

**UTILIZING SEMINAL PLASMA IN PORCINE NON-SURGICAL EMBRYO  
TRANSFER: EFFECTS ON REPRODUCTIVE OUTCOMES AND UTERINE  
TRANSCRIPTOME**

By

Sarah E. Stephens

A Thesis submitted to the Faculty of Graduate and Postdoctoral Studies of

The University of Manitoba

In partial fulfillment of requirements of the degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg, Canada

R3T 2N2

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## ABSTRACT

Embryo transfer (ET) is a reproductive biotechnology used to accelerate genetic gain and improve reproductive efficiency in livestock, but its application in swine remains limited due to technical challenges and inconsistent outcomes following non-surgical embryo transfer (NSET). Seminal plasma (SP) has been proposed as a strategy to improve uterine receptivity through immunomodulatory and gene regulatory effects, potentially enhancing pregnancy establishment and reproductive outcomes. These studies aimed to evaluate whether SP treatment of NSET recipients improves reproductive performance and alters uterine transcriptomic responses. In Experiment I, NSET was performed on control and SP-treated sows with Day 5 embryos and pregnancy rates and litter sizes were analyzed. In Experiment II, gilts were either untreated or treated with SP from different boar genetic lines at estrus and uterine gene expression profiles were compared. Seminal plasma treatment did not significantly affect pregnancy rates or litter size following NSET ( $P > 0.05$ ). However, transcriptomic analysis revealed pronounced, region-specific uterine responses to SP, with differential gene expression largely confined to the distal uterine horn. This response was characterized by activation of pathways associated with cell proliferation, DNA replication, and tissue remodeling, including upregulation of key cell cycle regulators (e.g., CDK1, PLK1, CCNB1, CDC20) and enrichment of transcriptional networks. In contrast, downregulated genes were strongly associated with steroid biosynthesis pathways. Minimal transcriptional differences were observed in the proximal and middle uterine regions, and SP derived from different genetic lines elicited similar uterine responses. Overall, SP induced clear molecular changes consistent with uterine priming for pregnancy; however, these changes were not translated into measurable improvements in reproductive performance under the conditions tested, indicating that further optimization of SP application strategies is required.

## ACKNOWLEDGEMENTS

There are many people and organizations that have contributed to the completion of this thesis and my master's program. Firstly, I would like to thank my advisor, Dr. C. Martin Nyachoti, for taking me on as his student and giving me the opportunity to research my interests. In addition, I would like to thank my advisory committee members; I thank Dr. Huigang Victor Lei, Dr. Louisa Zak, and Dr. Chengbo Yang for their expertise, significant contributions to my education, wonderful learning opportunities, and support. I would also like to thank Dr. Martijn Derks from Wageningen University and Research for his guidance in bioinformatics.

Furthermore, this project was made possible by Mitacs Accelerate funding and by technological support from Topigs Norsvin Canada Inc, Topigs Norsvin Research Center B.V., Carlo Genetics, and Genome Québec.

I want to extend heartfelt thanks to the support staff of the Department of Animal Science and the Glenlea Research Station swine unit; I would particularly like to thank Archie Isit for his impressive adaptability and technical assistance during my research trials and embryo transfer sessions. I would also like to thank my fellow graduate students and colleagues for their ongoing support and friendship during my program, Dr. Xiaoxiao Zhang, Madison Hildebrand, Larissa Scott, and Melanie Buckler.

Lastly, my program would not be completed without the love demonstrated by my whole family. My deepest thanks are extended to: John Stephens and Maria Marasco, my parents and biggest supporters; Sean Stephens, my brother and confidant; Avery Groeneveld, who is like a sister to me; and Lucas Barboza Segato, my partner, whose companionship and encouragement inspire me every day.

## TABLE OF CONTENTS

|                                                                                |             |
|--------------------------------------------------------------------------------|-------------|
| <b>ABSTRACT.....</b>                                                           | <b>ii</b>   |
| <b>ACKNOWLEDGEMENTS .....</b>                                                  | <b>iii</b>  |
| <b>TABLE OF CONTENTS.....</b>                                                  | <b>iv</b>   |
| <b>LIST OF TABLES.....</b>                                                     | <b>vii</b>  |
| <b>LIST OF FIGURES .....</b>                                                   | <b>viii</b> |
| <b>LIST OF ABBREVIATIONS .....</b>                                             | <b>ix</b>   |
| <b>CHAPTER ONE: GENERAL INTRODUCTION .....</b>                                 | <b>1</b>    |
| <b>CHAPTER TWO: LITERATURE REVIEW .....</b>                                    | <b>4</b>    |
| 2.1 INTRODUCTION.....                                                          | 4           |
| 2.2 A COMPARISON BETWEEN EMBRYO TRANSFER IN PORCINE AND BOVINE<br>SYSTEMS..... | 7           |
| 2.2.1 Development of bovine and porcine embryo transfer systems.....           | 7           |
| 2.2.2 Reproductive differences between species .....                           | 9           |
| 2.2.3 <i>In vitro</i> fertilization and ovum pick-up.....                      | 10          |
| 2.3 ISSUES FACING PORCINE EMBRYO TRANSFER.....                                 | 13          |
| 2.4 FUTURE DIRECTIONS FOR PORCINE EMBRYO TRANSFER .....                        | 15          |
| 2.4.1 Cryopreservation .....                                                   | 15          |
| 2.4.2 Cytokines and immune response .....                                      | 17          |
| 2.5 SEMINAL PLASMA TO IMPROVE EMBRYO SURVIVAL.....                             | 18          |

|                                                                                      |           |
|--------------------------------------------------------------------------------------|-----------|
| 2.5.1 What is seminal plasma? .....                                                  | 18        |
| 2.5.2 Effects of SP on the female reproductive tract and reproductive outcomes ..... | 19        |
| 2.6 TRANSCRIPTIONAL PROFILING BY RNA SEQUENCING IN SWINE<br>REPRODUCTION .....       | 22        |
| 2.6.1 Transcriptomics in reproduction research.....                                  | 23        |
| 2.6.2 Porcine reproductive transcriptomics research.....                             | 24        |
| 2.6.3 Challenges and the future of transcriptomics .....                             | 26        |
| <b>CHAPTER THREE: HYPOTHESES AND OBJECTIVES.....</b>                                 | <b>28</b> |
| <b>CHAPTER FOUR: EXPERIMENT I.....</b>                                               | <b>30</b> |
| 4.1 INTRODUCTION.....                                                                | 31        |
| 4.2 MATERIALS AND METHODS.....                                                       | 32        |
| 4.2.1 Seminal plasma preparation.....                                                | 32        |
| 4.2.2 Animals and experimental treatment .....                                       | 33        |
| 4.2.3 Embryo collection and transfer.....                                            | 37        |
| 4.2.4 Statistical analysis.....                                                      | 38        |
| 4.3 RESULTS .....                                                                    | 38        |
| 4.4 DISCUSSION .....                                                                 | 39        |
| 4.5 CONCLUSION .....                                                                 | 42        |
| <b>CHAPTER FIVE: EXPERIMENT II.....</b>                                              | <b>45</b> |
| 5.1 INTRODUCTION.....                                                                | 46        |

|                                              |           |
|----------------------------------------------|-----------|
| 5.2 MATERIALS AND METHODS.....               | 48        |
| 5.2.1 Seminal plasma preparation.....        | 48        |
| 5.2.2 Animals and pre-trial care.....        | 48        |
| 5.2.3 Experimental treatments .....          | 49        |
| 5.2.4 Sample collection .....                | 50        |
| 5.2.5 RNA-seq processing pipeline .....      | 52        |
| 5.3 RESULTS .....                            | 55        |
| 5.4 DISCUSSION .....                         | 63        |
| 5.5 CONCLUSION .....                         | 67        |
| <b>CHAPTER SIX: GENERAL DISCUSSION .....</b> | <b>68</b> |
| <b>CHAPTER SEVEN: CONCLUSION .....</b>       | <b>75</b> |
| <b>CHAPTER EIGHT: LITERATURE CITED .....</b> | <b>77</b> |
| <b>CHAPTER NINE: APPENDIX .....</b>          | <b>84</b> |

## LIST OF TABLES

|                                                                                                                                                                                                                                                                                       |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Table 2.1.</b> Global pig meat producing regions, average percentage of animals serviced with artificial insemination, and total AI million doses per year in these regions in 2011. Adapted from (Riesenbeck, 2011).....                                                          | 5  |
| <b>Table 4.1.</b> Non-surgical embryo transfer session dates with associated embryo numbers, pregnancy, farrowing, and litter size data (where applicable).....                                                                                                                       | 34 |
| <b>Table 4.2.</b> Back pressure test and standing reflex scoring system for estrus detection of sows and gilts using a teaser boar.....                                                                                                                                               | 36 |
| <b>Table 4.3.</b> Pregnancy rates among embryo transfer recipient sows from control (CON) and seminal plasma (SP) treatment groups. Analysis performed with Fisher's exact test ( $P < 0.05$ ) in R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria).....  | 39 |
| <b>Table 4.4.</b> Litter sizes among pregnant embryo transfer recipient sows from control (CON) and seminal plasma (SP) treatment groups. Analysis performed with Welch's T test ( $P < 0.05$ ) in R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria)..... | 39 |
| <b>Table 5.1.</b> Number of differentially expressed (DE) genes per comparison and uterine region using $P$ -value $< 0.05$ . The number of DE genes accounting for multiple testing (adjusted $P$ -value $< 0.05$ ) is given between parentheses .....                               | 58 |

## LIST OF FIGURES

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 2.1.</b> Breeders equation relating to genetic progress and selection variables in an animal breeding program. Adapted from (Decker and Courter, 2025).....                                                                                                                                                                                                                                                                                                        | 4  |
| <b>Figure 2.2.</b> Pregnancy, farrowing, and litter data of recipients after surgical and nonsurgical deep intra-uterine embryo transfer of vitrified-warmed porcine embryos. Procedures included surgical transfer of 30 embryos (S-30), non-surgical transfer of 30 embryos (NsDU-30), and non-surgical transfer of 40 embryos (NsDU-40). Different letters indicate significant differences between procedures ( $P < 0.004$ ). Adapted from (Martinez et al., 2015)..... | 16 |
| <b>Figure 4.1.</b> Schematic of the effects of seasonality (day length reduction) on hormone production and pregnancy. Adapted from (Bertoldo et al., 2012).....                                                                                                                                                                                                                                                                                                             | 41 |
| <b>Figure 5.1.</b> Sampling locations of the uterine endometrium explants taken from each animal. Figure created in BioRender.....                                                                                                                                                                                                                                                                                                                                           | 51 |
| <b>Figure 5.2.</b> Principal component analysis of RNA-seq samples based on overall gene expression profiles.....                                                                                                                                                                                                                                                                                                                                                            | 57 |
| <b>Figure 5.3.</b> Hierarchical clustering of RNA-seq samples based on transcriptome-wide expression profiles.....                                                                                                                                                                                                                                                                                                                                                           | 58 |
| <b>Figure 5.4.</b> Volcano plot showing differentially expressed genes up- or downregulated in gilts in the distal uterine horn treated with seminal plasma (SP_BOTH) versus control gilts.....                                                                                                                                                                                                                                                                              | 61 |

**LIST OF ABBREVIATIONS**

|             |                                                            |
|-------------|------------------------------------------------------------|
| <b>AI</b>   | Artificial insemination                                    |
| <b>CoA</b>  | Coenzyme A                                                 |
| <b>COC</b>  | Cumulus-oocyte complex                                     |
| <b>CON</b>  | Control group                                              |
| <b>D</b>    | Distal uterine sampling location                           |
| <b>DE</b>   | Differentially expressed                                   |
| <b>DGE</b>  | Differential gene expression                               |
| <b>E2F</b>  | Early 2 Factor transcription factors regulating cell cycle |
| <b>ET</b>   | Embryo transfer                                            |
| <b>FDR</b>  | False discovery rate                                       |
| <b>GnRH</b> | Gonadotropin-releasing hormone                             |
| <b>GO</b>   | Gene Ontology                                              |
| <b>IETS</b> | International Embryo Technology Society                    |
| <b>IVF</b>  | <i>In vitro</i> fertilization                              |
| <b>KEGG</b> | Kyoto Encyclopedia of Genes and Genomes                    |
| <b>LH</b>   | Lutenizing hormone                                         |
| <b>M</b>    | Mid-length uterine sampling location                       |

|                                           |                                                                                                |
|-------------------------------------------|------------------------------------------------------------------------------------------------|
| <b>MCM</b>                                | Minichromosome maintenance                                                                     |
| <b>miRNA</b>                              | Micro RNA                                                                                      |
| <b>MOET</b>                               | Multiple ovulation embryo transfer technology                                                  |
| <b>NSET</b>                               | Non-surgical embryo transfer                                                                   |
| <b>OPU</b>                                | Ovum pick-up                                                                                   |
| <b>P</b>                                  | Proximal uterine sampling location                                                             |
| <b>PCA</b>                                | Principle component analysis                                                                   |
| <b>PCOS</b>                               | Polycystic Ovary Syndrome; also referred to as Polyendocrine Metabolic Ovarian Syndrome (PMOS) |
| <b>PGE</b>                                | Prostaglandin E                                                                                |
| <b>PGF<sub>2</sub><math>\alpha</math></b> | Prostaglandin F <sub>2</sub> -alpha                                                            |
| <b>PSP1</b>                               | Porcine Seminal Plasma-1 spermadhesin                                                          |
| <b>PSP2</b>                               | Porcine Seminal Plasma-2 spermadhesin                                                          |
| <b>PCR</b>                                | Polymerase chain reaction                                                                      |
| <b>qPCR</b>                               | Quantitative PCR; also referred to as real-time PCR                                            |
| <b>RIN</b>                                | RNA Integrity Number                                                                           |
| <b>RNA-seq</b>                            | RNA sequencing                                                                                 |
| <b>RT-PCR</b>                             | Reverse transcription-PCR                                                                      |

|                               |                                                                 |
|-------------------------------|-----------------------------------------------------------------|
| <b>SP</b>                     | Seminal plasma                                                  |
| <b>SP_BOTH</b>                | Binary factor combining SP_SELF and SP_SIRE groups for analysis |
| <b>SP_SELF</b>                | Dam line seminal plasma treatment group                         |
| <b>SP_SIRE</b>                | Sire line seminal plasma treatment group                        |
| <b>TGF-<math>\beta</math></b> | Transforming growth factor- $\beta$                             |
| <b>TPM</b>                    | Transcripts per million                                         |

## CHAPTER ONE: GENERAL INTRODUCTION

Embryo transfer (ET) is a reproductive biotechnology used to accelerate genetic improvement and enhance reproductive efficiency in livestock by collecting embryos from genetically superior donor females and transferring them into synchronized recipients (Ciornei, 2022). ET is used extensively in a variety of livestock species at a commercial level; it has been successful in horses and present in the cattle industry for over forty years (Martinez et al., 2013). However, its applications remain limited in swine production systems. The development of ET in pigs has been slower due to anatomical and technical challenges, such as inefficient and inconsistent non-surgical ET (NSET) outcomes and limited success in repeated embryo collection from donor animals (Martinez et al., 2025; Larsen, 2024; Ewasiuk et al., 2025).

There are numerous benefits to developing porcine ET procedures, namely the efficient movement of genetic material while minimizing biosecurity risks and logistical constraints. Transporting embryos instead of live animals decreases disease transmission risk, lowers transportation costs, and avoids welfare concerns associated with live animal transport (Martinez et al., 2013). For widespread commercial adoption, ET procedures must be practical and feasible under field conditions while maintaining high standards of animal welfare.

Seminal plasma (SP) is a biologically active component of semen that influences the female reproductive tract through immune modulation, changes in gene expression, promotion of uterine receptivity to embryos, and regulation of reproductive physiology (Bogacki et al., 2020; Martinez et al., 2019b; Toledo-Guardiola et al., 2024). These effects create a uterine environment that supports implantation, embryo development, and pregnancy establishment, and evidence from pigs and other mammalian species demonstrates that SP can improve reproductive outcomes,

including conception, pregnancy, and farrowing rates (Schjenken and Robertson, 2020). Consequently, the incorporation of SP into embryo transfer protocols represents a promising strategy to enhance the success of NSET and potentially reduce the number of embryos required to achieve acceptable reproductive outcomes (Tajima et al., 2020).

Transcriptomics has become a key tool in porcine reproductive research for characterizing gene expression changes that regulate uterine function, conceptus development, and pregnancy establishment (Huang et al., 2015; Kim et al., 2015). In pigs, RNA sequencing and earlier microarray studies have identified thousands of differentially expressed genes across the estrous cycle and gestation, with major pathways involving steroid metabolism, cell cycle regulation, cell proliferation, and placental development, highlighting coordinated uterine remodeling and maternal–conceptus interactions (Huang et al., 2015; Kim et al., 2015). Network analyses have also revealed key pregnancy-associated genes involved in early uterine function and implantation processes (Kim et al., 2015). More recent transcriptomic work has provided better insights by identifying cell type–specific expression patterns within the endometrium and its cellular structures, and links these patterns to biosynthetic activity, differentiation, and developmental processes (Zeng et al., 2019). Additionally, endocrine signaling pathways have been implicated in regulating uterine receptivity and conceptus development during early pregnancy (Kim et al., 2004). Overall, these studies demonstrate the value of transcriptomics for understanding molecular regulation of reproduction in pigs, while also highlighting the continued need for higher-resolution spatial and temporal profiling of the endometrium during early gestation.

The overall objective of this dissertation was to optimize reproductive outcomes of NSET in pigs using SP as a treatment for embryo recipients. It was hypothesized that SP exposure at estrus would improve embryo transfer success, resulting in increased pregnancy rates and litter

sizes, and that SP would alter uterine gene expression patterns, with SP from different boar genetic lines eliciting distinct transcriptional responses. To test these hypotheses, the effects of SP treatment on pregnancy outcomes following Day 5 embryo transfer were evaluated, and transcriptomic analyses were conducted to characterize uterine gene expression changes, identify biological pathways influenced by SP exposure, and determine whether responses differed among SP genetic lines. Together, these studies aimed to improve understanding of the mechanisms through which SP influences uterine function and embryo transfer outcomes in pigs.

## CHAPTER TWO: LITERATURE REVIEW

### 2.1 INTRODUCTION

Embryo transfer (ET) is an important reproductive biotechnology used to accelerate genetic improvement and increase reproductive efficiency in livestock; the technique involves collecting embryos from genetically superior donor females and transferring them into synchronized recipient animals, allowing multiple offspring to be produced from genetically valuable individuals when combined with superovulation (MOET) (Ciornei, 2022). In both the dairy and beef cattle industry, production of multiple offspring from an elite female through superovulation followed by ET allows for an increased number of selection candidates to breed for the next generation, increasing selection intensity and contributing to overall genetic gain in a breeding population (Figure 2.1) (Decker and Courter, 2025). Additionally, genetic gain can be improved through ET by reducing the generation interval (average time for a male and female to produce their first offspring) of a population by allowing embryos from an elite female to gestate in other recipient animals (Figure 2.1).

$$\Delta G/\text{year} = \frac{r * s_g * i}{L}$$

$\Delta G$ : change per year in trait's genetic merit within the population  
 $r$ : accuracy of selection  
 $s_g$ : amount of genetic variation in the population  
 $i$ : selection intensity  
 $L$ : generation interval

**Figure 2.1.** Breeders equation relating to genetic progress and selection variables in an animal breeding program. Adapted from (Decker and Courter, 2025).

Currently, commercial swine breeding programs have achieved remarkable reproductive efficiency through the widespread adoption of artificial insemination (AI), with AI utilization reaching 80–90% in North America by 2000 and exceeding 95% in major European pig-producing countries by 2011 (Riesenbeck, 2011; Table 2.1). This technology has facilitated global dissemination of superior genetics, with AI centers in Denmark, the Netherlands, and Canada exporting semen to more than 35 countries (Riesenbeck, 2011). Coupled with advances in genetic selection and reproductive management, litter sizes have continued to increase, with European herds commonly achieving 18–20 pigs per litter in highly prolific genotypes and U.S. herds averaging 14–16 pigs per litter (Knox, 2025).

**Table 2.1.** Global pig meat producing regions, average percentage of animals serviced with artificial insemination, and total AI million doses per year in these regions in 2011. Adapted from (Riesenbeck, 2011).

| Region        | Mean AI usage (%) | Total AI doses/year (millions) |
|---------------|-------------------|--------------------------------|
| Asia          | 46.5 ± 28.9       | 41.5                           |
| Europe        | 87.9 ± 11.2       | 45.5                           |
| North America | 80.0 ± 10.0       | 45.2                           |
| South America | 83.3 ± 13.5       | 13.3                           |

In the pork industry, ET is currently being used for the distribution of germplasm (movement of breeding stock) to reduce biosecurity risk and could be a tool to improve animal welfare and minimize genetic lag, which is the time required for genetic improvement to pass from the nucleus level to commercial production (Martinez et al., 2013; Abell et al., 2010). At the nucleus level, all pigs bred are of the most elite genetics. However, due to the impacts of generation

interval on a breeding program and ease of relocating breeding stock onto new boar studs or sow farms, commercial farms tend to lag years behind the nucleus herds (Towers, 2015). ET may allow for the distribution of genetics directly from the nucleus level to commercial producers, thereby reducing the genetic lag on these farms. The present technology for ET is unable to improve selection intensity and reduce the generation interval due to the inability to collect embryos or oocytes repeatedly from donors, however ovum pick-up (OPU) and *in vitro* fertilization (IVF) methods are being explored to increase the number of selection candidates (Oltedal et al., 2024).

