E-06	5.03E-04	12
M39700	Androgen receptor signaling pathway	1.81E-06	5.03E-04	14
M39566	Regulation of Microtubule Cytoskeleton	4.06E-06	9.04E-04	10
M39591	Endoderm Differentiation	6.84E-06	1.27E-03	17
M151	Regulation of Androgen receptor activity	8.54E-06	1.32E-03	10
M13404	Control of Gene Expression by Vitamin D Receptor	9.51E-06	1.32E-03	7
M13266	Renal cell carcinoma	1.90E-05	2.35E-03	11
M39590	Energy Metabolism	2.56E-05	2.85E-03	9
M39597	MAPK Signaling Pathway	3.21E-05	3.21E-03	22
M39338	Angiopoietin Like Protein 8 Regulatory Pathway	3.46E-05	3.21E-03	15
M13191	Prostate cancer	3.90E-05	3.33E-03	12
M39520	Regulation of Actin Cytoskeleton	4.18E-05	3.33E-03	16
M1001	Rho cell motility signaling pathway	4.83E-05	3.39E-03	6
M39715	ErbB Signaling Pathway	4.88E-05	3.39E-03	12
M113	Role of Calcineurin-dependent NFAT signaling in lymphocytes	5.83E-05	3.82E-03	9
M23	Noncanonical Wnt signaling pathway	7.34E-05	4.54E-03	7
M10792	MAPK signaling pathway	9.19E-05	5.39E-03	22
M5193	Genes related to chemotaxis	1.08E-04	5.99E-03	8
M261	p53 pathway	1.38E-04	7.15E-03	9
M39669	Wnt Signaling	1.44E-04	7.15E-03	13
M12467	ErbB signaling pathway	1.48E-04	7.15E-03	11
M288	Notch-mediated HES/HEY network	1.73E-04	8.01E-03	8
M136	FoxO family signaling	2.00E-04	8.93E-03	8
M69	Reelin signaling pathway	2.75E-04	1.18E-02	6
M18306	Regulation of actin cytoskeleton	2.92E-04	1.20E-02	18
M295	Genes related to PIP3 signaling in cardiac myocytes	3.72E-04	1.48E-02	9
M207	Retinoic acid receptors-mediated signaling	4.10E-04	1.52E-02	6

M39445	PI3K-AKT-mTOR signaling pathway and therapeutic opportunities	4.10E-04	1.52E-02	6
M39866	Gastrin Signaling Pathway	4.28E-04	1.54E-02	12
M22006	Chromatin Remodeling by hSWI/SNF ATP-dependent Complexes	4.98E-04	1.73E-02	4
M39605	Circadian rhythm related genes	5.94E-04	2.01E-02	17
M12	RhoA signaling pathway	6.80E-04	2.08E-02	7
M266	N-cadherin signaling events	7.02E-04	2.08E-02	6
M638	Adherens junction	7.08E-04	2.08E-02	9
M39395	Sterol Regulatory Element-Binding Proteins (SREBP) signalling	7.08E-04	2.08E-02	9
M40	E2F transcription factor network	7.08E-04	2.08E-02	9
M281	Signaling events mediated by focal adhesion kinase	7.36E-04	2.10E-02	8
M39761	NRF2-ARE regulation	8.43E-04	2.35E-02	5
M39491	Leptin signaling pathway	9.52E-04	2.52E-02	9
M249	Class I PI3K signaling events mediated by Akt	9.72E-04	2.52E-02	6
M16801	Genes related to regulation of the actin cytoskeleton	9.72E-04	2.52E-02	6
P00048	PI3 kinase pathway	1.01E-03	2.56E-02	7
M7253	Focal adhesion	1.07E-03	2.64E-02	16
M39691	Brain-Derived Neurotrophic Factor (BDNF) signaling pathway	1.09E-03	2.64E-02	13
M39771	Type 2 papillary renal cell carcinoma	1.13E-03	2.69E-02	6
M6907	Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription in carcinoma cells	1.19E-03	2.75E-02	3
M5539	Axon guidance	1.30E-03	2.94E-02	12
P00057	Wnt signaling pathway	1.43E-03	3.13E-02	21

Table S4. ToppGene pathway output for genes with increased expression levels in embryos from low performing sires.

ID	Name	P-Value	FDR	Genes from Input
M189	Ribosome	1.46E-50	1.69E-47	60
M39495	Cytoplasmic Ribosomal Proteins	1.02E-46	5.88E-44	58
M39417	Electron Transport Chain (OXPHOS system in mitochondria)	6.21E-41	2.39E-38	58
M19540	Oxidative phosphorylation	1.65E-38	4.76E-36	62
M7272	Parkinson's disease	1.28E-35	2.95E-33	59
M13486	Huntington's disease	9.90E-30	1.91E-27	63
M16024	Alzheimer's disease	1.54E-29	2.55E-27	60
M39436	Oxidative phosphorylation	5.15E-27	7.44E-25	36
M39806	Nonalcoholic fatty liver disease	1.12E-20	1.43E-18	49
M39781	Mitochondrial complex I assembly model OXPHOS system	4.30E-20	4.96E-18	29
PW:0000034	oxidative phosphorylation	4.73E-20	4.97E-18	28
M4741	Systemic lupus erythematosus	4.83E-16	4.65E-14	40
M39881	Mitochondrial CIV Assembly	1.31E-12	1.16E-10	18
M2044	Spliceosome	4.31E-12	3.56E-10	33
M10680	Proteasome	2.19E-11	1.68E-09	19
M22026	Spliceosomal Assembly	2.87E-11	2.07E-09	11
M39639	Proteasome Degradation	4.31E-11	2.93E-09	22
M194	Proteasome Complex	2.01E-10	1.29E-08	12
MAP00190	MAP00190 Oxidative phosphorylation	7.81E-09	4.75E-07	16
M39885	Cellular Proteostasis	5.32E-07	3.07E-05	6
M17673	Cardiac muscle contraction	2.70E-06	1.49E-04	18
SMP00355	Mitochondrial Electron Transport Chain	8.28E-06	4.35E-04	8
M39386	Eukaryotic Transcription Initiation	1.08E-05	5.41E-04	12
M6981	Protein export	1.19E-05	5.71E-04	9
M39374	Histone Modifications	3.96E-05	1.83E-03	15
M39406	mRNA Processing	5.29E-05	2.35E-03	22
M39898	Mitochondrial CII Assembly	5.81E-05	2.49E-03	5
M1724	RNA polymerase	6.74E-05	2.78E-03	9
M39336	Pyrimidine metabolism	1.34E-04	5.33E-03	16
M5109	Pyrimidine metabolism	2.04E-04	7.84E-03	17
M39892	Mitochondrial CIII assembly	3.63E-04	1.35E-02	6
M963	RNA degradation	3.88E-04	1.40E-02	12
M18937	Nucleotide excision repair	4.63E-04	1.57E-02	10
M39847	Nucleotide Excision Repair	4.63E-04	1.57E-02	10

MAP03020	MAP03020 RNA polymerase	5.30E-04	1.75E-02	6
M39800	FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma	6.43E-04	2.06E-02	9
MAP03070	MAP03070 Type III secretion system	1.04E-03	3.13E-02	6
MAP00193	MAP00193 ATP synthesis	1.04E-03	3.13E-02	6
M39419	Dual hijack model of Vif in HIV infection	1.06E-03	3.13E-02	4
MAP00195	MAP00195 Photosynthesis	1.40E-03	4.04E-02	6

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