In addition to genetic improvement, ET offers practical advantages for livestock production and international breeding programs. Transporting embryos rather than live animals reduces transportation costs, minimizes disease transmission risk, and avoids animal welfare issues associated with long-distance transport (Martinez et al., 2013). Embryo transfer offers important biosecurity advantages by reducing the need to transport live animals, thereby minimizing the risk of introducing infectious diseases into breeding herds by reducing the entry of livestock trucks and new animals from entering properties (Martinez et al. 2013). This is particularly valuable in the swine industry, where diseases such as African swine fever, foot-and-mouth disease, and porcine reproductive and respiratory syndrome can have devastating consequences for herd productivity and global pork production (Knox, 2025). Additionally, there are internationally recognized procedures for the use of embryo transfer technology standards, such as those put forth by the International Embryo Technology Society (IETS); this facilitates the national and international exchange of high-value genetics (Martinez et al., 2025). The transfer of embryos into recipients is also an essential technology for advanced reproductive biotechnologies, including cloning, gene editing, and interspecies blastocyst complementation, where embryos are placed either in the oviduct or the uterine horn of the recipient (Martinez et al., 2019a). However, for widespread

commercial adoption, ET procedures must be practical and feasible under field conditions while maintaining high standards of animal welfare.

## **2.2 A COMPARISON BETWEEN EMBRYO TRANSFER IN PORCINE AND BOVINE SYSTEMS**

Comparing the well-established cattle embryo transfer industry with the developing swine embryo transfer industry can provide valuable insights for improving reproductive technologies in pigs. The widespread commercial success of embryo transfer in cattle demonstrates the potential benefits of optimized donor and recipient management, embryo handling procedures, and reproductive interventions (Ciornei, 2022; Mapletoft, 2013). Examining practices that have contributed to the efficiency and adoption of embryo transfer in cattle may help identify strategies to overcome current limitations in swine embryo transfer and accelerate its commercial implementation.

### **2.2.1 Development of bovine and porcine embryo transfer systems**

The first major reproductive biotechnology applied in cattle was AI, which allowed efficient dissemination of superior male genetics. However, genetic progress through females remained limited because natural reproduction typically produces only one calf per year from a cow (Ciornei, 2022). To address this limitation, superovulation together with non-surgical embryo transfer was developed in the late 1970s as a method to increase the number of offspring produced by genetically superior females and accelerate genetic progress in cattle populations (Ciornei, 2022).

Technological advances facilitated the adoption of ET in the cattle industry; improvements in superovulation protocols, embryo collection techniques, and synchronization of recipient cows allowed efficient embryo production and transfer under field conditions (Ciornei, 2022). The development of embryo cryopreservation was particularly important, as cryopreserved embryos could be stored and later transferred with pregnancy rates comparable to those obtained with fresh embryos (Mapletoft, 2013). Cryopreservation also enabled long-term storage and the international distribution of valuable genetic material, as *in vivo*-derived embryos with an intact zona pellucida can be washed to remove pathogens, making them effectively disease-free and suitable for international trade (Mapletoft, 2013). As a result, thousands of embryos are transported globally each year, supporting genetic improvement programs worldwide. Currently, around 300,000 *in vivo*-derived embryos are produced annually by donor cows, and over two million are produced using *in vitro* techniques, illustrating the widespread adoption of embryo technologies in the cattle industry (Viana, 2025).

In contrast, the development of embryo transfer in pigs has been slower due to anatomical and technical challenges. Early attempts at NSET in the 1950s were unsuccessful, while surgical methods such as laparotomy became the main technique for embryo collection and transfer during the 1960s (Martinez et al., 2013). Although non-surgical embryo transfer methods became commercially viable in cattle during the 1970s, similar approaches in pigs remained difficult to implement. In the 1990s, non-surgical transfer techniques for pigs were developed but showed limited success; later technological improvements led to the development of specialized equipment capable of depositing embryos directly into the uterine body of non-sedated sows, achieving farrowing rates of approximately 40% with more than seven piglets born per litter (Martinez et al., 2013). It has been established that the best results are achieved when non-surgically transferring

30 fresh embryos or 40 vitrified-warmed embryos, with farrowing rates of 30-90% and 50-70%, respectively (Martinez et al., 2025). Further advances focused on devices that could deposit embryos deeper into the uterine horns depending on the developmental stage of the embryos, improving the practical application of ET in pigs (Martinez et al., 2013).

### **2.2.2 Reproductive differences between species**

The reproductive anatomy of a cow makes *in vivo* embryo collection much easier than in sows. Cows have a relatively small uterine body and shorter uterine horns, which allows non-surgical embryo collection via flushing with a catheter possible (Larsen, 2024). In contrast, sows have extremely long (over one meter) and coiled uterine horns, making non-surgical embryo collection from donors difficult and complex (Larsen, 2024). These anatomical differences are a major reason why embryo transfer technologies are more easily applied in cattle than pigs.

Follicular dynamics also differ between species, as cows are a monotocous species (typically one oocyte ovulated each cycle) compared to the polytocous (multiple oocytes ovulated each cycle) sow. In cows, folliculogenesis and follicle recruitment occurs in a wave-like pattern during the estrous cycle; a cohort of follicles grows simultaneously, followed by a selection of a dominant follicle while the others undergo atresia (Evans, 2003). Multiple waves occur during the luteal and follicular phases, and the dominant follicle from the final wave ovulates (Evans, 2003). In pigs, follicular development is classified as continuous, with ongoing growth and atresia rather than distinct waves; many follicles develop into intermediate sizes and those destined to ovulate continue to grow during Days 14-16 of the estrous cycle, meaning that only late follicular phase oocytes can be fertilized in any estrous cycle (Evans, 2003).

These biological differences influence reproductive technologies; in cattle, superovulation can be applied to increase the number of ovulated follicles, allowing multiple embryos to be collected from a single donor (Noga and Looney, 2021). Since only one embryo is typically transferred per recipient cow, this greatly increases embryo transfer efficiency (Noga and Looney, 2021). In contrast, pigs require large numbers of embryos per transfer (approximately 25-30 for *in vivo*-produced fresh embryos), limiting the impact of superovulation and making embryo transfer less efficient compared to cattle (Martinez et al., 2025).

### **2.2.3 *In vitro* fertilization and ovum pick-up**

*In vitro* fertilization (IVF) is an important reproductive biotechnology used in livestock breeding. In cattle, IVF is highly developed and widely applied in commercial breeding programs, whereas in pigs the technology remains less efficient due to biological and technical challenges. Understanding the factors influencing oocyte quality, fertilization efficiency, and embryo development is essential for improving IVF outcomes in both species.

A key determinant of IVF success is oocyte developmental competence. In cattle, oocyte quality is strongly linked to the physiological state of the follicle from which the oocyte originates; oocytes derived from follicles in growing, plateau, or atretic phases show different developmental capacities (Sirard, 2018). Pre-ovulatory follicles may produce highly competent oocytes, although many follicles undergo atresia before ovulation (Sirard, 2018). Advances in molecular technologies have revealed that embryos produced by IVF differ from *in vivo* embryos at the transcriptomic and epigenetic levels, particularly in gene expression; these differences may contribute to slightly lower pregnancy rates and increased sensitivity to cryopreservation compared

with *in vivo* embryos (Sirard, 2018). Nevertheless, bovine IVF is still successful overall and plays a major role in global embryo production. A report of 2024 statistics for embryo production from Viana (2025) states that *in vitro*-produced embryos surpass traditional multiple ovulation and embryo transfer (MOET) systems (over two million embryos compared to under 300,000, respectively). In 2015, *in vitro*-produced embryos exceeded MOET-derived embryos for the first time, and by 2021 approximately 79.7 % of all bovine embryos were produced *in vitro* (Ciornei, 2022).

In contrast, IVF in pigs is less efficient and more difficult to standardize. Conventional methods for collecting oocytes, such as recovery from slaughterhouse ovaries or surgical retrieval, often produce embryos with low developmental competence and high levels of polyspermy (Gil et al., 2025). Polyspermy, the penetration of multiple sperm into a single oocyte, is a major limitation of porcine IVF and results in low proportions of normal monospermic zygotes, typically not more than 45% (Romar et al., 2019). Several factors contribute to this problem, including suboptimal oocyte maturation, high numbers of capacitated sperm around the oocyte, and culture systems that fail to replicate the physiological reproductive environment (Romar et al., 2019). Porcine oocytes are generally matured *in vitro* for approximately 42-44 hours, and culture conditions during this period strongly influence fertilization success and embryo development (Romar et al., 2019). One strategy to reduce polyspermy involves promoting zona pellucida hardening prior to fertilization; preincubation of oocytes in oviductal fluid before insemination can improve fertilization outcomes and increase blastocyst rates to around 40-45% (Romar et al., 2019). The presence of cumulus cells during fertilization may also help reduce polyspermy and support embryo development, while shortening the co-incubation time of sperm and oocytes can further decrease polyspermy rates (Oropeza-Moe et al., 2024).

Despite these improvements, porcine embryo culture systems remain suboptimal, and only about 15-30% of *in vitro*-matured oocytes develop to the blastocyst stage (Oropeza-Moe et al., 2024). Evaluation of embryo quality is also more challenging in pigs than in other species because porcine embryos contain high lipid levels that complicate morphological assessment; as a result, several parameters are used to assess IVF success, including monospermy rate, pronucleus formation, blastocyst development, and cell number (Romar et al., 2019).

Ovum pick-up (OPU) is a key technique for *in vitro* embryo production in cattle, with over 1.4 million transferred embryos derived from oocytes collected via this method (Viana, 2025). Transvaginal ultrasound-guided OPU is minimally invasive and widely used to retrieve cumulus-oocyte complexes (COCs) from live donor cows and is applicable across a broad range of donor types, including prepubertal calves, lactating and dry cows, and cows in early pregnancy up to three to four months (Chinarov, 2024; Galli et al., 2001). Hormonal stimulation is not required, although exogenous gonadotropins, particularly follicle stimulating hormone, can increase follicular growth and oocyte yield and has recently been used in around 54% of OPU collections globally (Viana, 2025). Collection frequency influences oocyte yield and quality, as twice-weekly collections maximize recovery and prevent the formation of dominant follicles which would otherwise cause atresia of subordinate oocytes (Galli et al., 2001). One collection a week yields fewer, lower-quality oocytes due to dominant follicle presence and cumulus degeneration; OPU with superovulation can increase per-session embryo yield but reduce cumulative production over time (Galli et al., 2001). Developmental competence of oocytes generally increases with donor age; however, prepubertal calves are valuable in breeding programs to accelerate genetic gain but may require laparotomy for oocyte collection (Galli et al., 2001). Bovine transvaginal OPU requires specialized equipment and expertise; donors are restrained, sedated, and receive epidural

anesthesia for rectal manipulation of the ovaries, and ultrasound probes with custom needle guides, aspiration needles, and vacuum pressures vary in protocols but should optimize recovery while minimizing oocyte damage (Galli et al., 2001). Although more technically demanding than superovulation, repeated OPU has minimal long-term side effects, except minor ovarian surface hardening in some donors (Galli et al., 2001).

In pigs, OPU is less established but shows potential; transvaginal OPU techniques adapted from cattle allows minimally invasive collection from live sows, and early studies report  $10.7 \pm 4.3$  oocytes per session, with animals returning to normal behavior within a day (Oltedal et al., 2024). The birth of piglets from OPU-derived oocytes in Japan in 2020 demonstrated feasibility, but anatomical challenges (discussed in section 2.2.2), donor variability, and handling requirements reduce efficiency and require further optimization for commercial adoption of the procedure (Oltedal et al., 2024; Oropeza-Moe et al., 2024).

## 2.3 ISSUES FACING PORCINE EMBRYO TRANSFER

Although progressive refinements in NSET techniques have improved outcomes – in some reports raising farrowing rates from 71.4% to 90% and average litter sizes from 6.9 to 9.0 piglets – important biological and technical limitations persist (Martinez et al., 2015). Commercial ET in pigs remains less advanced than in other species, primarily because *in vivo* embryo collection requires either donor slaughter or surgical intervention (Martinez et al., 2019a). Surgical collection does not impair future farrowing rates but negatively affects subsequent prolificacy of the donor sow (Martinez et al., 2019a). Presently, non-surgical collection is nearly impossible; collections from donors with surgically resected uterine tracts show lower embryo collection efficiency, with

an average of  $6.3 \pm 3.4$  embryos from resected tracts compared to  $17.7 \pm 2.2$  embryos collected after slaughter (Hazeleger and Kemp, 2001). Disease control remains a concern, as porcine parvovirus may contaminate follicular fluid from ovaries collected at slaughterhouses, and foot and mouth disease virus may adhere to or penetrate the zona pellucida (Oltedal et al., 2024). Thus, embryo collection remains one of the most limiting steps in porcine ET systems.

Moreover, NSET requires 25-30 fresh embryos per transfer – nearly double the number typically needed for surgical ET – likely due to less precise embryo placement compared with direct deposition into the tip of the uterine horn during surgery (Martinez et al., 2015). Increasing the number of embryos transferred has been shown to increase the number of viable fetuses at Day 30 of gestation and, consequently, the number of piglets born (Martinez et al., 2015). High embryo mortality represents a major biological constraint; approximately 40% of embryos produced through AI or natural breeding fail to develop to term, with significant losses occurring during the peri-implantation period (Days 15-30 of gestation) (Martinez et al., 2020b). Embryo mortality is even more pronounced in ET recipients, where approximately 70% of transferred embryos may not survive (Martinez et al., 2020b). Although fertilization rates in swine are high, early embryonic loss limits potential litter size; contributing factors include improper synchrony between conceptus and uterine development, failure of embryo signaling to recipient, and genetic influences including aneuploidy (Geisert and Schmitt, 2002; Jochems et al., 2023). Additionally, transferred embryos are typically allogenic to the recipient (genetic material entirely foreign), whereas naturally conceived embryos are hemi-allogenic (genetic material is half maternal, half foreign); though studies suggest the maternal immune system is locally downregulated to favor implantation and immune tolerance, immune-mediated rejection may contribute to embryo mortality (Bidarimath and Tayade, 2017; Martinez et al., 2020b). Evidence also suggests that hemi-allogenic conceptuses

are more advanced in development by Day 24 of gestation than fully allogenic embryos, indicating that embryos transferred may be disadvantaged compared to their natural counterparts (Martinez et al., 2020b).

Collectively, these biological, anatomical, and management-related factors continue to limit the efficiency and broader commercial viability of porcine NSET; further refinements to protocols are necessary to improve the acceptance of this procedure in the industry.

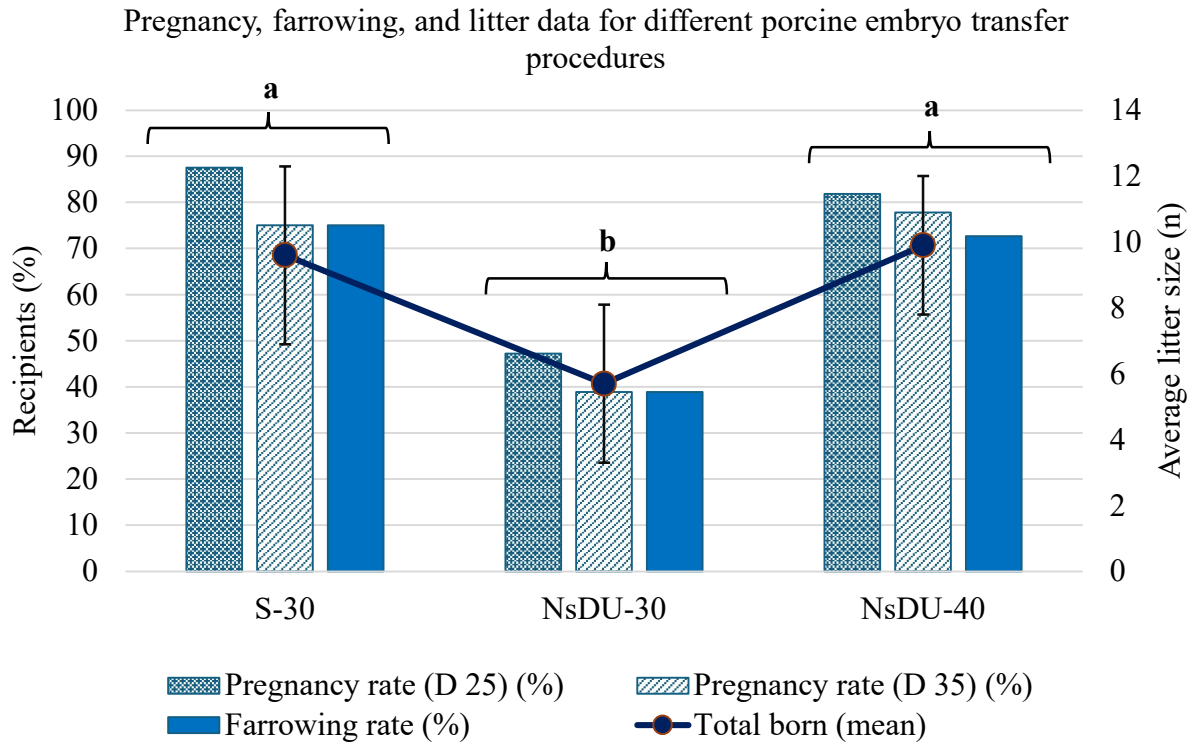
## **2.4 FUTURE DIRECTIONS FOR PORCINE EMBRYO TRANSFER**

Future directions in ET in pigs focus on improving embryo production efficiency, genetic selection, cryopreservation, and minimally invasive reproductive techniques. Advances in molecular technologies and reproductive management are expected to further increase the effectiveness of ET programs in both species.

### **2.4.1 Cryopreservation**

Commercial ET adoption has been slower due to biological challenges, particularly related to the practicalities of *in vivo* embryo collection and for embryos derived from IVF due to low blastocyst yields and poor embryo survival after cryopreservation (Gil et al., 2025). Pig embryos are highly sensitive to low temperatures, making conventional slow-freezing methods ineffective (Cuello et al., 2005). As a result, vitrification of *in vivo*-produced embryos followed by warming and surgical transfer have historically produced the best outcomes for *in vivo*-derived embryos,

however transfer by NSET is successful although less efficient as 40 embryos are transferred (Martinez et al., 2015) (Figure 2.2).



**Figure 2.2.** Pregnancy, farrowing, and litter data of recipients after surgical and nonsurgical deep intra-uterine embryo transfer of vitrified-warmed porcine embryos. Procedures included surgical transfer of 30 embryos (S-30), non-surgical transfer of 30 embryos (NsDU-30), and non-surgical transfer of 40 embryos (NsDU-40). Different letters indicate significant differences between procedures ( $P < 0.004$ ). Adapted from (Martinez et al., 2015).

Further improvements are still needed for the survival of IVF embryos after vitrification due to their increased sensitivity to cryopreservation injury (Martinez et al., 2015). Cryopreservation persists as an important research focus because the ability to store embryos long-

term would allow easier international transport of genetic material, indefinite preservation of valuable genetics, and recovery of superior genetics from diseased herds; successful cryopreservation combined with NSET is considered essential for widespread adoption of swine ET (Martinez et al., 2015). Recent research using vitrified *in vivo*-derived embryos that are warmed on-farm and having 40 blastocysts transferred, compared to the usual 25-30 fresh blastocysts has reported farrowing rates of 25–67% and litter sizes of 3–3.5 piglets, indicating that further optimization of vitrification and transfer protocols is required, especially considering greater numbers of embryos are required for NSET compared to surgical ET (Gil et al., 2025).

#### **2.4.2 Cytokines and immune response**

Another area of research focuses on maternal immune response to the embryo. Studies on cytokines and inflammatory processes suggest that immune signaling may influence embryo survival and implantation success (Martinez et al., 2020a). Hormonal effects of estrogen produced by the developing embryo and progesterone produced by the corpora lutea also have a purpose in maternal recognition; estrogens prevent luteolysis and promote progesterone production, which triggers endometrial secretions and aids in the development of the embryo (Waclawik et al., 2017). Several prostaglandins, including prostaglandin F<sub>2</sub>-alpha (PGF<sub>2</sub>α), contribute to implantation processes such as angiogenesis, trophoblast adhesion, and cell proliferation, is now recognized as a potential mediator of conceptus–endometrial communication (Waclawik et al., 2017). Investigating molecular interactions between the maternal uterus and the allogenic embryo, as well as gene expression changes in the uterus during the peri-implantation period, may help identify strategies to improve ET success rates (Martinez et al., 2019a).

## **2.5 SEMINAL PLASMA TO IMPROVE EMBRYO SURVIVAL**

Improving the efficiency of NSET, especially when using cryopreserved embryos, is critical for its commercial application, as acceptable pregnancy and farrowing outcomes currently require the transfer of more than 30 vitrified-warmed embryos (Tajima et al., 2020). Enhancing uterine receptivity and reproductive success with fewer embryos is therefore desirable. An understanding of how seminal plasma and other seminal components found in whole semen during natural mating modulate the female reproductive tract may help address these fertility limitations and improve NSET outcomes (Alvarez-Rodriguez et al., 2020).

### **2.5.1 What is seminal plasma?**

Seminal plasma is the fluid portion of semen that surrounds spermatozoa and supports sperm transport, protection, nutrition, gamete interaction, and pregnancy establishment (Bogacki et al., 2020). In boars, SP represents approximately 95% of the ejaculate volume and is composed of secretions from mainly the accessory sex glands, such as the vesicular glands, prostate, bulbourethral glands, as well as the testis and epididymis (Parrilla et al., 2020). Specific to boars, the ejaculate is released in distinct fractions: a sperm-rich fraction containing most spermatozoa and relatively little SP, an intermediate fraction, and a post-sperm-rich fraction that contributes the greatest volume but contains few sperm cells (Toledo-Guardiola et al., 2024).

In addition to its role as a transport medium, SP contains bioactive molecules, including proteins, that influence the female reproductive tract. Components of SP deposited in the lower female reproductive tract can easily reach the upper tract and induce molecular and cellular changes in the endometrium (Bogacki et al., 2020). These responses include cytokine release,

increased angiogenesis (development of new blood vessels), altered cell proliferation, and the expression of implantation-related factors that promote maternal immune tolerance of the hemi-allogeneic embryo; the female response to SP is influenced by its composition, and evidence suggests that different semen components exert region-specific effects throughout the reproductive tract, including alterations in cellular proliferation (Toledo-Guardiola et al., 2024).

### **2.5.2 Effects of seminal plasma on the female reproductive tract and reproductive outcomes**

Seminal plasma exerts a wide range of effects on the female reproductive tract that extend beyond sperm transport and fertilization. Following insemination, SP interacts with epithelial and immune cells within the reproductive tract, triggering molecular and cellular responses that influence fertility, implantation, pregnancy establishment, and subsequent fetal and placental development (Schjenken and Robertson, 2020).

A primary effect of SP is the induction of a transient inflammatory response; in pigs, SP exposure increases endometrial inflammation, recruits inflammatory leukocytes, stimulates cytokine production, and alters the expression of more than 1,600 transcripts associated with immune function and cellular processes (Martinez et al., 2019b; Toledo-Guardiola et al., 2024). Similar inflammatory responses have been reported in rodents, humans, dogs, horses, and other livestock species, indicating that SP-induced immune activation is a conserved response among mammals (Schjenken and Robertson, 2020). Following SP exposure, neutrophils, macrophages, and dendritic cells are recruited to the reproductive tract, where they contribute to pathogen defense, regulation of the reproductive tract environment, and preparation of the uterus for embryo implantation (Robertson and Moldenhauer, 2014).

Despite inducing inflammation early in gestation, SP promotes maternal immune tolerance rather than immune rejection. Immunoregulatory molecules present in SP, including prostaglandin E (PGE) and transforming growth factor- $\beta$  (TGF- $\beta$ ), facilitate tolerance to paternal antigens and the developing hemi-allogeneic embryo (Bogacki et al., 2020). Regulatory T cells are activated within the endometrium and contribute to the establishment of a pregnancy-supportive immune environment (Robertson and Moldenhauer, 2014). Studies in mice have shown that the initial inflammatory response is rapidly followed by anti-inflammatory and immune-tolerant states, a pattern that appears consistent across several mammalian species (Schjenken and Robertson, 2020).

Seminal plasma also influences reproductive physiology of the female. Exposure to SP has been shown to trigger ovulation, shorten the interval between the luteinizing hormone (LH) surge and ovulation, and enhance corpus luteum development, leading to increased progesterone production in pigs (Toledo-Guardiola et al., 2024; Alvarez-Rodriguez et al., 2020; Schjenken and Robertson, 2020). Additionally, SP promotes uterine vascularization, supporting placental development and pregnancy establishment (Jalali et al., 2014).

At the molecular level, SP modifies endometrial gene expression and cellular activity. Whole semen induces the greatest transcriptomic response; however, sperm-free SP can also activate pathways associated with cell proliferation and tissue remodeling that prepare the uterus for early embryo development (Alvarez-Rodriguez et al., 2020). Endometrial cell proliferation is critical for uterine receptivity and embryo implantation in pigs (Bogacki et al., 2020). By Day 6 after insemination, SP-induced changes include activation of cell proliferation pathways and suppression of immune-response and steroid-biosynthesis pathways, reflecting a transition toward a uterine environment that supports pregnancy (Bogacki et al., 2020).

These effects are mediated by numerous bioactive SP components, including cytokines, prostaglandins, sex steroid hormones, spermadhesins, and extracellular vesicles that regulate immune signaling and gene expression within the female reproductive tract (Schjenken and Robertson, 2020). In boars, spermadhesins comprise approximately 90% of SP proteins, and specific spermadhesins such as Porcine Seminal Plasma-1 and -2 (PSP1 and PSP2, respectively) have been shown to induce uterine inflammation and immune-cell recruitment (Dostálová et al., 1994; Garcia et al., 2008).

Although SP is not essential for reproduction, its supplementation has been shown to improve reproductive outcomes, including increased farrowing, conception, and pregnancy rates in pigs in suboptimal breeding conditions. SP supplementation has been reported to increase farrowing rates in pigs from 70% to 81% during AI protocols, while the addition of SP to cryopreserved sperm can improve conception and pregnancy rates (Flowers and Esbenshade, 1993; Okazaki et al., 2009). Additionally, the absence of SP is associated with reduced fertilization and embryo development rates, as well as increased post-implantation fetal losses (Martinez et al., 2019b). Furthermore, SP supplementation has been shown to improve farrowing rates and litter sizes (Bogacki et al., 2020). Similar benefits have been observed in other species; SP exposure improves implantation and pregnancy rates in humans and is routinely incorporated into rodent embryo transfer protocols due to its importance for implantation and embryo development (Martinez et al., 2019b; Schjenken and Robertson, 2020). In pigs, AI prior to surgical or non-surgical embryo transfer has improved the survival of vitrified-warmed embryos, enabling successful pregnancies and live piglet production from as few as 10–15 transferred embryos (Tajima et al., 2020). Though these results may be obtained by increasing the overall number of embryos in the recipient and improving pregnancy signaling to the sow, this could indicate that the

presence of SP in semen can be utilized successfully to lessen the number of embryos needed for transfer.

## **2.6 TRANSCRIPTIONAL PROFILING BY RNA SEQUENCING IN SWINE REPRODUCTION**

Transcriptomics is the study of the transcriptome, the complete set of RNA molecules expressed within a cell or tissue at a given time. Because the transcriptome reflects which genes are actively being transcribed, it plays a central role in determining cellular identity, function, development, and responses to environmental stimuli (Wang et al., 2009). Transcriptomic analyses aim to identify RNA species, characterize gene structures, and quantify changes in gene expression across biological conditions, providing valuable insight into gene regulation, development, and disease processes (Wang et al., 2009; Kukurba and Montgomery, 2015).

RNA sequencing (RNA-seq) has become the dominant technology for transcriptome analysis due to its ability to provide unbiased, genome-wide measurement of gene expression. RNA sequencing can be used to identify genes that are active at specific developmental stages or physiological conditions, as well as to detect expression changes resulting from treatments, environmental factors, or disease states; these data provide insight into biological pathways, regulatory networks, fertility, reproduction, and associated disorders (Kulshrestha et al., 2025). In addition, RNA-seq continues to improve understanding of transcriptome evolution and the molecular mechanisms underlying phenotypic variation (Marguerat and Bähler, 2010).

Beyond identifying individual differentially expressed genes, transcriptomic data can be used for gene set enrichment and pathway analyses, allowing researchers to investigate

coordinated biological processes and networks rather than isolated genes (Marguerat and Bähler, 2010). Transcriptomic results can also guide downstream proteomic studies by identifying candidate pathways and genes for further investigation. Furthermore, approaches such as spatial transcriptomics enable the characterization of location-specific gene expression patterns within tissues, providing additional context for understanding cellular interactions and tissue organization (Kulshrestha et al., 2025). RNA sequencing has proven to be a highly reliable method for gene expression analysis, demonstrating strong agreement with quantitative polymerase chain reaction (qPCR), although targeted validation remains valuable when conclusions depend on a small number of genes or subtle expression differences (Coenye, 2021).

### **2.6.1 Transcriptomics in reproduction research**

Transcriptomics has become a powerful tool in reproduction research by enabling comprehensive characterization of gene expression changes across reproductive tissues and developmental stages. In humans, transcriptomic approaches have provided key insights into assisted reproductive technologies, including preimplantation genetic testing and chromosomal aneuploidy screening, as well as mechanisms underlying male infertility through analysis of spermatogenesis and testicular microenvironment abnormalities (Zhao and Tong, 2025). In female reproduction, transcriptome profiling has been used to identify oocyte abnormalities, oocyte stage-specific gene expression patterns in the zona pellucida, and to uncover gene variants associated with Polycystic Ovary Syndrome (PCOS) risk (Kulshrestha et al., 2025). Additionally, studies of the endometrial transcriptome have revealed hormone-driven gene expression changes across the menstrual cycle, dysregulation associated with endometriosis, and altered immune signaling

involving cytokines, growth factors, and progesterone resistance that contribute to disease progression and reproductive dysfunction (Kirschen et al., 2023). Transcriptomics has also advanced understanding of implantation failure, maternal–fetal communication, and immune cell regulation within the uterus, including links between regulatory T cells and recurrent pregnancy loss and PCOS (Kirschen et al., 2023). It has further been applied to uterine fibroids, where distinct gene and micro RNA (miRNA) expression profiles help distinguish pathological states (Kirschen et al., 2023).

In livestock species, transcriptomics is increasingly used to evaluate embryo development and reproductive efficiency, such as comparing *in vivo*-derived and IVF embryos in cattle and identifying dysregulated genes associated with oocyte pickup and *in vitro* production success (Nadeem and Javed, 2022). In buffalo, transcriptome analyses have helped clarify molecular pathways regulating ovarian follicle development, particularly those involving immune-related gene networks (Nadeem and Javed, 2022). Collectively, these applications demonstrate how transcriptomics bridges molecular mechanisms and reproductive outcomes across both human medicine and animal science.

### **2.6.2 Porcine reproductive transcriptomics research**

Transcriptomic reproduction research in pigs has been widely used to characterize uterine gene expression dynamics across gestation and the estrous cycle, particularly during the peri-implantation period. Early studies using microarray and reverse transcription-PCR (RT-PCR) approaches identified extensive differential gene expression in the endometrium across Days 15, 26, and 50 of gestation, with up to 1,649 annotated genes differing between Meishan and Yorkshire

breeds (Huang et al., 2015). These genes were enriched in pathways related to steroid metabolism, cell cycle regulation, cell proliferation, and maternal placenta development, highlighting key biological processes underlying uterine remodeling and conceptus–maternal interactions. Similarly, global gene expression profiling on Day 12 of gestation identified 6,991 differentially expressed genes, with network analyses revealing key pregnancy-associated hub genes such as *ALPPL2*, *RANBP17*, *NF1B*, *SPPI*, and *CST6*, which were subsequently validated using RT-PCR (Kim et al., 2015).

More recent transcriptomic approaches have provided insights into tissue- and cell-type–specific uterine regulation. Low-input RNA-seq of distinct endometrial components, including luminal epithelium, glandular epithelium, blood vessels, and stroma, on Day 14 of gestation identified 3,262 differentially expressed genes in whole tissue, with gene ontology (GO) analyses indicating enrichment for biosynthetic processes, cell growth, differentiation, and developmental pathways (Zeng et al., 2019). Earlier work has also highlighted the importance of endocrine signaling in early pregnancy, identifying estrogen receptor-beta as of potential importance to estrogen action in the endometrium and describing the function of TGF- $\beta$  signaling in conceptus development and uterine receptivity during the peri-implantation period (Kim et al., 2004). Despite these advances, spatial transcriptomic information along the length of the porcine endometrium, particularly at early cycle or gestation stages such as Day 5, remains limited, representing an important gap in current knowledge.

### 2.6.3 Challenges and the future of transcriptomics

Spatial transcriptomics offers opportunities for reproductive biology by preserving spatial context while profiling gene expression, but several technical and analytical challenges currently limit its application. Compared with single-cell RNA sequencing, bulk transcriptomics generally has lower sensitivity, making it more difficult to detect low-abundance transcripts, non-coding RNAs, and rare cell populations that may be biologically important in reproductive tissues (Zhang et al., 2023). Its use also depends on high-quality tissue preservation and involves significant cost and specialized instrumentation. In addition, computational challenges remain substantial, particularly in accurate cell segmentation and in modeling complex cell–cell communication networks, including long-range signaling interactions that are critical for processes such as implantation and uterine remodeling (Zhang et al., 2023).

Despite these limitations, rapid methodological and computational advances are expected to expand the utility of transcriptomics in reproductive research. Improvements in RNA capture efficiency, machine learning–based image analysis, and more sophisticated models are anticipated to improve the accessibility and interpretability of spatial transcriptomic data (Zhang et al., 2023). A major future direction is the integration of RNA-seq with proteomics, which together provide complementary information on transcript abundance and functional protein output; this is particularly important given the relatively weak correlation between RNA and protein levels and the role of post-transcriptional regulation (Wang et al., 2014). More broadly, future transcriptomics applications are expected to move toward dynamic analyses of cellular processes such as gamete development and embryogenesis within tissue environments (Zhang et al., 2023). Integration with additional omics approaches, including epigenomics and functional genomics, will further enhance

understanding of gene regulation and reproductive function, and the identification of key genes involved in fertility, development, and disease (Zhang et al., 2023).

### **CHAPTER THREE: HYPOTHESES AND OBJECTIVES**

The studies in this dissertation tested the following hypotheses:

1. Seminal plasma (SP) treatment of embryo recipient sows will improve reproductive outcomes of non-surgical embryo transfer (NSET).
2. Seminal plasma treatment will alter gene expression patterns in the uterine transcriptome of gilts and different genetic lines of SP will have distinct transcriptional responses.

The overall objective was to optimize reproductive outcomes of an NSET protocol using SP to treat embryo transfer recipients.

The specific objectives were:

1. To determine whether treatment of recipient sows with SP at natural estrus influences pregnancy rates and litter size following Day 5 embryo transfer, compared with untreated recipient sows (naïve uterus controls).
2. To determine the effects of SP exposure at estrus on the porcine uterine transcriptome and its potential implications for embryo transfer success by:
  - a. Evaluating global gene expression patterns along the uterine horn following seminal plasma treatment compared with a naïve uterus;
  - b. Assessing whether SP from different boar genetic lines elicits distinct uterine transcriptional responses;

- c. Identifying the key genes and biological pathways affected by each treatment to improve understanding of the mechanisms through which SP may influence uterine function and embryo transfer outcomes in pigs.

## CHAPTER FOUR: EXPERIMENT I

### Effects of seminal plasma treatment of recipient sows on non-surgical embryo transfer outcomes

**Sarah E. Stephens<sup>1</sup>, Louisa J. Zak<sup>2</sup>, Huaigang Lei<sup>3</sup>, C. Martin Nyachoti<sup>1</sup>**

<sup>1</sup> Department of Animal Science, University of Manitoba, Winnipeg, Canada;

<sup>2</sup> Topigs Norsvin Research Center B.V., 's-Hertogenbosch, The Netherlands;

<sup>3</sup> Topigs Norsvin Canada Inc, Oak Bluff, Canada.

**S.E. Stephens:** methodology, trial conduction, data collection and analysis, writing – original draft.

**L.J. Zak:** conceptualization, methodology, writing – review and editing.

**H. Lei:** conceptualization, methodology, writing – review and editing.

**C.M. Nyachoti:** supervision, project administration, writing – review and editing.

## 4.1 INTRODUCTION

Embryo transfer (ET) is a reproductive biotechnology used to accelerate genetic improvement and enhance reproductive efficiency in livestock through the collection of embryos from genetically superior donor females and their transfer into synchronized recipients (Ciornei, 2022). This technology is well established in several livestock species and has been used successfully in horses and cattle for more than 40 years (Martinez et al., 2013). However, widespread application of ET in swine production remains limited due to anatomical and technical challenges, including inconsistent success of non-surgical embryo transfer (NSET) and difficulties associated with repeated embryo collection from donor females (Martinez et al., 2025; Larsen, 2024; Ewasiuk et al., 2025).

Interest has grown in strategies that improve uterine receptivity and embryo survival following transfer. Seminal fluid contains numerous bioactive components, including proteins, minerals, and lipids, in addition to spermatozoa (Schjenken and Robertson, 2020). Evidence indicates that seminal plasma (SP), the fluid portion of semen not containing spermatozoa, plays an important regulatory role in reproduction in pigs and other species. In sows, SP treatment shortens the interval between the luteinizing hormone (LH) surge and ovulation, advances ovulation, and promotes corpus luteum development and progesterone production (Martinez et al., 2020a). Seminal plasma also induces endometrial gene expression changes associated with maternal immunity, cytokine signaling, cell-cycle regulation, hormone responsiveness, and embryo development, while altering gene expression in Day 6 blastocysts related to development and implantation (Martinez et al., 2020a). Furthermore, components of SP deposited in the lower reproductive tract can easily access the upper reproductive tract and induce changes within the endometrium (Bogacki et al., 2020).

The potential application of SP to improve ET outcomes is supported by evidence that artificial insemination (AI) prior to surgical or non-surgical embryo transfer enhances the survival of vitrified-warmed embryos, resulting in successful pregnancies and live piglet production from as few as 10–15 transferred embryos (Tajima et al., 2020). Although these outcomes may partly reflect an increased number of embryos present within the recipient and enhanced pregnancy signaling, they suggest that SP-associated factors may contribute to improved embryo survival and establishment of pregnancy.

Therefore, this study evaluated whether exposure of multiparous sows to SP at estrus improves reproductive outcomes following transfer of Day 5 embryos. Specifically, the effects of seminal plasma treatment on pregnancy rates and litter size are compared with those of a naïve uterus to determine whether SP exposure enhances reproductive performance, which may provide a practical strategy for improving embryo transfer efficiency in pigs.

## **4.2 MATERIALS AND METHODS**

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee, and pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

### **4.2.1 Seminal plasma preparation**

Raw ejaculates (with sperm motility and normality > 70%) from proven Large White boars were transferred to sterile 50 mL centrifuge tubes (Celltreat, Ayer, MA, USA) under aseptic

conditions and centrifuged at 25°C in 800 G for 10 minutes (protocol taken from Pedrosa et al., 2021). Seminal plasma was extracted and stored in sterile 50 mL centrifuge tubes (Celltreat, Ayer, MA, USA) at -20°C until the experimental target volume was reached. Seminal plasma was thawed at room temperature and pooled before being divided into 80 mL doses (QuickTip Flexitube; Minitube International, Tiefenbach, Germany). All prepared doses were then stored at -20°C until required for experimental use.

#### **4.2.2 Animals and experimental treatment**

A total 96 Landrace x Large White F1 sows from the Glenlea Research Station (University of Manitoba) sow barn herd were selected for the experiment. Sixty-four sows were selected as embryo donors and 32 sows were selected as embryo recipients over the course of eight embryo transfer sessions (Table 4.1), where each session consisted of eight donor and four recipient animals. Out of the recipient sows selected each session, two were randomly assigned to the control (CON) group and two were randomly assigned to the seminal plasma treatment (SP) group. Animals were housed in individual crates and were subjected to boar exposure twice per day three days after weaning to record and track behavioural estrus duration until the end of their estrus. Animals were selected for the study based on previous reproductive performance (no less than 11 piglets from previous litter), good health status, and parity; sows from second parity and onwards were considered for donors, and sows from third parity and onwards were considered for recipients, as per recommendations by the International Embryo Technology Society (IETS) porcine embryo transfer manual (Martinez et al., 2025).

**Table 4.1.** Non-surgical embryo transfer sessions with associated embryo numbers, pregnancy, farrowing, and litter size data (where applicable).

| Session date | Sample number | Treatment | Embryos transferred (n) | Pregnant <sup>a</sup> (Y/N) | Farrowed (Y/N) | Litter size (n) |
|--------------|---------------|-----------|-------------------------|-----------------------------|----------------|-----------------|
| Dec-2024     | 1             | CON       | 30                      | Y                           | Y              | 4               |
| Dec-2024     | 2             | CON       | 29                      | N                           | N              | 0               |
| Dec-2024     | 3             | SP        | 30                      | N                           | N              | 0               |
| Dec-2024     | 4             | SP        | 30                      | Y                           | Y              | 8               |
| Feb-2025     | 5             | SP        | 29                      | Y                           | Y              | 5               |
| Feb-2025     | 6             | CON       | 30                      | N                           | N              | 0               |
| Feb-2025     | 7             | SP        | 30                      | N                           | N              | 0               |
| Feb-2025     | 8             | CON       | 30                      | Y                           | Y              | 11              |
| Mar-2025     | 9             | CON       | 29                      | Y                           | Y              | 8               |
| Mar-2025     | 10            | SP        | 30                      | N                           | N              | 0               |
| Mar-2025     | 11            | CON       | 30                      | N                           | N              | 0               |
| Mar-2025     | 12            | SP        | 30                      | N                           | N              | 0               |
| May-2025     | 13            | CON       | 30                      | Y                           | Y              | 8               |
| May-2025     | 14            | SP        | 29                      | Y                           | Y              | 5               |
| May-2025     | 15            | CON       | 30                      | Y                           | Y              | 3               |
| May-2025     | 16            | SP        | 30                      | N                           | N              | 0               |
| Sep-2025     | 17            | SP        | 31                      | N                           | N              | 0               |
| Sep-2025     | 18            | SP        | 31                      | N                           | N              | 0               |
| Sep-2025     | 19            | CON       | 31                      | N                           | N              | 0               |
| Sep-2025     | 20            | CON       | 31                      | N                           | N              | 0               |
| Nov-2025     | 21            | SP        | 26                      | N                           | N              | 0               |
| Nov-2025     | 22            | SP        | 27                      | Y                           | Y              | 6               |
| Nov-2025     | 23            | CON       | 27                      | N                           | N              | 0               |
| Nov-2025     | 24            | CON       | 27                      | N                           | N              | 0               |
| Jan-2026     | 25            | SP        | 28                      | N                           | N              | 0               |
| Jan-2026     | 26            | CON       | 28                      | N                           | N              | 0               |
| Jan-2026     | 27            | SP        | 28                      | N                           | N              | 0               |
| Jan-2026     | 28            | CON       | 29                      | N                           | N              | 0               |
| Apr-2026     | 29            | CON       | 28                      | N                           | N              | 0               |
| Apr-2026     | 30            | SP        | 28                      | N                           | N              | 0               |
| Apr-2026     | 31            | SP        | 28                      | N                           | N              | 0               |
| Apr-2026     | 32            | CON       | 29                      | N                           | N              | 0               |

<sup>a</sup> Pregnancy confirmed by the presence of behavioural estrus and percutaneous ultrasound on Day 35.

Animals were selected based on estrus behaviour with an aim to achieve asynchronous transfer between donors and recipients (outlined in Angel et al., 2014). Donor sows were selected four days after weaning with a behavioural estrus score of 6-8 (Table 4.2) and were treated with 0.75 mL of human Chorionic Gonadotropin (Chorulon; Intervet Inc., Rahway, NJ, USA) followed by 5 mL of 0.9% saline (PosiFlush; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) via intramuscular injection to synchronize ovulation. Donors were inseminated (intracervical) during their natural behavioural estrus (Day 0) at 24 and 36 hours after hormone administration; a third insemination was performed if sows were still in behavioural estrus (scores 8-10; Table 4.2) at 60 hours post injection.

Recipient sows were selected five days after weaning with a behavioural estrus score of 10 (Table 4.2) and were assigned to treatment groups. Sows assigned to the **SP** group received two intracervical seminal plasma doses, one at the time of natural estrus and again 24 hours later. Sows in the **CON** group were untreated.

**Table 4.2.** Back pressure test and standing reflex scoring system for estrus detection of sows and gilts using a teaser boar<sup>a</sup>.

| Estrus score | Description                        | Details                                                                                                                                                                                                                |
|--------------|------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 6            | Fairly quickly but with help       | <p>Animal hears the boar approaching and responds with interest.</p> <p>Brief standing reflex can be obtained with back pressure stimulation.</p>                                                                      |
| 8            | No difficulty, but not lasting     | <p>Animal shows interest in the boar and is restless when close by.</p> <p>Animal shows standing reflex which lasts for about a minute.</p> <p>With back pressure, animal immediately shows standing reflex again.</p> |
| 10           | Automatic, lasting several minutes | <p>Animal shows full standing reflex, lasting up to 5 minutes or longer.</p> <p>Even after the boar moves away, standing reflex continues.</p> <p>No back pressure is required to trigger standing reflex.</p>         |

<sup>a</sup> Scale developed based on estrus descriptions from Soede et al. (1995) and Glencorse et al. (2025).

#### 4.2.3 Embryo collection and transfer

On Day 5, donor sows were sedated with intramuscular Stresnil (1 mL/20 kg; Elanco Pharmaceuticals, Indianapolis, IN, USA) and Nerfasin 100 (0.80 mL/20 kg; Dechra Pharmaceuticals, Northwich, England) and then euthanized by captive bolt pistol followed by exsanguination for 10 minutes, and reproductive tracts were retrieved immediately. Embryos were collected by flushing the distal part of each uterine horn with 40 mL of warmed (38°C) Tyrode's lactate-HEPES-polyvinyl alcohol medium (taken from Martinez et al., 2025). Each uterine horn was held vertically in a clamp and, using an 18-gauge needle attached to a 50 mL syringe, it was inserted into uterine lumen close to the utero-tubal junction. At a length of 40 cm distal from the utero-tubal lumen, the uterine horn was cut and fluid containing the embryos was collected in warmed sterile tubes (Celltreat, Ayer, MA, USA) and placed in a water bath set to 38°C until embryo searching. Each reproductive tract was assessed for anatomical abnormalities that may influence embryo performance, such as the presence of ovarian cysts.

Each tube of flushed medium was evaluated for the presence and stage of embryos, according to the guidelines in the IETS porcine embryo transfer manual (Martinez et al., 2025). Embryos classified from compacted morula to full blastocyst stages and of good to excellent quality (Martinez et al., 2025) were then assigned to each transfer recipient, so that embryos from each donor and embryo stages were evenly distributed across embryo recipients in each transfer. Intrauterine embryo transfer catheters (DeepBlue; Minitube International, Tiefenbach, Germany) were placed in recipients according to the guidelines in the IETS porcine transfer manual (Martinez et al., 2025) and between 26 to 31 embryos were transferred to each sow (Table 4.1). Following transfer, recipient sows were individually housed until first pregnancy check (up to 35 days) before

returning to the barn's gestation herd. Pregnancy was confirmed by the presence of behavioural estrus and percutaneous ultrasound.

Pregnancy data were collected for each recipient sow, followed by farrowing rates and litter sizes, if applicable (Table 4.1).

#### 4.2.4 Statistical analysis

All statistical analysis was performed on R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria) using the default and *performance* packages with default settings unless otherwise stated; P-values  $< 0.05$  were considered significant. Pregnancy rate data were analyzed using Fisher's exact test and a Cochran-Mantel-Haenszel test with session as a fixed effect was used to confirm the results. Litter size data among the animals that became pregnant were analyzed using Welch's T test and a generalized linear model with session as a fixed effect was performed as a supporting check.

### 4.3 RESULTS

Pregnancy rates did not differ significantly between the **CON** and **SP** groups when analyzed by Fisher's exact test ( $P > 0.05$ , Table 4.3). This result was consistent with the Cochran–Mantel–Haenszel test controlling for session, which detected no significant treatment effect ( $P = 1.00$ ). The estimated common odds ratio was 0.67, although this estimate should be interpreted with caution because there was no evidence of a statistically significant treatment effect when controlling for session.

**Table 4.3.** Pregnancy rates among embryo transfer recipient sows from control (**CON**) and seminal plasma (**SP**) treatment groups. Analysis performed with Fisher's exact test ( $P < 0.05$ ) in R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria).

| Treatment  | Pregnant/total (n) | Pregnancy rates (%) | P-value |
|------------|--------------------|---------------------|---------|
| <b>CON</b> | 5/16               | 31.3                | 1.00    |
| <b>SP</b>  | 4/16               | 25.0                |         |

Litter size data did not differ significantly between the **CON** and **SP** groups when analyzed with Welch's T test ( $P > 0.05$ ; Table 4.4); this result was consistent with results obtained by generalized linear model with session as a fixed effect ( $P = 0.665$ ).

**Table 4.4.** Litter sizes among pregnant embryo transfer recipient sows from control (**CON**) and seminal plasma (**SP**) treatment groups. Analysis performed with Welch's T test ( $P < 0.05$ ) in R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria).

| Treatment  | Pregnant (n) | Mean litter size $\pm$ SD | P-value |
|------------|--------------|---------------------------|---------|
| <b>CON</b> | 5            | $6.80 \pm 3.27$           | 0.641   |
| <b>SP</b>  | 4            | $6.00 \pm 1.41$           |         |

#### 4.4 DISCUSSION

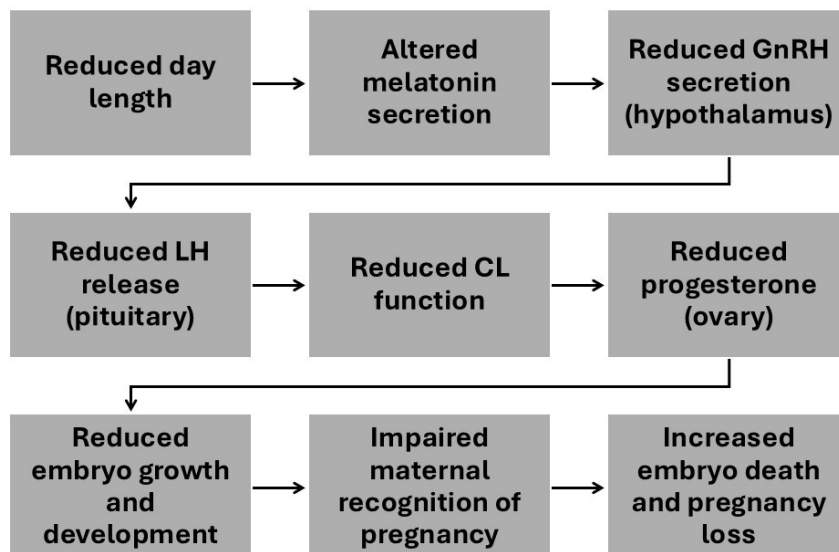
The objective of this study was to evaluate whether exposure of recipient sows to SP at estrus could improve reproductive outcomes following NSET. Based on previous research demonstrating that SP modulates the uterine environment through effects on immune regulation and reproductive physiology, it was hypothesized that SP treatment would enhance pregnancy

establishment and litter size. However, no significant differences were observed between the SP-treated and control groups for either pregnancy rate or litter size. These results suggest that, under the conditions of the present study, SP exposure at the time of estrus did not improve NSET outcomes.

Although SP has been shown to promote uterine receptivity and improve reproductive performance in several studies, the absence of significant treatment effects may be due to limitations associated with the experimental design. A primary limitation was the modest sample size, with only 16 sows assigned to each treatment group; this treatment size was based on 70% farrowing rates previously achieved to obtain appropriate statistical power. Due to the various factors that may play a role in ET success, including variability associated with reproductive traits, this group size may have been too low to detect relatively small but biologically meaningful differences in pregnancy rate or litter size for this experimental set up.

Additionally, embryo transfers were conducted over multiple sessions involving only a few animals at a time. As a result, seasonal influences may have contributed to variation in reproductive performance among recipients, although all treatments were represented in all sessions. Limitations in human resources and number of animals available at any given session may have also contributed to the variation in reproductive performance, especially since donors were slaughtered on-farm as opposed to in an abattoir. Seasonal infertility is a well-documented challenge in swine production and has been associated with prolonged wean-to-estrus intervals, delayed ovulation, and reduced farrowing rates, particularly during late summer and early autumn (Bertoldo et al., 2012). These reproductive effects can be influenced by seasonal changes in melatonin secretion that alter hypothalamic gonadotropin-releasing hormone (GnRH) release and subsequently affect luteinizing hormone (LH) pulse frequency and amplitude. Alterations in LH

secretion may impair ovulation, corpus luteum formation, and progesterone production, ultimately creating a less favorable uterine environment for embryo survival and development (Figure 4.1) (Bertoldo et al., 2012). Consequently, seasonal variation and seasonal changes to fertility may have masked potential treatment effects of SP.



**Figure 4.1.** Schematic of the effects of seasonality (day length reduction) on hormone production and pregnancy. Adapted from (Bertoldo et al., 2012).

Variation in personnel throughout the study may have also contributed to experimental inconsistency. Changes in staff increase the likelihood of variation in animal handling, embryo collection procedures, embryo evaluation, and embryo transfer techniques, all of which can influence reproductive outcomes. Furthermore, this study was conducted under commercial production conditions rather than in a controlled laboratory environment. While this increases the practical relevance of the findings, it also introduces additional sources of variability. Embryos

may have been exposed to non-ideal handling conditions, including temperature fluctuations during collection and transfer procedures, as well as environmental contaminants such as dust and insects; although these factors were managed to the best of our team's abilities, these are commonly present in commercial barn facilities. These factors may negatively affect embryo viability and could partially explain differences between the present findings and previously published studies conducted under more controlled conditions (Martinez et al., 2025).

Despite the lack of significant treatment effects observed in this study, the biological mechanisms by which SP influences the female reproductive tract are documented in other studies. Seminal plasma has been shown to promote immune tolerance, influence ovulation and luteal function, and improve reproductive outcomes in a variety of species (Martinez et al., 2020a). This study attempted to utilize SP in a commercial NSET program; after the results obtained from this study, additional research is warranted to further evaluate its potential application in commercial NSET programs. Future studies should utilize larger sample sizes and implement greater control over environmental and management factors. Standardizing personnel involvement and minimizing exposure of embryos to temperature fluctuations and environmental contaminants may also reduce experimental variation. Such studies would help clarify whether SP treatment can reliably improve pregnancy rates, litter size, and overall NSET efficiency under commercial swine production conditions.

## **4.5 CONCLUSION**

This study evaluated whether exposure of recipient sows to seminal plasma at estrus could improve reproductive outcomes following NSET of Day 5 embryos. Although seminal plasma has been shown to influence uterine receptivity, immune regulation, and reproductive physiology, no

significant differences were observed between seminal plasma-treated and control sows for either pregnancy rate or litter size. These findings suggest that seminal plasma treatment alone did not improve embryo transfer outcomes under the conditions of the present study. However, the established biological effects of seminal plasma on the female reproductive tract and its reported benefits in other reproductive technologies indicate that further investigation is warranted. Future studies incorporating larger sample sizes and greater control of environmental and management variables may help clarify the potential role of seminal plasma as a strategy to improve the efficiency and commercial application of NSET in pigs.

## TRANSITION STATEMENT

Experiment I aimed to assess the effects of seminal plasma (SP) treatment on farrowing rates and litter sizes of embryo transfer recipient sows in a non-surgical embryo transfer (NSET) protocol. This experiment identified that there did not appear to be any significant effects from SP on these reproductive outcomes in SP-treated sows compared to the control group. Experiment II intended to determine the effects of two distinct genetic lines of SP on the global uterine transcriptome and assess the differential gene expression at three locations along the reproductive tracts of gilts. Specifically, Experiment II aimed to identify key gene pathways in the endometrium at various locations following SP treatment that could impact reproductive success of NSET recipients.

## CHAPTER FIVE: EXPERIMENT II

### Global uterine transcriptome response to seminal plasma in gilts

**Sarah E. Stephens<sup>1</sup>, Martijn F.L. Derks<sup>2</sup>, Louisa J. Zak<sup>3</sup>, Huaigang Lei<sup>4</sup>, C. Martin  
Nyachoti<sup>1</sup>**

<sup>1</sup> Department of Animal Science, University of Manitoba, Winnipeg, Canada;

<sup>2</sup> Animal Breeding and Genomics, Wageningen University and Research, Wageningen, The  
Netherlands;

<sup>3</sup> Topigs Norsvin Research Center B.V., 's-Hertogenbosch, The Netherlands;

<sup>4</sup> Topigs Norsvin Canada Inc, Oak Bluff, Canada.

**S.E. Stephens:** methodology, trial conduction, data collection and transcriptomic analysis, writing – original draft.

**M.F.L. Derks:** methodology, transcriptomic analysis.

**L.J. Zak:** conceptualization, methodology, writing – review and editing.

**H. Lei:** conceptualization, methodology, data collection, writing – review and editing.

**C.M. Nyachoti:** supervision, project administration, writing – review and editing.

## 5.1 INTRODUCTION

Embryo transfer (ET) is a reproductive biotechnology that promotes genetic improvement and reproductive efficiency in livestock through the collection of embryos from genetically superior donor females and their transfer into synchronized recipient animals (Ciornei, 2022). The technology is widely implemented commercially across several livestock species and has been successfully utilized in horses and the cattle industry for more than four decades (Martinez et al., 2013). Despite these advances, adoption of ET in swine production remains comparatively limited. Progress has been hindered by anatomical and technical constraints, including variable success of non-surgical embryo transfer (NSET), and challenges associated with repeated embryo collection from donor females (Martinez et al., 2025; Larsen, 2024; Ewasiuk et al., 2025).

Seminal fluid contains many constituents, like proteins, minerals, and lipids, in addition to spermatozoa (Schjenken and Robertson, 2020). Seminal plasma (SP) has been shown to play a significant regulatory role in reproduction in pigs, as well as other species like mice and humans. In sows, SP treatment shortens the lutenizing hormone (LH) peak-to-ovulation interval, advances ovulation, and supports luteal and progesterone development (Martinez et al., 2020a). SP also induces endometrial gene expression changes associated with maternal immunity, embryo development, cytokine signaling, cell cycle regulation, and hormone response, and alters gene expression in Day 6 blastocysts related to development and implantation, indicating potential to improve ET outcomes (Martinez et al., 2020a). Prostatomes in boar seminal fluid are extracellular vesicles that support sperm motility and influence the female tract response to male antigens by eliciting gene expression changes in the endometrial epithelial cells to produce transcripts related to chemokines and cytokines (Schjenken and Robertson, 2020). Additionally, SP-derived exosomes stimulate endometrial transcripts affecting conception, implantation, embryo and fetal

development, offspring survival, and long-term health (Schjenken and Robertson, 2020). SP treatment at estrus promotes a receptive reproductive environment, modulating endometrial gene expression and improving farrowing rates and litter size (Alvarez-Rodriguez et al., 2020; Bogacki et al., 2020). Evidence suggests that seminal plasma deposited within the lower reproductive tract can easily access the upper reproductive tract and induce changes in the endometrium (Bogacki et al. 2020).

This study evaluated whether exposure of gilts to seminal plasma at estrus can modulate the uterine environment at Day 5 after onset of behavioural estrus (Day 0). This coincides with the timing of the estrus cycle when sows would act as a recipient in embryo transfer protocols (Martinez et al., 2025) to potentially improve embryo transfer outcomes. We aimed to determine whether seminal plasma treatment alters global gene expression patterns along the uterine horn and elicits transcriptional responses distinct from those observed in a naïve uterus. To assess potential spatial differences in uterine responses, samples were collected from three locations along the uterine horn. Attention was given to potential differences in uterine responses elicited by SP derived from two distinct genetic lines to determine whether SP genetics influences endometrial transcriptional activity. Finally, we aimed to identify the key genes and biological pathways affected by each treatment to better understand the physiological mechanisms underlying these effects and to inform optimized strategies for improving embryo transfer efficiency in pigs.

## **5.2 MATERIALS AND METHODS**

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee, and pigs were cared for according to the guidelines of the Canadian Council on Animal Care (2009).

### **5.2.1 Seminal plasma preparation**

Raw ejaculates (with sperm motility and normality > 70%) from Large White boars of two different genetic lines (a sire line and a dam line) were transferred to sterile 50 mL centrifuge tubes (Celltreat, Ayer, MA, USA) under aseptic conditions and centrifuged at 25°C in 800 G for 10 minutes (protocol taken from Pedrosa et al., 2021). Seminal plasma was extracted and stored in sterile 50 mL centrifuge tubes (Celltreat, Ayer, MA, USA) at -20°C until the experimental target volume was reached. Seminal plasma was thawed at room temperature and pooled according to genetic line before being divided into 80 mL doses (QuickTip Flexitube; Minitube International, Tiefenbach, Germany). All prepared doses were then stored at -20°C until required for experimental use.

### **5.2.2 Animals and pre-trial care**

Thirty hybrid Landrace x Large White F1 gilts at 22 weeks of age were housed in groups at the Glenlea Research Station (University of Manitoba). The gilts received conventional feed and husbandry, including routine vaccination and ear tagging. Body condition scoring for each gilt was monitored throughout the experiment to ensure proper development and nutrition. Boar exposure

twice daily was started at 24 weeks of age, and standing estrus detection was performed for 30 days after the initial boar exposure. Animals were synchronized with oral progesterone altrenogest (5 mL, Regumate 0.22%; Intervet International B.V., Boxmeer, the Netherlands) at the onset of pubertal estrus. Following withdrawal, gilts entered the experiment at onset of behavioural estrus.

### 5.2.3 Experimental treatments

Animals were allocated to treatment groups based on pedigree (littermates equally distributed between treatments) and onset of behavioural estrus (staggered randomly). Gilts were assigned to one of the following three treatments (Appendix Table 9.1):

1. Control (**CON**) (n=10) - no seminal plasma administered at estrus.
2. **SP\_SELF** (n=10) - received one dose of seminal plasma (intracervical administration) at estrus and another dose 24 hours later (from dam line boars).
3. **SP\_SIRE** (n=10) - received one dose of seminal plasma (intracervical administration) at estrus and another dose 24 hours later (from sire line boars).

Standing response to back pressure test in the presence of a teaser boar was recorded to determine onset of behavioural estrus.

Five days after onset of estrus, gilts were sedated with intramuscular Stresnil (1 mL/20 kg; Elanco Pharmaceuticals, Indianapolis, IN, USA) and Nerfasin 100 (0.80 mL/20 kg; Dechra Pharmaceuticals, Northwich, England) and then euthanized by captive bolt pistol followed by exsanguination for ten minutes, and reproductive tracts were retrieved immediately. Corpora lutea

counts were compared among experimental groups prior to RNA sequencing (RNA-seq) analysis to assess baseline equivalence of fertility between treatment groups.

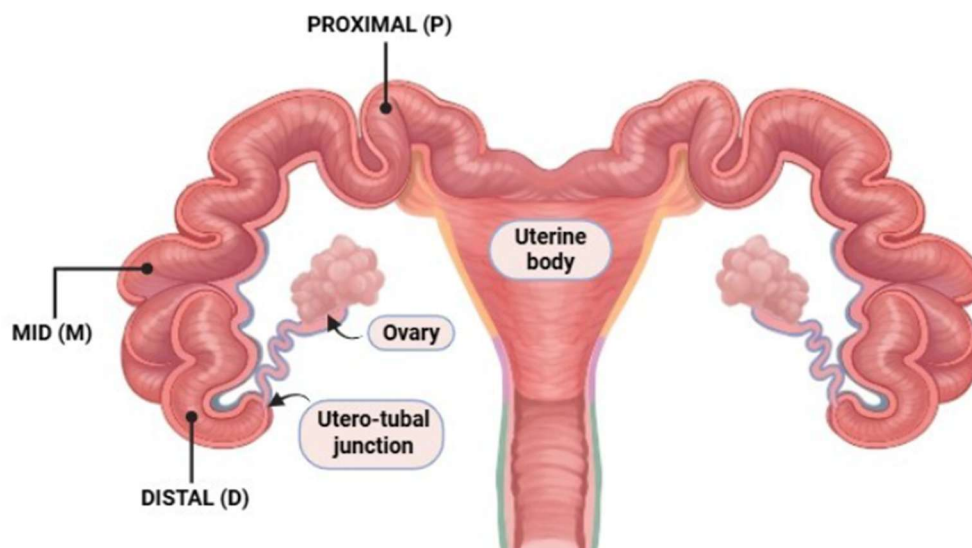
### ***Data analysis for baseline fertility***

Using R version 4.4.2 (The R Foundation for Statistical Computing; Vienna, Austria), a generalized linear model was fitted with Poisson distribution and a Chi-Square test was performed.

### **5.2.4 Sample collection**

To assess potential spatial differences in uterine response, samples were collected from three locations along the uterine horn: 10 cm from the utero-tubal junction, where Day 5 embryos are naturally located and NSET embryos are ideally deposited; the mid-horn region, a potential site of NSET embryo deposition; and 10 cm from the uterine septum, where localized effects of seminal plasma may occur (Bazer and Johnson, 2024; Bazer, 2013). Uterine endometrial explants (approximately 0.5 x 0.5 cm) were taken from the left uterine horn of each animal at three locations (Figure 5.1):

1. Proximal (P) – 10 cm away from septum of uterine body.
2. Mid (M) – mid-length of uterine horn.
3. Distal (D) – 10 cm away from utero-tubal junction.



**Figure 5.1.** Sampling locations of the uterine endometrium explants taken from each animal.

Figure created with BioRender.com.

Tissue collection was performed with disposable supplies or tools cleaned thoroughly with RNaseZap (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA), and explants were thinly sheared using a sterilized #10 scalpel blade (Meiyi; Huaiyin Medical Instruments Co., Ltd., Huayin Jiangsu P.R., China) before storage in sterile nuclease-free microcentrifuge tubes (MTC Bio, Sayreville, NJ, USA) filled with 1.5 mL of RNeasy lysis buffer (Qiagen, Crawley, UK). Tubes were promptly transferred to a 4°C refrigerator for overnight storage. 24 hours following refrigeration, samples were transferred to a -80°C freezer for long-term storage.

Samples were shipped to Genome Québec (Montréal, Canada) for RNA extraction and sequencing; RNA Integrity Number (RIN) values and sequencing libraries were provided for each sample.

### 5.2.5 RNA-seq processing pipeline

Raw RNA-seq reads were processed using the nf-core/rnaseq pipeline (v3.14.0) implemented in Nextflow (Patel et al., 2026), which provides a standardized and reproducible workflow for RNA-seq analysis. Paired-end FASTQ files were provided to the pipeline through a sample sheet specifying sample identifiers, read files, and library strandedness. Libraries were paired-end and the strandedness of the libraries was automatically inferred by the pipeline. Where necessary, FASTQ files originating from the same sample were merged prior to analysis.

#### *Quality control and read preprocessing*

Initial quality control of raw sequencing reads was performed using FastQC (Simons, 2010), and results were summarized across samples using MultiQC (Ewels et al., 2016). Adapter sequences and low-quality bases were removed using trimalore (Krueger, 2026). Potential contamination from non-target genomes was removed using BBSplit (Bushnell, 2014), and ribosomal RNA reads were filtered using SortMeRNA (Kopylova et al., 2012).

#### *Read alignment and expression quantification*

Reads were aligned to the *Sus scrofa* reference genome (Sscrofa11.1) using the splice-aware aligner STAR (Dobin et al., 2013). Gene annotation from Ensembl release 115 was used during alignment and quantification. Following alignment, transcript and gene expression levels were quantified using RSEM (Li and Dewey, 2011), which estimates expression while accounting for multi-mapping reads and transcript isoforms. Aligned reads were sorted and indexed using

SAMtools (Danecek et al., 2021), and duplicate reads were identified using Picard MarkDuplicates (Broad Institute, 2019).

### ***Transcript assembly and coverage tracks***

Transcript assembly and transcript abundance estimation were additionally performed using StringTie (Pertea et al., 2015). Genome-wide coverage tracks were generated from alignment files using BEDTools (Quinlan and Hall, 2010) and converted to bigWig format using bedGraphToBigWig (University of California Santa Cruz, 2006) for visualization in genome browsers.

### ***Quality assessment***

Several quality control metrics were computed to assess data quality and alignment performance. These included RNA-seq specific metrics calculated using RSeQC (Wang et al., 2012), alignment statistics generated by Qualimap (Okonechnikov et al., 2015), duplicate rate estimation using dupRadar (Sayols et al., 2016), and library complexity estimation using Preseq (Daley et al., 2022). Quality control metrics, gene biotype composition, strand-specificity, and sample similarity measures were summarized across samples using MultiQC (Ewels et al., 2016).

### ***Downstream analysis and differential expression analysis***

Gene-level expected counts generated by RSEM (Li and Dewey, 2011) were used for downstream analyses, including normalization and differential gene expression analysis using DESeq2 (Love et al., 2014). Differential expression analysis was performed in R using DESeq2 (Love et al., 2014) on gene-level expected counts obtained from RSEM (Li and Dewey, 2011). Analyses were conducted separately for the proximal, middle, and distal uterine horn samples. One outlier sample (8L) was excluded from all three tissue-specific analyses. Count values were rounded to integers prior to model fitting.

For each tissue, two DESeq2 (Love et al., 2014) models were fitted. The first model used a three-level treatment factor (**CON**, **SP\_SELF**, **SP\_SIRE**) to test the contrasts **SP\_SELF** vs **CON**, **SP\_SIRE** vs **CON**, and **SP\_SELF** vs **SP\_SIRE**. A second model used a binary factor in which **SP\_SELF** and **SP\_SIRE** were combined into a single SP group (**SP\_BOTH**), allowing the comparison of both SP groups vs **CON**. DESeq2 (Love et al., 2014) was used with default settings for normalization, dispersion estimation, and fitting of negative binomial generalized linear models. Statistical significance was assessed using Wald tests, and P-values were adjusted for multiple testing using the Benjamini–Hochberg method.

Variance-stabilized transformed expression values were used for exploratory analysis and visualization. Principal component analysis was performed separately for each tissue and treatment model. Volcano plots were generated using EnhancedVolcano (Blighe et al., 2018), and heatmaps were produced for the top 50 genes ranked by adjusted P-value using pheatmap (Kolde, 2025).

### 5.3 RESULTS

No significant differences in mean corpora lutea counts among groups were detected (Poisson regression, likelihood ratio  $\chi^2$  test,  $P = 0.2654$ ), indicating that ovulation rates were similar across **CON** ( $22.5 \pm 3.7$ ), **SP\_SELF** ( $19.3 \pm 3.2$ ), and **SP\_SIRE** ( $21.7 \pm 4.3$ ). Tissue samples had RIN values ranging from 7.5 to 9.8 following RNA extraction.

#### *Sequencing output and mapping statistics*

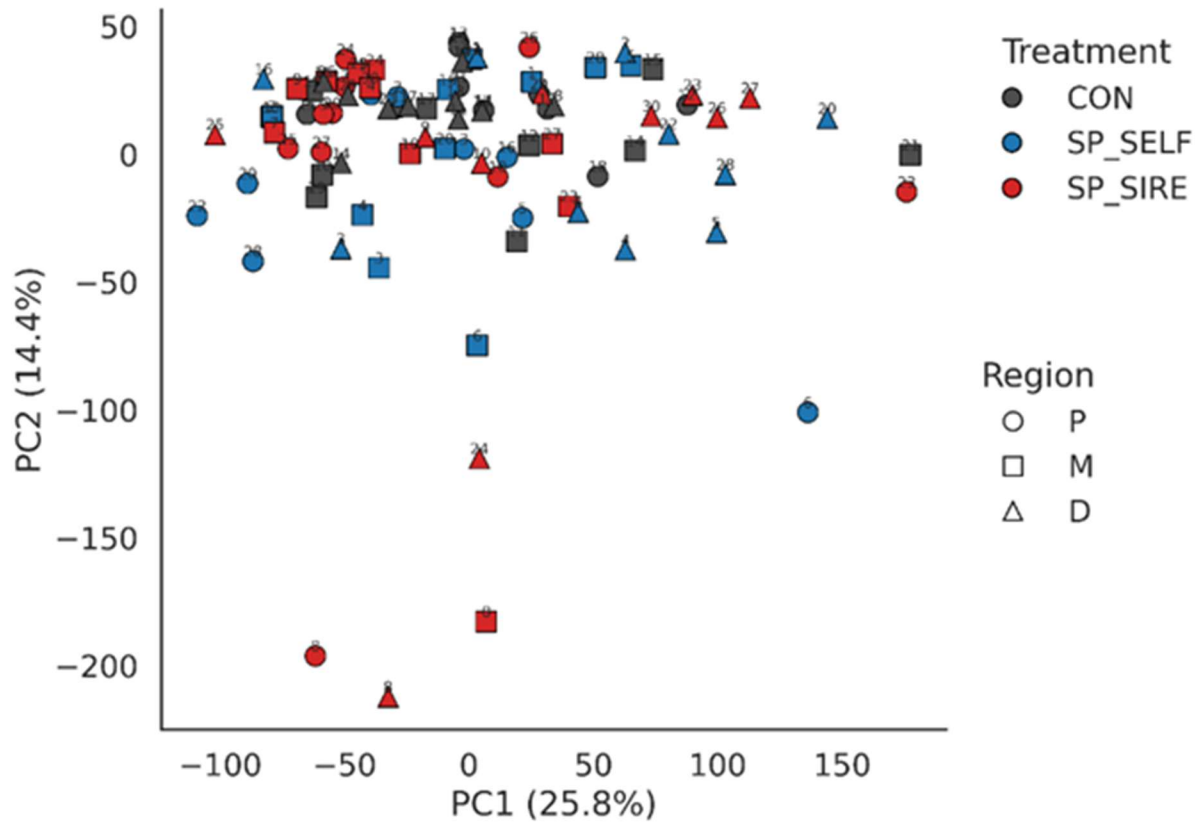
RNA sequencing produced an average of 47.7 million paired-end reads per sample. Following quality control and alignment, an average of 96.4% of reads successfully mapped to the *Sus scrofa* reference genome (Sscrofa11.1; range: 93.0–98.1%), with 93.6% of these reads mapping uniquely (range: 90.0–95.0%) (Appendix Table 9.2). Gene-level quantification performed using RSEM (Li and Dewey, 2011) indicated that 93.6% of reads were uniquely assigned to annotated genes (range: 91.7–96.8%), while 1.6% mapped to multiple genes (range: 1.2–3.8%) based on the Ensembl v115 annotation (Appendix Table 9.3). Across all samples, 13,836 genes exhibited detectable expression (median transcripts per million (TPM) > 1 across samples).

#### *Global transcriptome structure*

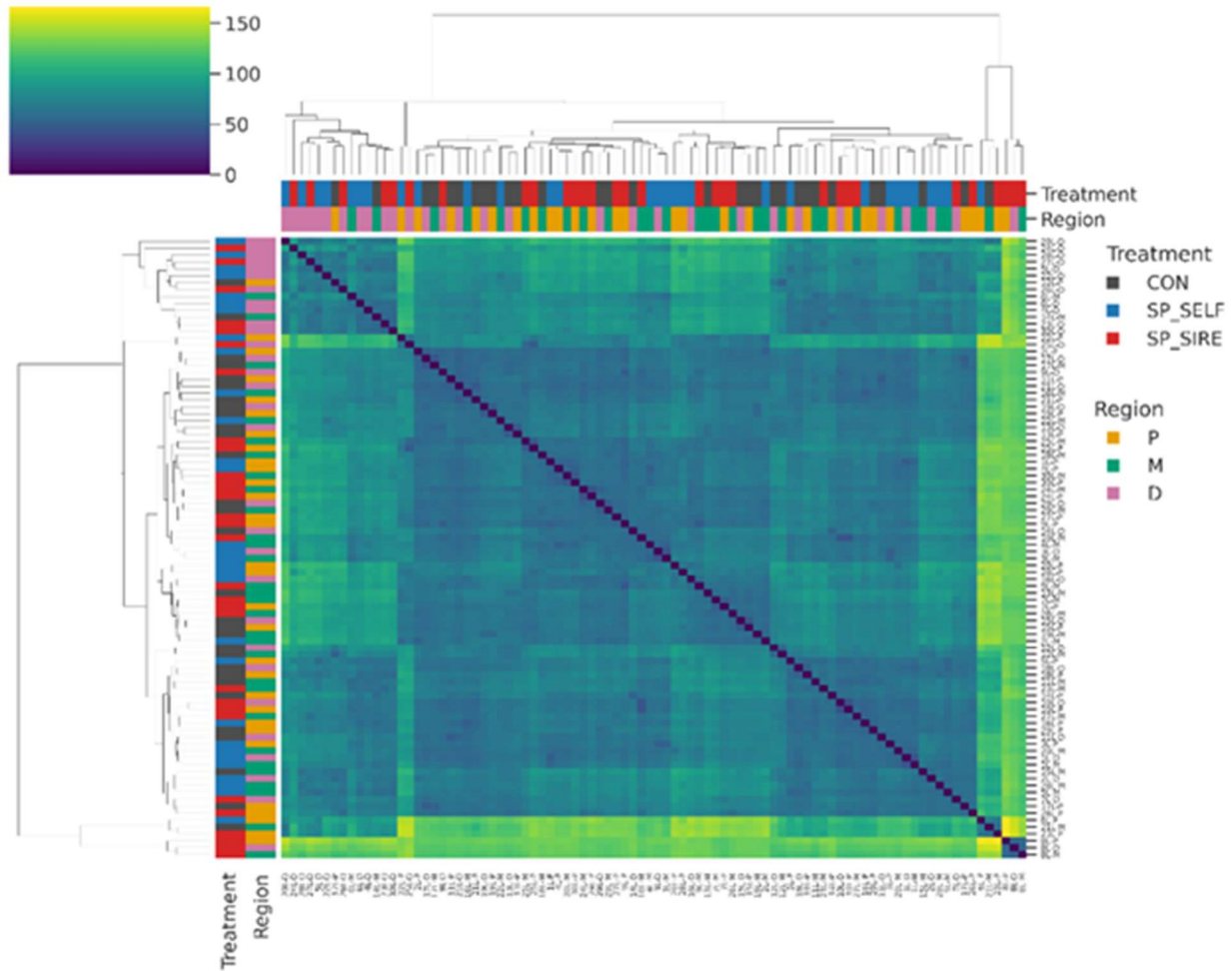
Principle component analysis (PCA) and hierarchical clustering of global gene expression profiles showed no clear separation of samples by uterine region (proximal, middle, distal) or treatment group (**CON**, **SP\_SELF**, **SP\_SIRE**) (Figure 5.2). Samples from different uterine regions

overlapped in transcriptomic space, indicating that neither anatomical location nor treatment was a major driver of global transcriptional variation.

In contrast, sample 8 (8L) separated distinctly along principal component 2 (Figure 5.2) and showed low similarity to all other samples in the distance heatmap (Figure 5.3). This sample contained 96 genes with high expression (median TPM > 20) that were minimally expressed in all other samples (median TPM < 2). Gene Ontology (GO) enrichment analysis identified significant overrepresentation of immune-related processes, including leukocyte chemotaxis, immune system process, and inflammatory response (Appendix Table 9.4), suggesting a localized inflammatory response consistent with a subclinical infection despite no clinical signs at sampling. Due to its strong transcriptomic divergence and immune-associated signature, sample 8 was excluded from downstream differential expression analyses to avoid confounding treatment effects.



**Figure 5.2.** Principal component analysis of RNA-seq samples based on overall gene expression profiles.



**Figure 5.3.** Hierarchical clustering of RNA-seq samples based on transcriptome-wide expression profiles.

### *Differential gene expression across uterine regions*

Differential expression analysis showed marked transcriptional differences between treatment groups primarily in the distal uterine horn, while minimal to no differential expression was observed in the proximal or middle regions (Table 5.1). In the distal region, many genes were differentially expressed (DE) between controls and seminal plasma-exposed animals, particularly in the combined comparison (CON vs SP\_BOTH), which identified 1,912 significant DE genes

(adjusted  $P < 0.05$ ). A similar pattern was observed for **CON** vs **SP\_SELF** (1,488 DE genes), whereas only 45 DE genes were detected for **CON** vs **SP\_SIRE**. In contrast, the proximal and middle regions showed almost no differential expression, indicating that transcriptional responses to seminal plasma are largely localized to the distal uterine horn. Direct comparisons between **SP\_SELF** and **SP\_SIRE** revealed no DE genes in the distal or middle regions and only one gene in the proximal region, indicating highly similar transcriptional responses between the two treatments.

**Table 5.1.** Number of differentially expressed (DE) genes per comparison and uterine region using  $P$ -value  $< 0.05$ . The number of DE genes accounting for multiple testing (adjusted  $P$ -value  $< 0.05$ ) is given between parentheses.

| Region              | Comparison         | Upregulated | Downregulated | Total       |
|---------------------|--------------------|-------------|---------------|-------------|
| <b>Proximal (P)</b> | CON vs SP_BOTH     | 823 (0)     | 627 (0)       | 1450 (0)    |
|                     | CON vs SP_SELF     | 1086 (0)    | 766 (0)       | 1852 (0)    |
|                     | CON vs SP_SIRE     | 379 (0)     | 464 (0)       | 843 (0)     |
|                     | SP_SELF vs SP_SIRE | 386 (0)     | 538 (1)       | 924 (1)     |
| <b>Mid (M)</b>      | CON vs SP_BOTH     | 466 (2)     | 827 (0)       | 1293 (2)    |
|                     | CON vs SP_SELF     | 369 (2)     | 471 (0)       | 840 (2)     |
|                     | CON vs SP_SIRE     | 537 (1)     | 1261 (3)      | 1798 (4)    |
|                     | SP_SELF vs SP_SIRE | 573 (0)     | 422 (0)       | 995 (0)     |
| <b>Distal (D)</b>   | CON vs SP_BOTH     | 2907 (1346) | 2286 (566)    | 5193 (1912) |
|                     | CON vs SP_SELF     | 2641 (1019) | 2061 (469)    | 4702 (1488) |
|                     | CON vs SP_SIRE     | 2034 (38)   | 1222 (7)      | 3256 (45)   |
|                     | SP_SELF vs SP_SIRE | 260 (0)     | 193 (0)       | 453 (0)     |

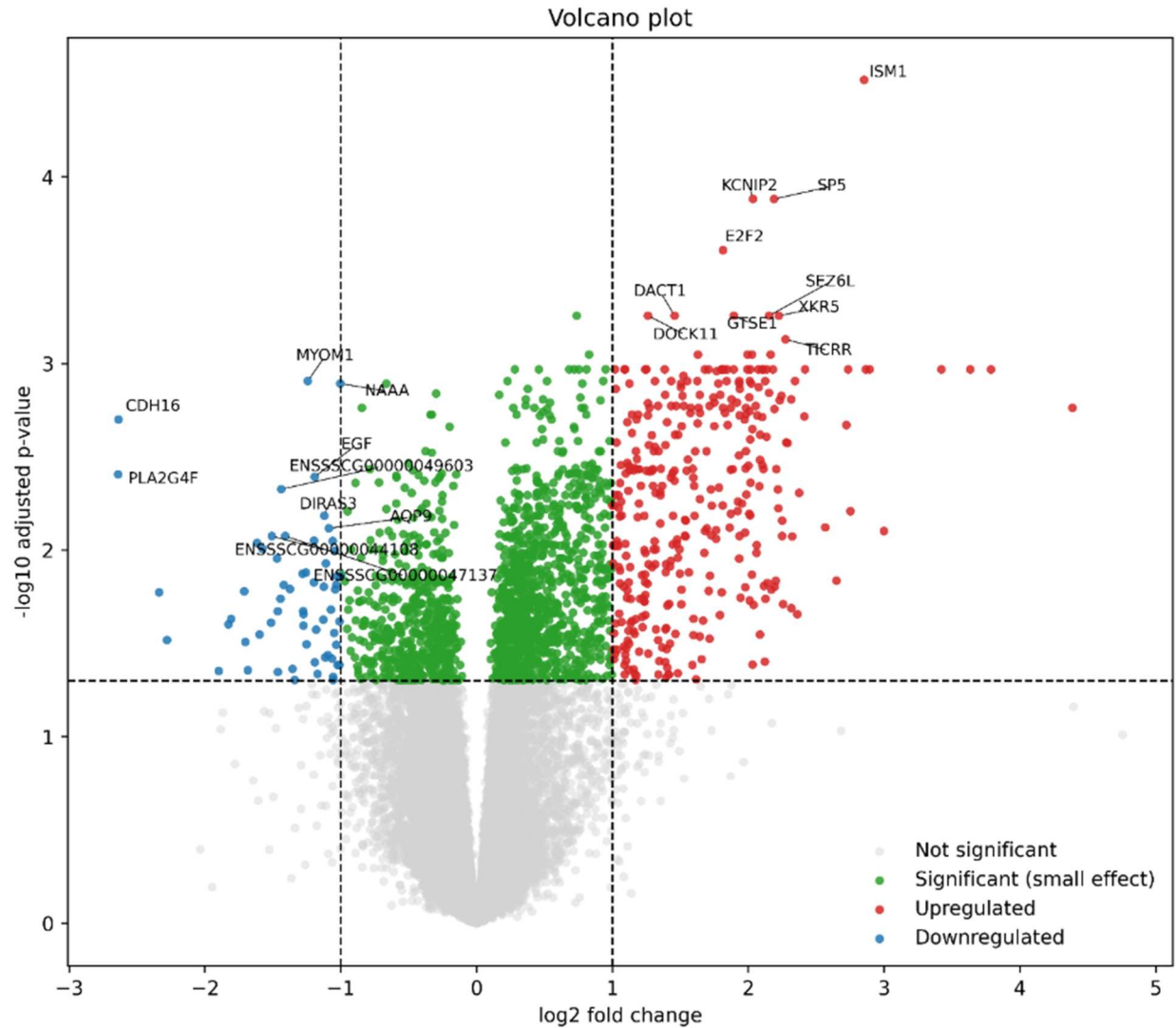
Overlap of DE genes in the distal uterine comparisons showed that the combined treatment response largely reflects that of the **SP\_SELF** group (Appendix Figure 9.1). A total of 1,211 genes

were shared between **CON vs SP\_BOTH** and **CON vs SP\_SELF**, indicating that most genes identified in the combined analysis were already present in the **SP\_SELF** comparison. In contrast, **CON vs SP\_BOTH** had 656 unique genes, while 239 genes were unique to **CON vs SP\_SELF**. Only 38 genes were shared across all three comparisons. No genes were uniquely differentially expressed in **CON vs SP\_SIRE**, and only seven genes overlapped between **CON vs SP\_BOTH** and **CON vs SP\_SIRE**. These results indicate that transcriptional differences in the distal uterine horn were primarily driven by **SP\_SELF**, with minimal contribution from **SP\_SIRE**. However, at the nominal (unadjusted) p-value level, there is broader overlap with **SP\_SIRE**, whereas after multiple testing correction (adjusted P-value), the number of DE genes in **CON vs SP\_SIRE** was reduced.

### ***Functional enrichment analysis of distal uterine response to seminal plasma***

Enrichment analysis of differentially expressed genes was performed using ShinyGO (Ge et al., 2020), with a background set of 11,957 genes expressed in uterine tissue (median TPM > 2). Functional enrichment of DE genes in the distal uterine horn indicated a strong, coordinated transcriptional response to seminal plasma exposure (Figure 5.4). Among the 1,370 genes upregulated in seminal plasma-treated gilts, both GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed significant enrichment for cell cycle regulation, DNA replication, and chromosome dynamics (Appendix Table 9.5). The most significantly enriched GO biological processes included chromosome organization, cell cycle process, chromosome segregation, and mitotic cell cycle, all with very strong significance (false discovery rate (FDR) <

$10^{-25}$ ). Consistently, KEGG pathways enriched include cell cycle, DNA replication, base excision repair, and homologous recombination.



**Figure 5.4.** Volcano plot showing differentially expressed genes up- or downregulated in gilts in the distal uterine horn treated with seminal plasma (SP\_BOTH) versus control gilts.

In contrast, the 566 genes downregulated in SP\_BOTH were primarily enriched for metabolic and transport-related processes (Appendix Table 9.6). GO enrichment analysis highlighted pathways related to sterol, steroid, and cholesterol metabolism, including sterol metabolic process, cholesterol biosynthetic process, and steroid biosynthetic process. In addition, processes associated with lipid metabolism and transport, as well as ion transport, were significantly enriched among downregulated genes. KEGG analysis further supported these findings, identifying enrichment for steroid biosynthesis and lysosome pathways. These results suggest a coordinated downregulation of lipid metabolic activity and transport functions in response to seminal plasma.

### ***Network analysis***

Network analysis of upregulated genes revealed a highly interconnected module dominated by key regulators of the cell cycle and DNA replication (Appendix Table 9.7). The top hub genes, including *CDK1*, *PLK1*, *CCNB1*, *CDC20*, and members of the MCM (minichromosome maintenance) complex, represent essential components of cell cycle progression, DNA synthesis, and mitotic division. In addition, transcriptional regulators such as *FOXMI* and *E2F8* were identified within this network, indicating activation of canonical proliferation pathways. Transcription factor enrichment analysis identified E2F and MYC family regulators as key drivers of the observed proliferative response.

Network analysis of downregulated genes revealed a highly interconnected module centered on cholesterol biosynthesis (Appendix Table 9.8). The top hub genes, including *HMGCR*, *HMGCS1*, *FDFT1*, *SQLE*, *LSS*, and *CYP51A1*, represent key enzymes of the mevalonate pathway

responsible for cholesterol production. In addition, regulatory genes such as *INSIG1* and *INSIG2*, which control sterol homeostasis, were also part of this network.

## 5.4 DISCUSSION

In this study, we investigated the effects of SP exposure at time of estrus on the uterine transcriptome of gilts at Day 5 after onset of estrus, which is the time when they would have received embryos if they were recipient gilts in an embryo transfer protocol, with a particular focus on spatial variation along the uterine horn and potential differences between boar genetic lines. Our results demonstrate that SP induces a strong and highly localized transcriptional response in the distal uterine horn, characterized by activation of proliferative pathways and suppression of lipid and steroid metabolism. In contrast, minimal transcriptional changes were observed in the proximal and middle regions, and only limited differences were detected between SP derived from different genetic lines. Together, these findings provide important insights into how SP modulates the uterine environment and suggest potential mechanisms through which it may influence embryo survival following embryo transfer.

A key finding of this study is the spatial specificity of the uterine response to SP. Despite uniform treatment of the reproductive tract, the transcriptional response was almost exclusively confined to the distal uterine horn. This is particularly interesting given that, in pigs, embryos enter the uterus around Day 5 and are distributed along the uterine horns thereafter; the distal region is therefore likely to represent one of the first sites of embryo–maternal interaction (Bazer and Johnson, 2024; Bazer, 2013). The pronounced transcriptional activation observed in this region suggests that SP primes the uterus in a location-specific manner, potentially enhancing its

receptivity at sites where early embryo signaling and implantation-related processes are initiated (Martinez et al., 2019b). The absence of a strong response in proximal and middle regions further indicates that SP-induced effects are not uniformly distributed but instead reflect localized signaling or differential tissue sensitivity.

At the functional level, SP exposure induced a striking upregulation of genes involved in cell cycle progression, DNA replication, and chromosomal organization. The identification of key regulators such as *CDK1*, *PLK1*, *CCNB1*, and *FOXM1*, together with enrichment of *E2F* and *MYC* transcription factor targets, indicates activation of canonical proliferative programs. This suggests that SP promotes cellular proliferation and tissue remodeling in the distal endometrium. Such processes are likely essential for preparing the uterine lining for embryo attachment and subsequent placental development (Bogacki et al., 2020). Increased epithelial and stromal cell proliferation may contribute to endometrial thickening, glandular development, and enhanced secretory capacity, all of which are critical for establishing a supportive environment for early embryos (Kelleher et al., 2019).

In parallel, SP treatment resulted in coordinated downregulation of genes involved in cholesterol, sterol, and steroid biosynthesis. The strong enrichment of the mevalonate pathway, including key enzymes such as *HMGCR*, *SQLE*, and *CYP51A1*, indicates a systemic suppression of lipid metabolic activity. Given that cholesterol is the precursor for steroid hormones, this finding suggests a reduced capacity for local steroidogenesis in the uterus following SP exposure (Miller and Auchus, 2011). This shift away from metabolic and endocrine activity toward a proliferative state may reflect a reallocation of cellular resources to support tissue remodeling and early pregnancy establishment. Alternatively, it may indicate a regulatory mechanism through which SP modulates the hormonal microenvironment to fine-tune embryo–maternal interactions.

Interestingly, previous studies have shown that SP can influence systemic progesterone dynamics and luteal function, suggesting that local and systemic endocrine effects may be coordinated (Martinez et al., 2019b).

Another important observation is the limited transcriptional difference between SP derived from different boar genetic lines. Despite the expectation that variation in seminal plasma composition between genetic lines could lead to differential uterine responses, our results indicate that both SP treatments induce highly similar transcriptional profiles. Since Hutchison et al. (1974) describes that mitochondrial DNA is maternally inherited, it was hypothesized, although not directly tested, that SP originating from the dam line may be immunologically more similar to the recipient than SP from a sire line, as the F1 gilt genotype does not include paternal genetic contributions from the sire line evaluated. This genetic relationship provided a biological basis for investigating whether SP from different genetic backgrounds elicits distinct uterine transcriptomic responses. The lack of significant differences suggests that the core bioactive components responsible for modulating the uterine environment are conserved across these lines, or that their effects converge on common downstream pathways. The slightly stronger signal observed for the **SP\_SELF** treatment compared to **SP\_SIRE** may reflect subtle quantitative differences in SP composition or compatibility with the recipient genotype, but these effects appear minor relative to the overall SP-induced response. To our knowledge, the effects of different SP genetic lines on uterine transcriptome have not been previously published.

The strong immune-related transcriptional signature observed in one outlier sample highlights the sensitivity of the uterine transcriptome to inflammatory processes. Although this sample was excluded from downstream analyses, its profile underscores the importance of controlling for subclinical infections or immune activation in reproductive studies, as these factors

can profoundly influence gene expression and potentially confound treatment effects. More broadly, it is well established that SP plays a role in modulating the maternal immune system, promoting tolerance to paternal antigens and facilitating successful pregnancy establishment (Shen et al., 2023). While our analysis primarily identified proliferative and metabolic pathways, it is likely that immune-related processes are also involved but may be more subtle or temporally restricted.

From an applied perspective, our findings have important implications for improving NSET outcomes in pigs. The high rate of embryonic loss associated with NSET remains a major limitation for its widespread use, and insufficient uterine receptivity is considered a key contributing factor (Martinez et al., 2020b; Geisert and Schmitt, 2002). The ability of SP to induce a proliferative and remodeling response in the distal uterus suggests that it may enhance the uterine environment at a critical time point for embryo survival. By promoting tissue readiness and potentially modulating local endocrine and immune conditions, SP treatment could improve implantation success and reduce early embryonic mortality (Bogacki et al., 2020). However, it is important to note that our study did not directly assess reproductive outcomes such as pregnancy rate or litter size. Future studies integrating transcriptomic data with phenotypic measures of fertility will be essential to establish the functional relevance of these molecular changes.

Several limitations of this study should be considered. First, the sample size per treatment group was relatively modest, which may limit the detection of subtle transcriptional differences, particularly between SP sources. Second, sampling was performed at a single time point (Day 5), providing a snapshot of the uterine response but not capturing its temporal flow. Third, while RNA-seq provides valuable insights into gene expression changes, it does not directly measure protein

abundance or functional activity. Complementary approaches such as single cell RNA sequencing, proteomics, or histological analyses would help to validate and extend our findings.

## **5.5 CONCLUSION**

In conclusion, this study demonstrates that seminal plasma exposure at estrus induces a strong, spatially localized transcriptional response in the distal uterus of gilts, characterized by activation of proliferative pathways and suppression of lipid and steroid metabolism. These findings support a role for SP in priming the uterine environment for early pregnancy and provide a molecular framework for understanding how SP treatment could improve embryo transfer success. Further research integrating molecular, physiological, and reproductive outcomes will be crucial to translate these insights into practical applications in pig breeding and reproductive biotechnology.

## CHAPTER SIX: GENERAL DISCUSSION

Embryo transfer (ET) has become an important reproductive biotechnology for accelerating genetic improvement and increasing reproductive efficiency in livestock production systems. While ET has been successfully implemented on a commercial scale in several species such as cattle, its widespread application in the swine sector remains limited by anatomical and technical challenges that contribute to inconsistent outcomes, particularly following non-surgical embryo transfer (NSET) (Martinez et al., 2013; Martinez et al., 2025; Larsen, 2024; Ewasiuk et al., 2025). Embryonic loss represents one of the greatest barriers to improving ET efficiency in pigs, with approximately 70% of transferred embryos failing to survive following transfer (Martinez et al., 2020b). Factors contributing to this loss include inadequate synchrony between embryonic development and the uterine environment, insufficient embryo–maternal signaling, genetic abnormalities, and potential immune-mediated responses against the allogenic embryo (Geisert and Schmitt, 2002; Bidarimath and Tayade, 2017; Jochems et al., 2023). Consequently, identifying strategies that enhance uterine receptivity and support embryo survival remains a critical area of research for advancing the commercial application of ET in swine.

Seminal plasma (SP) has emerged as a possible solution for modulating the uterine environment of embryo recipients due to its array of bioactive molecules capable of influencing immune regulation, angiogenesis, cellular proliferation, and the expression of implantation-related factors within the female reproductive tract (Toledo-Guardiola et al., 2024). Previous studies have demonstrated that SP treatment can improve reproductive outcomes under certain conditions, including increased conception, pregnancy, and farrowing rates in pigs (Flowers and Esbenshade, 1993; Okazaki et al., 2009). Building upon this foundation, the objectives of the research in this thesis were to determine whether SP treatment could improve reproductive outcomes following

NSET and to characterize the molecular mechanisms through which SP alters the uterine environment. Collectively, the results of these studies demonstrate that although SP induced substantial transcriptional changes within the uterus, particularly in pathways associated with cellular proliferation and tissue remodeling, these molecular responses were not accompanied by significant improvements in pregnancy rate or litter size under the conditions evaluated. These findings suggest that although SP induces meaningful molecular changes in the uterus, such changes do not necessarily translate into measurable phenotypic outcomes, particularly for complex traits such as fertility.

A major finding of the present research was that SP induced substantial changes in uterine gene expression, confirming that SP is biologically active and capable of modifying the uterine environment. Previous studies have demonstrated that SP influences uterine physiology through effects on immune regulation, cytokine signaling, angiogenesis, and endometrial remodeling (Martinez et al., 2019b; Toledo-Guardiola et al., 2024). The results of Experiment II build on these observations by providing molecular evidence that SP exposure causes a transcriptional response within the endometrium. Notably, genes associated with cell cycle progression, DNA replication, chromosomal organization, and cellular proliferation were significantly upregulated following SP treatment, such as the *E2F2* and *FOXM1*. Activation of these pathways suggests that SP promotes tissue remodeling and endometrial development, processes that are critical for establishing a uterine environment capable of supporting embryo survival and implantation (Bogacki et al., 2020; Kelleher et al., 2019). In contrast, genes involved in cholesterol metabolism and steroid biosynthesis were downregulated, indicating a shift in cellular activity away from metabolic functions and toward proliferative processes. Together, these findings support that SP acts as a biological signal that prepares the uterus for the establishment of pregnancy.

An equally important observation was the highly localized nature of the uterine response. Despite exposure of the entire reproductive tract to SP, transcriptional changes were observed almost exclusively within the distal uterine horn. This spatial specificity is particularly interesting because porcine embryos enter the uterus around Day 5 of development and subsequently distribute throughout the uterine horns, making the distal region one of the earliest sites of embryo–maternal interaction (Bazer, 2013; Bazer and Johnson, 2024). The activation of proliferative pathways within this region suggests that SP may prime specific areas of the uterus for subsequent conceptus signaling and implantation-related events (Martinez et al., 2019b). Furthermore, the limited response observed in the proximal and middle uterine regions indicates that SP-induced effects are not uniformly distributed throughout the reproductive tract but instead may reflect localized signaling mechanisms or differences in tissue sensitivity. These findings provide further evidence that SP alters uterine biology in a manner consistent with enhanced uterine receptivity and preparation for pregnancy, supporting previous reports that seminal plasma contributes to maternal adaptation prior to embryo implantation (Toledo-Guardiola et al., 2024; Bogacki et al., 2020). Interestingly, existing transcriptomic studies in pigs have largely focused on whole uterine tissues or later stages of pregnancy, including Days 12–15 and onwards of gestation, with limited attention given to regional differences in uterine gene expression (Huang et al., 2015; Kim et al., 2015; Zeng et al., 2019). Consequently, the highly localized response observed in the distal uterine horn suggests that the porcine uterus may exhibit greater spatial specialization than previously appreciated. The present findings identify an important knowledge gap regarding region-specific uterine responses during the early stages of pregnancy establishment.

Although our results suggest that there were substantial transcriptional changes induced by SP in Experiment II, these molecular responses did not appear as significant improvements in

pregnancy rate or litter size following NSET in Experiment I. This finding raises an important point regarding the relationship between uterine molecular changes and reproductive success which suggests that uterine molecular biology is one component for the establishment of a pregnancy. The disconnect between molecular responses and fertility outcomes is not entirely unexpected given the multifactorial nature of reproductive success following porcine ET. Previous studies have identified embryo quality, recipient synchrony, embryo–maternal communication, and immune tolerance as critical determinants of embryo survival (Geisert and Schmitt, 2002; Bidarimath and Tayade, 2017; Jochems et al., 2023). Additionally, transferred embryos are entirely allogenic to the recipient, unlike naturally conceived embryos, which are hemi-allogenic and develop in the presence of seminal components during mating (Martinez et al., 2020b). Therefore, although SP may partially replicate elements of natural reproductive signaling, it is unlikely to fully compensate for the absence of interactions that normally occur between the conceptus and maternal environment.

Another possibility is that the molecular responses induced by SP did not reach a biological threshold necessary to produce measurable improvements in fertility. The transcriptomic results obtained suggest that SP promotes cellular proliferation, tissue remodeling, and other processes associated with uterine preparation for pregnancy. However, improvements in uterine receptivity alone may be insufficient to overcome other limitations affecting embryo survival following transfer. Therefore, it is possible that SP enhances uterine preparedness without generating changes large enough to influence pregnancy establishment or litter size under the conditions evaluated. This interpretation is consistent with the variable nature of reproductive success and highlights the challenge of translating molecular evidence of biological activity into observable improvements in fertility.

To create successful commercial embryo transfer outcomes, embryo quality, embryo handling procedures, recipient synchronization, transfer technique, and environmental conditions all need to be optimized to establish a pregnancy and achieve acceptable numbers of piglets. Embryo characteristics such as developmental stage, abnormalities, and degeneration have been identified as important determinants of ET success, such that embryos at the morula and blastocyst stage result in optimal pregnancy outcomes. Additionally, environment in which embryo collection and transfer occur also contribute to success, including temperature fluctuations and environmental contaminants commonly encountered under commercial production settings (Martinez et al., 2025). Recipient synchronization is another critical factor, as current recommendations suggest that recipients should be in estrus approximately 24 hours after donors for optimal transfer of Day 5 and 6 embryos (Angel et al., 2014) and seasonal influences also may contribute to success of ET as seasonal infertility has been associated with delayed ovulation, altered endocrine function, and reduced reproductive performance in pigs (Bertoldo et al., 2012). These findings also reinforce comparisons made between bovine and porcine ET systems. Whereas cattle ET routinely achieves commercial success with the transfer of a single embryo, porcine ET remains constrained by the need for large embryo numbers and high embryonic loss (Martinez et al., 2025). Improvements in uterine receptivity alone are unlikely to overcome the biological limitations unique to pigs, highlighting the need for continued optimization across all components of ET protocols.

From a commercial perspective, SP is an attractive tool to improve NSET outcomes because it is a natural component of reproduction, relatively inexpensive, and readily available within existing artificial insemination systems. Its ability to induce molecular changes associated with uterine remodeling and receptivity suggests potential applications for recipient preparation

and NSET optimization. However, despite these biological effects, SP treatment did not improve pregnancy rate or litter size in the present study. Therefore, while SP shows promise as a modulator of the uterine environment, additional research is needed to determine whether these molecular responses can be translated into consistent improvements in reproductive performance before routine commercial use can be recommended. The findings of Experiments I and II contribute to the growing body of literature surrounding porcine ET and SP biology. This work demonstrates that SP induces substantial and region-specific transcriptional responses within the Day 5 porcine uterus despite failing to improve pregnancy rates or litter size under the conditions evaluated. Additionally, it provides insight into the spatial uterine responses of early gestation and highlights the complex relationship between molecular indicators of uterine receptivity and reproductive outcomes. These findings suggest that successful implementation of porcine ET will likely require the optimization of multiple biological and management factors rather than reliance on a single intervention.

Future research should focus on establishing clearer links between SP-induced molecular changes and reproductive outcomes by combining transcriptomic analyses with fertility measurements in the same animals. Additional studies examining multiple time points following SP exposure would help determine the duration and progression of uterine responses, along with analysis of blood samples to determine hormonal baselines for each animal throughout treatment. Furthermore, validation of transcriptomic findings through proteomic, histological, and immunohistochemical approaches would provide a more comprehensive understanding of the biological mechanisms involved. Research aimed at optimizing SP application, including dose, timing, and frequency may also improve its effectiveness; additional research on the effects of different genetic lines of SP on the transcriptome and on reproductive outcomes is also warranted.

to support the results obtained from this experiment. Finally, larger-scale field trials conducted under commercial ET conditions will be necessary to determine whether the molecular effects of SP can be translated into meaningful improvements in NSET efficiency and reproductive performance.

## CHAPTER SEVEN: CONCLUSION

Embryo transfer has considerable potential to accelerate genetic improvement and enhance reproductive efficiency in the swine industry, but its widespread adoption remains limited by challenges associated with embryo survival and the inconsistent success of non-surgical embryo transfer. Given the documented effects of seminal plasma on uterine physiology and immune regulation, this thesis investigated whether SP treatment could improve reproductive outcomes, like farrowing rates and litter sizes, following NSET and examined the underlying effects of SP on the uterine transcriptome.

The results demonstrated that SP exposure at estrus did not significantly improve pregnancy rate or litter size following NSET. However, SP induced substantial and highly localized transcriptomic changes within the distal uterine horn, including the upregulation of pathways involved in cell proliferation, DNA replication, and tissue remodeling. These findings confirm that SP is biologically active and influences the uterine environment in a manner consistent with enhanced preparation for pregnancy. Nevertheless, the molecular changes observed were not accompanied by measurable improvements in fertility under the conditions evaluated, highlighting the complexity of embryo survival and pregnancy establishment following ET.

Collectively, this research advances our understanding of SP-mediated uterine responses and provides new insight into the molecular mechanisms through which SP may influence reproductive function in pigs. While the results do not currently support the routine use of SP treatment to improve NSET outcomes, they identify promising biological pathways and topics for future investigation. Further research integrating molecular, physiological, and reproductive

measurements will be necessary to determine whether SP-based strategies can ultimately contribute to improved embryo transfer success in commercial swine production.

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## CHAPTER NINE: APPENDIX

**Table 9.1.** List of endometrium sample ID numbers and their respective treatments.

| Sample ID | Treatment |
|-----------|-----------|
| 1L        | SP_SELF   |
| 2L        | SP_SELF   |
| 3L        | SP_SELF   |
| 4L        | SP_SELF   |
| 5L        | SP_SELF   |
| 6L        | SP_SELF   |
| 7L        | SP_SIRE   |
| 8L        | SP_SIRE   |
| 9L        | SP_SIRE   |
| 10L       | SP_SIRE   |
| 11L       | CON       |
| 12L       | CON       |
| 13L       | CON       |
| 14L       | CON       |
| 15L       | CON       |
| 16L       | SP_SELF   |
| 17L       | CON       |
| 18L       | CON       |
| 19L       | CON       |
| 20L       | SP_SELF   |
| 21L       | CON       |
| 22L       | SP_SELF   |
| 23L       | SP_SIRE   |
| 24L       | SP_SIRE   |
| 25L       | SP_SIRE   |
| 26L       | SP_SIRE   |
| 27L       | SP_SIRE   |
| 28L       | SP_SELF   |
| 29L       | CON       |
| 30L       | SP_SIRE   |

**Table 9.2.** Mapping statistics for each gilt endometrium sample provided by MultiQC report (Ewals et al., 2016). Sample name listed as sample ID number-location in uterine horn (D = Distal; M = Mid; P = Proximal).

| Sample Name | Duplication | 5'-3' bias | M Aligned | Alignable | Proper Pairs | Error rate | Reads mapped | % Mapped | % Proper pairs | Total seqs | Aligned | Uniq aligned |
|-------------|-------------|------------|-----------|-----------|--------------|------------|--------------|----------|----------------|------------|---------|--------------|
| 1L-D        | 25.90%      | 1.12       | 25.2M     | 94.50%    | 66.10%       | 0.31%      | 50.3M        | 96.20%   | 96.20%         | 52.3M      | 96.20%  | 93.20%       |
| 1L-M        | 27.80%      | 1.14       | 26.1M     | 94.40%    | 64.40%       | 0.30%      | 52.2M        | 96.10%   | 96.10%         | 54.3M      | 96.10%  | 93.10%       |
| 1L-P        | 27.40%      | 1.12       | 26.1M     | 93.30%    | 64.10%       | 0.31%      | 52.2M        | 95.40%   | 95.40%         | 54.7M      | 95.40%  | 92.30%       |
| 2L-D        | 26.00%      | 1.15       | 23.1M     | 92.20%    | 65.20%       | 0.31%      | 46.3M        | 94.60%   | 94.60%         | 48.9M      | 94.60%  | 91.70%       |
| 2L-M        | 26.80%      | 1.1        | 24.4M     | 94.30%    | 65.10%       | 0.32%      | 48.8M        | 96.00%   | 96.00%         | 50.8M      | 96.00%  | 92.90%       |
| 2L-P        | 27.50%      | 1.14       | 23.9M     | 93.10%    | 64.00%       | 0.32%      | 47.9M        | 95.20%   | 95.20%         | 50.3M      | 95.20%  | 92.20%       |
| 3L-D        | 27.60%      | 1.1        | 24.0M     | 94.70%    | 64.30%       | 0.32%      | 48.1M        | 96.30%   | 96.30%         | 49.9M      | 96.30%  | 93.00%       |
| 3L-M        | 26.50%      | 1.07       | 21.1M     | 95.00%    | 65.30%       | 0.31%      | 42.2M        | 96.50%   | 96.50%         | 43.7M      | 96.50%  | 93.20%       |
| 3L-P        | 26.80%      | 1.13       | 24.1M     | 94.00%    | 64.90%       | 0.31%      | 48.1M        | 95.80%   | 95.80%         | 50.2M      | 95.80%  | 92.60%       |
| 4L-D        | 30.70%      | 1.09       | 25.2M     | 94.80%    | 61.70%       | 0.31%      | 50.4M        | 96.30%   | 96.30%         | 52.3M      | 96.30%  | 93.10%       |
| 4L-M        | 29.20%      | 1.09       | 25.7M     | 94.90%    | 63.40%       | 0.32%      | 51.4M        | 96.40%   | 96.40%         | 53.3M      | 96.40%  | 93.30%       |
| 4L-P        | 26.00%      | 1.12       | 21.8M     | 94.50%    | 66.10%       | 0.31%      | 43.5M        | 96.20%   | 96.20%         | 45.3M      | 96.20%  | 93.20%       |
| 5L-D        | 30.10%      | 1.06       | 27.4M     | 95.50%    | 62.70%       | 0.31%      | 54.9M        | 96.80%   | 96.80%         | 56.7M      | 96.80%  | 93.70%       |
| 5L-M        | 27.50%      | 1.12       | 25.4M     | 94.10%    | 64.90%       | 0.30%      | 50.8M        | 95.90%   | 95.90%         | 53.0M      | 95.90%  | 93.00%       |
| 5L-P        | 27.30%      | 1.1        | 22.7M     | 93.80%    | 64.50%       | 0.32%      | 45.5M        | 95.70%   | 95.70%         | 47.5M      | 95.70%  | 92.60%       |
| 6L-D        | 27.20%      | 1.13       | 20.0M     | 95.90%    | 65.50%       | 0.27%      | 40.0M        | 97.10%   | 97.10%         | 41.2M      | 97.10%  | 93.90%       |
| 6L-M        | 28.80%      | 1.06       | 24.9M     | 94.80%    | 62.80%       | 0.28%      | 49.7M        | 96.30%   | 96.30%         | 51.6M      | 96.40%  | 92.80%       |
| 6L-P        | 28.80%      | 1.02       | 24.1M     | 96.30%    | 64.20%       | 0.27%      | 48.2M        | 97.40%   | 97.40%         | 49.4M      | 97.40%  | 94.20%       |
| 7L-D        | 26.50%      | 1.11       | 25.2M     | 95.70%    | 66.40%       | 0.31%      | 50.5M        | 97.10%   | 97.10%         | 52.0M      | 97.10%  | 94.10%       |
| 7L-M        | 27.40%      | 1.14       | 23.8M     | 96.10%    | 65.40%       | 0.32%      | 47.6M        | 97.30%   | 97.30%         | 48.9M      | 97.30%  | 94.20%       |
| 7L-P        | 28.40%      | 1.14       | 21.4M     | 94.50%    | 63.90%       | 0.31%      | 42.8M        | 96.20%   | 96.20%         | 44.5M      | 96.20%  | 93.20%       |
| 8L-D        | 25.80%      | 1.09       | 18.9M     | 96.80%    | 65.60%       | 0.34%      | 37.7M        | 97.80%   | 97.80%         | 38.6M      | 97.80%  | 93.80%       |
| 8L-M        | 26.60%      | 1.06       | 23.3M     | 95.90%    | 64.90%       | 0.33%      | 46.6M        | 97.20%   | 97.20%         | 47.9M      | 97.20%  | 93.50%       |
| 8L-P        | 27.00%      | 1.11       | 23.8M     | 95.70%    | 63.80%       | 0.32%      | 47.6M        | 97.10%   | 97.00%         | 49.1M      | 97.10%  | 92.90%       |
| 9L-D        | 26.50%      | 1.12       | 23.0M     | 96.20%    | 66.30%       | 0.32%      | 46.1M        | 97.40%   | 97.30%         | 47.3M      | 97.40%  | 94.30%       |
| 9L-M        | 26.40%      | 1.14       | 18.2M     | 97.00%    | 66.50%       | 0.31%      | 36.5M        | 97.90%   | 97.90%         | 37.2M      | 98.00%  | 94.70%       |

|              |        |      |       |        |        |       |        |        |        |        |        |        |
|--------------|--------|------|-------|--------|--------|-------|--------|--------|--------|--------|--------|--------|
| <b>9L-P</b>  | 29.00% | 1.13 | 27.1M | 96.70% | 64.30% | 0.31% | 54.1M  | 97.70% | 97.70% | 55.4M  | 97.80% | 94.60% |
| <b>10L-D</b> | 28.30% | 1.13 | 25.8M | 95.40% | 64.20% | 0.31% | 51.6M  | 96.80% | 96.70% | 53.3M  | 96.80% | 93.60% |
| <b>10L-M</b> | 29.60% | 1.11 | 25.6M | 97.30% | 63.90% | 0.31% | 51.2M  | 98.10% | 98.00% | 52.2M  | 98.10% | 94.90% |
| <b>10L-P</b> | 28.70% | 1.15 | 24.9M | 96.50% | 64.10% | 0.31% | 49.9M  | 97.50% | 97.50% | 51.1M  | 97.60% | 94.30% |
| <b>11L-D</b> | 25.10% | 1.16 | 19.0M | 94.60% | 67.00% | 0.30% | 38.0M  | 96.20% | 96.20% | 39.4M  | 96.20% | 93.30% |
| <b>11L-M</b> | 27.90% | 1.05 | 23.4M | 95.60% | 64.30% | 0.31% | 46.8M  | 96.90% | 96.80% | 48.3M  | 96.90% | 93.50% |
| <b>11L-P</b> | 27.50% | 1.16 | 22.2M | 96.10% | 65.40% | 0.30% | 44.5M  | 97.30% | 97.30% | 45.7M  | 97.30% | 94.20% |
| <b>12L-D</b> | 26.10% | 1.15 | 18.2M | 95.90% | 66.20% | 0.33% | 36.3M  | 97.20% | 97.20% | 37.4M  | 97.20% | 94.00% |
| <b>12L-M</b> | 28.70% | 1.14 | 22.5M | 96.20% | 64.10% | 0.33% | 45.0M  | 97.40% | 97.40% | 46.2M  | 97.40% | 94.20% |
| <b>12L-P</b> | 27.30% | 1.14 | 21.8M | 96.30% | 65.60% | 0.32% | 43.6M  | 97.40% | 97.40% | 44.8M  | 97.50% | 94.40% |
| <b>13L-D</b> | 25.90% | 1.13 | 21.9M | 95.40% | 66.50% | 0.32% | 43.9M  | 96.80% | 96.80% | 45.3M  | 96.90% | 93.80% |
| <b>13L-M</b> | 27.70% | 1.15 | 18.7M | 95.90% | 64.80% | 0.32% | 37.4M  | 97.20% | 97.20% | 38.5M  | 97.20% | 94.00% |
| <b>13L-P</b> | 22.90% | 1.13 | 15.7M | 95.80% | 69.40% | 0.32% | 31.4M  | 97.10% | 97.10% | 32.4M  | 97.20% | 94.10% |
| <b>14L-D</b> | 30.70% | 1.14 | 26.8M | 95.90% | 61.10% | 0.30% | 53.5M  | 97.20% | 97.20% | 55.0M  | 97.20% | 93.20% |
| <b>14L-M</b> | 29.80% | 1.09 | 28.0M | 97.30% | 63.90% | 0.29% | 56.0M  | 98.10% | 98.10% | 57.0M  | 98.10% | 95.00% |
| <b>14L-P</b> | 25.20% | 1.15 | 19.3M | 95.40% | 67.30% | 0.30% | 38.6M  | 96.80% | 96.80% | 39.9M  | 96.80% | 93.70% |
| <b>15L-D</b> | 26.40% | 1.13 | 19.4M | 96.10% | 66.70% | 0.32% | 38.8M  | 97.30% | 97.30% | 39.8M  | 97.30% | 94.40% |
| <b>15L-M</b> | 26.40% | 1.17 | 24.4M | 96.30% | 66.70% | 0.31% | 48.9M  | 97.40% | 97.40% | 50.1M  | 97.50% | 94.50% |
| <b>15L-P</b> | 27.90% | 1.13 | 22.3M | 95.30% | 64.70% | 0.32% | 44.7M  | 96.70% | 96.70% | 46.2M  | 96.70% | 93.70% |
| <b>16L-D</b> | 28.10% | 1.14 | 22.3M | 95.90% | 65.00% | 0.31% | 44.7M  | 97.10% | 97.10% | 46.0M  | 97.20% | 94.20% |
| <b>16L-M</b> | 28.90% | 1.14 | 25.7M | 95.70% | 64.20% | 0.30% | 51.4M  | 97.10% | 97.00% | 53.0M  | 97.10% | 94.10% |
| <b>16L-P</b> | 28.60% | 1.13 | 23.9M | 97.00% | 65.00% | 0.31% | 47.8M  | 97.90% | 97.90% | 48.8M  | 97.90% | 94.90% |
| <b>17L-D</b> | 32.50% | 1.14 | 57.1M | 93.70% | 59.90% | 0.32% | 114.3M | 95.70% | 95.60% | 119.5M | 95.70% | 92.60% |
| <b>17L-M</b> | 27.30% | 1.14 | 22.8M | 95.60% | 65.30% | 0.32% | 45.7M  | 97.00% | 97.00% | 47.1M  | 97.00% | 93.90% |
| <b>17L-P</b> | 23.40% | 1.2  | 18.0M | 92.40% | 67.50% | 0.32% | 36.1M  | 94.80% | 94.80% | 38.0M  | 94.80% | 91.80% |
| <b>18L-D</b> | 26.30% | 1.17 | 19.6M | 96.00% | 66.70% | 0.31% | 39.1M  | 97.30% | 97.20% | 40.2M  | 97.30% | 94.30% |
| <b>18L-M</b> | 28.10% | 1.12 | 23.8M | 95.90% | 64.70% | 0.32% | 47.7M  | 97.20% | 97.20% | 49.1M  | 97.20% | 94.10% |
| <b>18L-P</b> | 26.20% | 1.12 | 23.5M | 96.30% | 66.70% | 0.31% | 47.0M  | 97.40% | 97.40% | 48.3M  | 97.50% | 94.40% |
| <b>19L-D</b> | 28.00% | 1.14 | 24.6M | 95.30% | 64.90% | 0.31% | 49.2M  | 96.80% | 96.70% | 50.9M  | 96.80% | 93.80% |
| <b>19L-M</b> | 26.90% | 1.13 | 22.6M | 96.20% | 66.10% | 0.31% | 45.2M  | 97.40% | 97.30% | 46.4M  | 97.40% | 94.30% |

|              |        |      |       |        |        |       |       |        |        |       |        |        |
|--------------|--------|------|-------|--------|--------|-------|-------|--------|--------|-------|--------|--------|
| <b>19L-P</b> | 26.00% | 1.15 | 19.5M | 94.90% | 65.90% | 0.31% | 39.1M | 96.60% | 96.50% | 40.5M | 96.60% | 93.30% |
| <b>20L-D</b> | 24.10% | 1.13 | 18.1M | 95.00% | 68.20% | 0.30% | 36.2M | 96.50% | 96.50% | 37.5M | 96.50% | 93.60% |
| <b>20L-M</b> | 28.50% | 1.13 | 24.0M | 96.70% | 64.90% | 0.32% | 48.1M | 97.70% | 97.70% | 49.2M | 97.70% | 94.70% |
| <b>20L-P</b> | 26.10% | 1.15 | 16.5M | 92.40% | 64.90% | 0.33% | 33.0M | 94.70% | 94.70% | 34.9M | 94.70% | 91.70% |
| <b>21L-D</b> | 27.30% | 1.14 | 24.2M | 95.10% | 64.90% | 0.31% | 48.5M | 96.60% | 96.60% | 50.2M | 96.60% | 93.40% |
| <b>21L-M</b> | 24.90% | 1.14 | 18.2M | 95.80% | 67.90% | 0.29% | 36.3M | 97.00% | 97.00% | 37.4M | 97.00% | 94.10% |
| <b>21L-P</b> | 28.50% | 1.13 | 22.9M | 96.20% | 64.50% | 0.31% | 45.8M | 97.30% | 97.30% | 47.0M | 97.30% | 94.30% |
| <b>22L-D</b> | 22.20% | 1.1  | 8.5M  | 96.80% | 70.50% | 0.32% | 17.0M | 97.90% | 97.90% | 17.4M | 97.90% | 94.80% |
| <b>22L-M</b> | 26.10% | 1.14 | 18.4M | 94.50% | 66.10% | 0.32% | 36.8M | 96.30% | 96.20% | 38.2M | 96.30% | 93.40% |
| <b>22L-P</b> | 27.80% | 1.09 | 21.3M | 96.90% | 65.40% | 0.34% | 42.6M | 97.80% | 97.80% | 43.6M | 97.90% | 94.70% |
| <b>23L-D</b> | 27.60% | 1.14 | 25.8M | 93.90% | 64.70% | 0.29% | 51.6M | 95.70% | 95.70% | 53.9M | 95.80% | 92.90% |
| <b>23L-M</b> | 25.90% | 1.13 | 18.3M | 95.50% | 66.50% | 0.29% | 36.6M | 96.80% | 96.80% | 37.9M | 96.80% | 93.70% |
| <b>23L-P</b> | 25.60% | 1.12 | 20.6M | 95.80% | 67.10% | 0.28% | 41.1M | 97.00% | 96.90% | 42.4M | 97.00% | 93.90% |
| <b>24L-D</b> | 30.70% | 0.95 | 25.7M | 96.90% | 61.00% | 0.36% | 51.3M | 97.90% | 97.90% | 52.4M | 97.90% | 93.60% |
| <b>24L-M</b> | 26.90% | 1.12 | 24.7M | 95.20% | 65.10% | 0.31% | 49.4M | 96.70% | 96.70% | 51.1M | 96.70% | 93.50% |
| <b>24L-P</b> | 25.80% | 1.16 | 21.7M | 93.90% | 65.50% | 0.31% | 43.5M | 95.80% | 95.80% | 45.4M | 95.80% | 92.60% |
| <b>25L-D</b> | 28.40% | 1.11 | 23.8M | 96.70% | 64.80% | 0.32% | 47.5M | 97.70% | 97.70% | 48.6M | 97.70% | 94.60% |
| <b>25L-M</b> | 25.50% | 1.12 | 21.2M | 96.80% | 67.60% | 0.31% | 42.4M | 97.80% | 97.80% | 43.4M | 97.80% | 94.80% |
| <b>25L-P</b> | 29.50% | 1.11 | 25.1M | 95.60% | 63.30% | 0.31% | 50.1M | 96.90% | 96.90% | 51.7M | 97.00% | 93.80% |
| <b>26L-D</b> | 25.50% | 1.07 | 21.5M | 94.60% | 66.30% | 0.30% | 42.9M | 96.20% | 96.20% | 44.6M | 96.20% | 93.10% |
| <b>26L-M</b> | 27.50% | 1.12 | 24.9M | 95.00% | 64.80% | 0.32% | 49.9M | 96.50% | 96.50% | 51.7M | 96.50% | 93.50% |
| <b>26L-P</b> | 25.90% | 1.09 | 22.1M | 95.00% | 66.30% | 0.31% | 44.1M | 96.60% | 96.50% | 45.7M | 96.60% | 93.60% |
| <b>27L-D</b> | 25.00% | 1.17 | 19.8M | 90.10% | 64.90% | 0.30% | 39.6M | 93.00% | 93.00% | 42.5M | 93.00% | 90.00% |
| <b>27L-M</b> | 27.80% | 1.16 | 25.8M | 96.40% | 65.60% | 0.30% | 51.5M | 97.50% | 97.50% | 52.8M | 97.50% | 94.60% |
| <b>27L-P</b> | 25.60% | 1.12 | 20.0M | 95.30% | 66.80% | 0.31% | 40.0M | 96.70% | 96.70% | 41.3M | 96.70% | 93.70% |
| <b>28L-D</b> | 27.80% | 1.11 | 25.5M | 92.90% | 63.80% | 0.30% | 51.0M | 95.10% | 95.10% | 53.6M | 95.10% | 92.10% |
| <b>28L-M</b> | 22.90% | 1.15 | 16.3M | 93.20% | 68.20% | 0.31% | 32.5M | 95.30% | 95.30% | 34.1M | 95.30% | 92.30% |
| <b>28L-P</b> | 27.60% | 1.1  | 19.6M | 90.60% | 62.50% | 0.33% | 39.1M | 93.40% | 93.30% | 41.9M | 93.40% | 90.20% |
| <b>29L-D</b> | 26.50% | 1.16 | 22.6M | 92.90% | 64.70% | 0.30% | 45.1M | 95.10% | 95.00% | 47.5M | 95.10% | 92.00% |
| <b>29L-M</b> | 25.40% | 1.13 | 17.0M | 92.90% | 65.40% | 0.31% | 34.1M | 95.00% | 95.00% | 35.8M | 95.00% | 91.80% |

|              |        |      |       |        |        |       |       |        |        |       |        |        |
|--------------|--------|------|-------|--------|--------|-------|-------|--------|--------|-------|--------|--------|
| <b>29L-P</b> | 26.70% | 1.15 | 24.5M | 94.20% | 65.00% | 0.30% | 49.0M | 95.90% | 95.90% | 51.1M | 96.00% | 92.80% |
| <b>30L-D</b> | 26.20% | 1.11 | 20.0M | 94.30% | 65.90% | 0.30% | 40.0M | 96.00% | 96.00% | 41.6M | 96.00% | 93.10% |
| <b>30L-M</b> | 26.40% | 1.11 | 22.9M | 93.30% | 64.70% | 0.31% | 45.9M | 95.40% | 95.30% | 48.1M | 95.40% | 92.10% |
| <b>30L-P</b> | 27.00% | 1.14 | 23.0M | 93.30% | 64.50% | 0.31% | 46.1M | 95.30% | 95.30% | 48.3M | 95.30% | 92.30% |

**Table 9.3.** Gene-level quantification using RSEM (Li and Dewey, 2011) based on Ensembl v115 for all samples.

| Sample | Aligned uniquely to a gene | Aligned to multiple genes | Unalignable reads | % Aligned uniquely to a gene | % Aligned to multiple genes | % Unalignable reads |
|--------|----------------------------|---------------------------|-------------------|------------------------------|-----------------------------|---------------------|
| 1L-D   | 16585734                   | 288432                    | 982373            | 92.9                         | 1.6                         | 5.5                 |
| 1L-M   | 17334576                   | 307967                    | 1048568           | 92.7                         | 1.6                         | 5.6                 |
| 1L-P   | 17336793                   | 297848                    | 1263950           | 91.7                         | 1.6                         | 6.7                 |
| 2L-D   | 15279567                   | 274386                    | 1310666           | 90.6                         | 1.6                         | 7.8                 |
| 2L-M   | 16386263                   | 281566                    | 1007726           | 92.7                         | 1.6                         | 5.7                 |
| 2L-P   | 15913654                   | 281457                    | 1202191           | 91.5                         | 1.6                         | 6.9                 |
| 3L-D   | 15986372                   | 291378                    | 918935            | 93.0                         | 1.7                         | 5.3                 |
| 3L-M   | 14233126                   | 274066                    | 762654            | 93.2                         | 1.8                         | 5.0                 |
| 3L-P   | 16250892                   | 289044                    | 1054769           | 92.4                         | 1.6                         | 6.0                 |
| 4L-D   | 17091988                   | 320231                    | 957335            | 93.0                         | 1.7                         | 5.2                 |
| 4L-M   | 17354062                   | 308411                    | 952733            | 93.2                         | 1.7                         | 5.1                 |
| 4L-P   | 14596153                   | 239984                    | 858437            | 93.0                         | 1.5                         | 5.5                 |
| 5L-D   | 18851875                   | 363897                    | 907483            | 93.7                         | 1.8                         | 4.5                 |
| 5L-M   | 17008949                   | 294590                    | 1085934           | 92.5                         | 1.6                         | 5.9                 |
| 5L-P   | 15266943                   | 281883                    | 1024983           | 92.1                         | 1.7                         | 6.2                 |
| 6L-D   | 13708722                   | 239466                    | 599417            | 94.2                         | 1.6                         | 4.1                 |
| 6L-M   | 16660083                   | 314853                    | 938804            | 93.0                         | 1.8                         | 5.2                 |
| 6L-P   | 16436316                   | 321284                    | 637201            | 94.5                         | 1.8                         | 3.7                 |
| 7L-D   | 16649627                   | 303194                    | 759443            | 94.0                         | 1.7                         | 4.3                 |
| 7L-M   | 16007157                   | 286599                    | 661678            | 94.4                         | 1.7                         | 3.9                 |
| 7L-P   | 14312697                   | 257099                    | 841242            | 92.9                         | 1.7                         | 5.5                 |
| 8L-D   | 12237093                   | 384561                    | 417435            | 93.8                         | 2.9                         | 3.2                 |
| 8L-M   | 15056921                   | 437342                    | 668748            | 93.2                         | 2.7                         | 4.1                 |

|       |          |        |         |      |     |     |
|-------|----------|--------|---------|------|-----|-----|
| 8L-P  | 15661728 | 481820 | 719576  | 92.9 | 2.9 | 4.3 |
| 9L-D  | 15412694 | 274082 | 622389  | 94.5 | 1.7 | 3.8 |
| 9L-M  | 12200256 | 200768 | 381384  | 95.4 | 1.6 | 3.0 |
| 9L-P  | 18101311 | 308599 | 620658  | 95.1 | 1.6 | 3.3 |
| 10L-D | 17722347 | 314447 | 860659  | 93.8 | 1.7 | 4.6 |
| 10L-M | 17546030 | 306617 | 503624  | 95.6 | 1.7 | 2.7 |
| 10L-P | 17043032 | 301549 | 623172  | 94.9 | 1.7 | 3.5 |
| 11L-D | 12620550 | 215566 | 738132  | 93.0 | 1.6 | 5.4 |
| 11L-M | 15953390 | 296495 | 756736  | 93.8 | 1.7 | 4.4 |
| 11L-P | 14936773 | 252849 | 612643  | 94.5 | 1.6 | 3.9 |
| 12L-D | 12171582 | 222780 | 524964  | 94.2 | 1.7 | 4.1 |
| 12L-M | 14892957 | 279360 | 605234  | 94.4 | 1.8 | 3.8 |
| 12L-P | 14594952 | 262223 | 570528  | 94.6 | 1.7 | 3.7 |
| 13L-D | 14467039 | 268137 | 711570  | 93.7 | 1.7 | 4.6 |
| 13L-M | 12465792 | 211859 | 543009  | 94.3 | 1.6 | 4.1 |
| 13L-P | 10251323 | 177730 | 460713  | 94.1 | 1.6 | 4.2 |
| 14L-D | 17532958 | 304890 | 758718  | 94.3 | 1.6 | 4.1 |
| 14L-M | 18593226 | 335747 | 534545  | 95.5 | 1.7 | 2.7 |
| 14L-P | 12941225 | 223358 | 632145  | 93.8 | 1.6 | 4.6 |
| 15L-D | 12958977 | 217303 | 528072  | 94.6 | 1.6 | 3.9 |
| 15L-M | 16186575 | 290797 | 637691  | 94.6 | 1.7 | 3.7 |
| 15L-P | 14882351 | 260801 | 753932  | 93.6 | 1.6 | 4.7 |
| 16L-D | 15001790 | 236651 | 653277  | 94.4 | 1.5 | 4.1 |
| 16L-M | 17194505 | 289927 | 777521  | 94.2 | 1.6 | 4.3 |
| 16L-P | 16112801 | 281137 | 508393  | 95.3 | 1.7 | 3.0 |
| 17L-D | 37770887 | 669966 | 2582607 | 92.1 | 1.6 | 6.3 |
| 17L-M | 15220262 | 266463 | 704875  | 94.0 | 1.6 | 4.4 |

|       |          |        |         |      |     |     |
|-------|----------|--------|---------|------|-----|-----|
| 17L-P | 11809452 | 210595 | 987759  | 90.8 | 1.6 | 7.6 |
| 18L-D | 12931149 | 226052 | 549960  | 94.3 | 1.6 | 4.0 |
| 18L-M | 15701109 | 276318 | 681133  | 94.3 | 1.7 | 4.1 |
| 18L-P | 15625586 | 292466 | 614467  | 94.5 | 1.8 | 3.7 |
| 19L-D | 16259169 | 291518 | 822545  | 93.6 | 1.7 | 4.7 |
| 19L-M | 14979117 | 275206 | 610606  | 94.4 | 1.7 | 3.8 |
| 19L-P | 12742718 | 234571 | 693498  | 93.2 | 1.7 | 5.1 |
| 20L-D | 12124863 | 214626 | 652746  | 93.3 | 1.7 | 5.0 |
| 20L-M | 16029836 | 277191 | 564600  | 95.0 | 1.6 | 3.3 |
| 20L-P | 11033059 | 184539 | 922164  | 90.9 | 1.5 | 7.6 |
| 21L-D | 16158028 | 275666 | 852202  | 93.5 | 1.6 | 4.9 |
| 21L-M | 12378414 | 220469 | 555416  | 94.1 | 1.7 | 4.2 |
| 21L-P | 15397000 | 255345 | 625464  | 94.6 | 1.6 | 3.8 |
| 22L-D | 5483760  | 101172 | 181743  | 95.1 | 1.8 | 3.2 |
| 22L-M | 12133114 | 203989 | 712905  | 93.0 | 1.6 | 5.5 |
| 22L-P | 14392365 | 252409 | 468991  | 95.2 | 1.7 | 3.1 |
| 23L-D | 17318537 | 298450 | 1142952 | 92.3 | 1.6 | 6.1 |
| 23L-M | 12613867 | 235711 | 602576  | 93.8 | 1.8 | 4.5 |
| 23L-P | 14315675 | 257596 | 642317  | 94.1 | 1.7 | 4.2 |
| 24L-D | 16913331 | 348626 | 553953  | 94.9 | 2.0 | 3.1 |
| 24L-M | 16407366 | 275541 | 835756  | 93.7 | 1.6 | 4.8 |
| 24L-P | 14273416 | 241913 | 946425  | 92.3 | 1.6 | 6.1 |
| 25L-D | 15918864 | 273171 | 559920  | 95.0 | 1.6 | 3.3 |
| 25L-M | 14242731 | 243987 | 480080  | 95.2 | 1.6 | 3.2 |
| 25L-P | 16955574 | 296410 | 787931  | 94.0 | 1.6 | 4.4 |
| 26L-D | 14422202 | 284478 | 837385  | 92.8 | 1.8 | 5.4 |
| 26L-M | 16564808 | 304222 | 892692  | 93.3 | 1.7 | 5.0 |

|       |          |        |         |      |     |     |
|-------|----------|--------|---------|------|-----|-----|
| 26L-P | 14700447 | 277967 | 781453  | 93.3 | 1.8 | 5.0 |
| 27L-D | 13349172 | 228627 | 1491820 | 88.6 | 1.5 | 9.9 |
| 27L-M | 17477773 | 309712 | 659345  | 94.7 | 1.7 | 3.6 |
| 27L-P | 13494254 | 240396 | 679499  | 93.6 | 1.7 | 4.7 |
| 28L-D | 16941256 | 331803 | 1317330 | 91.1 | 1.8 | 7.1 |
| 28L-M | 10733665 | 193435 | 795717  | 91.6 | 1.7 | 6.8 |
| 28L-P | 13153374 | 245318 | 1388563 | 89.0 | 1.7 | 9.4 |
| 29L-D | 15010782 | 265127 | 1169620 | 91.3 | 1.6 | 7.1 |
| 29L-M | 11378395 | 214894 | 888731  | 91.2 | 1.7 | 7.1 |
| 29L-P | 16380983 | 291148 | 1031895 | 92.5 | 1.6 | 5.8 |
| 30L-D | 13307750 | 234024 | 824640  | 92.6 | 1.6 | 5.7 |
| 30L-M | 15208164 | 253259 | 1111786 | 91.8 | 1.5 | 6.7 |
| 30L-P | 15510497 | 259163 | 1134685 | 91.8 | 1.5 | 6.7 |

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**Table 9.4.** GO enrichment results on 96 genes highly expressed in sample 8 (8L) (TPM >20) and low to no median expression in all other samples (TPM <2).

| Enrichment FDR | Genes (#) | Pathway Genes | Fold Enrichment | Pathway                                                                              |
|----------------|-----------|---------------|-----------------|--------------------------------------------------------------------------------------|
| 3.32E-09       | 11        | 166           | 17.38905622     | GO:0030595 Leukocyte chemotaxis                                                      |
| 3.72E-13       | 16        | 247           | 16.99865047     | GO:0060326 Cell chemotaxis                                                           |
| 1.44E-12       | 17        | 334           | 13.35653693     | GO:0006935 Chemotaxis                                                                |
| 1.45E-12       | 17        | 336           | 13.27703373     | GO:0042330 Taxis                                                                     |
| 3.64E-10       | 14        | 283           | 12.98174323     | GO:0050900 Leukocyte migration                                                       |
| 3.72E-13       | 21        | 552           | 9.983242754     | GO:0006954 Inflammatory response                                                     |
| 2.52E-10       | 20        | 719           | 7.299490032     | GO:0034097 Response to cytokine                                                      |
| 2.52E-10       | 20        | 720           | 7.289351852     | GO:1901652 Response to peptide                                                       |
| 3.20E-19       | 36        | 1304          | 7.244631902     | GO:0006952 Defense response                                                          |
| 1.50E-10       | 21        | 785           | 7.020063694     | GO:0140546 Defense response to symbiont                                              |
| 3.34E-17       | 34        | 1351          | 6.604120405     | GO:0006955 Immune response                                                           |
| 6.11E-13       | 27        | 1100          | 6.441136364     | GO:0051707 Response to other organism                                                |
| 6.11E-13       | 27        | 1103          | 6.423617407     | GO:0043207 Response to external biotic stimulus                                      |
| 1.23E-09       | 21        | 889           | 6.198818898     | GO:0098542 Defense response to other organism                                        |
| 2.87E-09       | 21        | 933           | 5.906484459     | GO:0040011 Locomotion                                                                |
| 4.43E-12       | 27        | 1221          | 5.802825553     | GO:0044419 Biological process involved in interspecies interaction between organisms |

|          |    |      |             |                                          |
|----------|----|------|-------------|------------------------------------------|
| 1.21E-17 | 38 | 1725 | 5.780772947 | GO:0009605 Response to external stimulus |
| 4.33E-18 | 41 | 2007 | 5.36077894  | GO:0002376 Immune system process         |
| 2.74E-12 | 40 | 2900 | 3.61954023  | GO:0006950 Response to stress            |

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**Table 9.5.** KEGG pathways enriched for genes upregulated in the SP\_BOTH group (n=1370).

| Enrichment<br>FDR | Genes (#) | Pathway<br>Genes | Fold Enrichment | Pathways                    |
|-------------------|-----------|------------------|-----------------|-----------------------------|
| 2.10E-13          | 52        | 153              | 3.3             | Cell cycle                  |
| 5.10E-10          | 20        | 35               | 5.7             | DNA<br>replication          |
| 1.20E-06          | 19        | 43               | 4.2             | Base excision repair        |
| 1.50E-05          | 33        | 123              | 2.6             | Spliceosome                 |
| 3.90E-05          | 18        | 49               | 3.6             | Fanconi anemia pathway      |
| 4.60E-04          | 10        | 21               | 4.8             | Mismatch<br>repair          |
| 9.50E-04          | 13        | 40               | 3.6             | Homologous recombination    |
| 3.60E-02          | 21        | 107              | 2.1             | Nucleocytoplasmic transport |

**Table 9.6.** KEGG pathways enriched for genes downregulated in the SP\_BOTH group (n=566).

| Enrichment<br>FDR | Genes (#) | Pathway Genes | Fold Enrichment | Pathways             |
|-------------------|-----------|---------------|-----------------|----------------------|
| 2.10E-02          | 16        | 130           | 3               | Lysosome             |
| 2.80E-02          | 5         | 20            | 8.3             | Steroid biosynthesis |

**Table 9.7.** Node degree in STRING network for top 30 genes upregulated in the SP\_BOTH group (n=1346).

| Node # | Identifier              | Node_degree |
|--------|-------------------------|-------------|
| CDK1   | 9823.ENSSSCP00000044214 | 167         |
| PLK1   | 9823.ENSSSCP00000023889 | 151         |
| EXO1   | 9823.ENSSSCP00000072686 | 148         |
| KIF11  | 9823.ENSSSCP00000011160 | 148         |
| CDCA8  | 9823.ENSSSCP00000037810 | 147         |
| CDC20  | 9823.ENSSSCP00000004268 | 144         |
| MAD2L1 | 9823.ENSSSCP00000031508 | 144         |
| BUB1B  | 9823.ENSSSCP00000022619 | 141         |
| RAD51  | 9823.ENSSSCP00000005138 | 138         |
| BUB1   | 9823.ENSSSCP00000072094 | 136         |
| TOP2A  | 9823.ENSSSCP00000018518 | 136         |
| CCNB1  | 9823.ENSSSCP00000023163 | 135         |
| CDC45  | 9823.ENSSSCP00000030798 | 135         |
| CCNB2  | 9823.ENSSSCP00000004945 | 133         |
| NUF2   | 9823.ENSSSCP00000006752 | 131         |
| ESPL1  | 9823.ENSSSCP00000000280 | 125         |
| BIRC5  | 9823.ENSSSCP00000042198 | 124         |
| KIF2C  | 9823.ENSSSCP00000004247 | 124         |
| CENPA  | 9823.ENSSSCP00000037748 | 123         |
| KIF23  | 9823.ENSSSCP00000005344 | 123         |
| TTK    | 9823.ENSSSCP00000068925 | 122         |
| MCM4   | 9823.ENSSSCP00000047671 | 120         |
| DLGAP5 | 9823.ENSSSCP00000005435 | 118         |
| KIF20A | 9823.ENSSSCP00000053967 | 118         |

|       |                         |     |
|-------|-------------------------|-----|
| UBE2C | 9823.ENSSSCP00000007910 | 118 |
| NDC80 | 9823.ENSSSCP00000067142 | 117 |
| ASPM  | 9823.ENSSSCP00000040351 | 116 |
| PBK   | 9823.ENSSSCP00000010328 | 116 |
| TYMS  | 9823.ENSSSCP00000042649 | 116 |

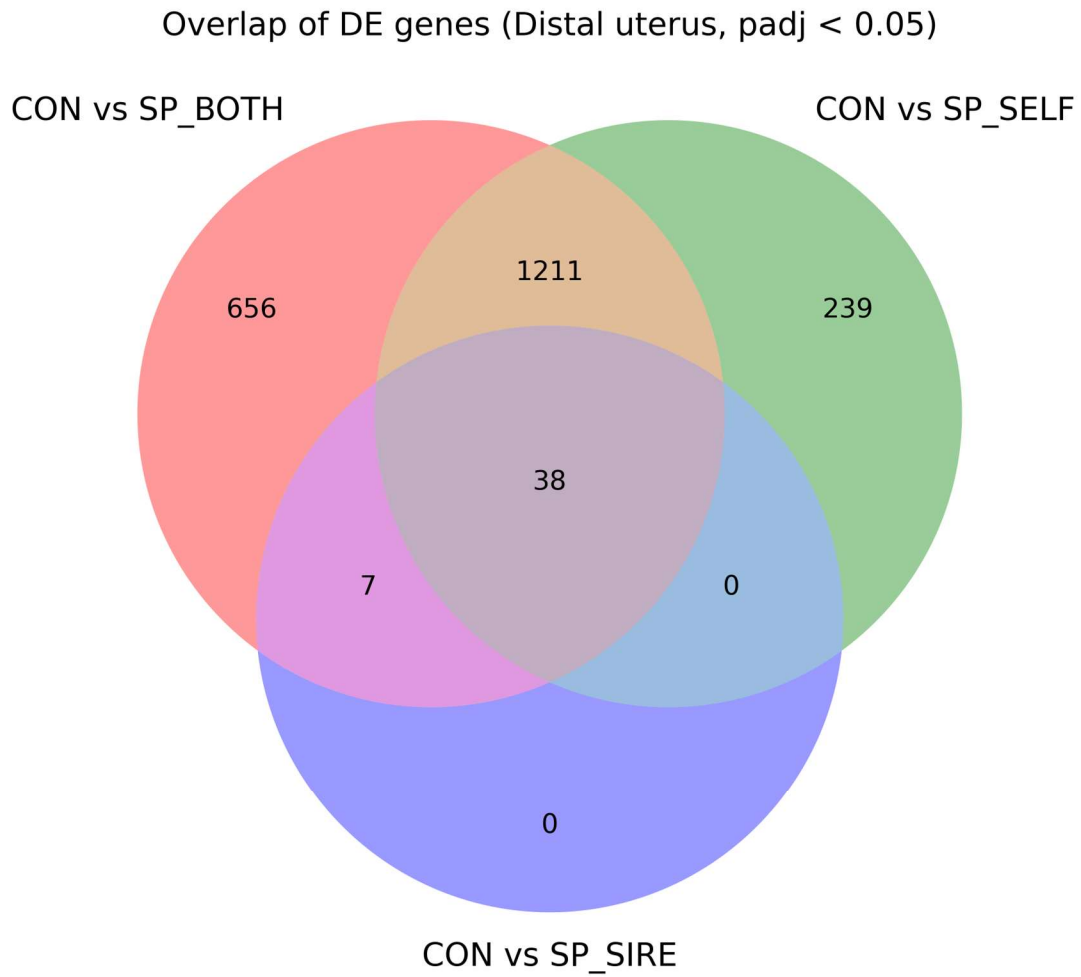
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**Table 9.8.** Node degree in STRING network for the top 30 genes downregulated in the SP\_BOTH group (n=566).

| Node #   | Identifier              | Node_degree |
|----------|-------------------------|-------------|
| HMGCR    | 9823.ENSSSCP00000053515 | 31          |
| NSDHL    | 9823.ENSSSCP00000046863 | 31          |
| FDFT1    | 9823.ENSSSCP00000033099 | 30          |
| CYP51A1  | 9823.ENSSSCP00000041656 | 28          |
| HMGCS1   | 9823.ENSSSCP00000017880 | 28          |
| DHCR7    | 9823.ENSSSCP00000024599 | 27          |
| SQLE     | 9823.ENSSSCP00000006378 | 26          |
| HSD17B7  | 9823.ENSSSCP00000006756 | 25          |
| SC5D     | 9823.ENSSSCP00000026883 | 25          |
| IDI1     | 9823.ENSSSCP00000025683 | 24          |
| FDPS     | 9823.ENSSSCP00000006940 | 23          |
| LSS      | 9823.ENSSSCP00000035138 | 23          |
| MSMO1    | 9823.ENSSSCP00000036001 | 23          |
| MVK      | 9823.ENSSSCP00000010598 | 23          |
| CH25H    | 9823.ENSSSCP00000058740 | 22          |
| MVD      | 9823.ENSSSCP00000037983 | 22          |
| SIGMAR1  | 9823.ENSSSCP00000054205 | 22          |
| TM7SF2   | 9823.ENSSSCP00000013829 | 22          |
| HMGCS2   | 9823.ENSSSCP00000034427 | 21          |
| PMVK     | 9823.ENSSSCP00000006964 | 21          |
| INSIG1   | 9823.ENSSSCP00000036426 | 20          |
| ACAT2    | 9823.ENSSSCP00000047700 | 19          |
| LAMTOR1  | 9823.ENSSSCP00000073191 | 17          |
| ATP6V1C2 | 9823.ENSSSCP00000009203 | 16          |

|        |                         |    |
|--------|-------------------------|----|
| EBP    | 9823.ENSSSCP00000055785 | 16 |
| FAXDC2 | 9823.ENSSSCP00000045749 | 16 |
| ERG28  | 9823.ENSSSCP00000073238 | 15 |
| PTK2   | 9823.ENSSSCP00000057598 | 15 |
| TSC1   | 9823.ENSSSCP00000072801 | 15 |

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**Figure 9.1.** Differentially expressed gene counts (using adjusted p-value to account for multiple testing; padj < 0.05) in the distal uterus by comparison.