VARIATION IN EMBRYONIC SPACING IN THE MOUSE:

ELUCIDATING THE GENETIC CONTRIBUTION

A Dissertation

by

SAMANTHA ANN FLETCHER

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Deborah Threadgill
Committee Members,	David Threadgill
	Penny Riggs
	Clare Gill
	James Womack
Interdisciplinary Program Chair,	David Threadgill

May 2019

Major Subject: Genetics

Copyright 2019 Samantha A. Fletcher

ABSTRACT

Even embryonic spacing and migration, the movement and positioning of embryos in the uterine horn prior to implantation, has been previously characterized as the even arrangement of embryos along a single uterine horn. This preimplantation process has been described as a conserved, tightly regulated phenomenon important for healthy pregnancies in many multiparous species including swine, sheep, goats, rabbits, mice, and rats. In addition, spacing and migration is thought to play a significant role in early pregnancy of non-polytocous species, such as humans and bovine. Abnormalities in spacing and migration can result in growth restriction, morbidity, mortality, and abortion/reabsorption in many polytocous species. In humans, uneven spacing and migration of embryos have been suggested to contribute to ectopic pregnancies, placental previa, sharing of the placenta, and twin-twin transfusion syndrome, which can result in the increased risk of growth restriction, malformations of the heart, perinatal mortality, and embryo morbidity. Using a mouse model, illumination of genetic components associated with uneven spacing provides insight into mechanisms and pathways that drive pre- and peri-implantation events.

To elucidate genetic mechanisms associated with embryonic spacing and migration, C57BL/6J, C3HeB/FeJ, and C3H/HeJ strains of mice were used (to define the model), in which, C57BL/6J represented a population of mice with evenly spaced embryos, whereas C3HeB/FeJ and C3H/HeJ represented populations with unevenly spaced embryos. Pedigree analysis suggested that both maternal and embryonic genetic contributions affect this process with an emphasis on a maternal effect and dominance gene action. Several other assays were conducted

in order to determine if any physiological mechanisms contribute to uneven embryo spacing, including sex of the embryo, parity (primigravida vs. multigravida pregnancies), and timing of implantation. None of the tested physiological mechanisms explained the uneven spacing observed in the two C3H strains. Additionally, quantitative trait loci analysis was performed on data collected from 12 recombinant inbred lines, generated from C57BL/6J and C3H/HeJ. These data led us to several regions of interest on chromosomes 13 and 9. Additionally, RNAseq, led to further elucidation of differentially expressed genes from E3.5 embryos. Furthermore, future research will refine the understanding of the genetic mechanism(s) in embryo spacing and migration are controlled.

DEDICATION

To my daughter, may this show you to never give up on your dreams and never give in to those that doubt you. To Mr. Ron Feltes, for always believing in me, guiding me, and fostering my love of science and genetics, without you, I would not be where I am now. In memoriam of my grandfather, thank you for always encouraging me and for being an amazing role model. I hope you are proud.

ACKNOWLEDGEMENTS

I would like to thank to my parents for always being there to guide, support, & encourage and for always giving me the tough love I sometimes needed, without you I would not be the woman I am today. To my husband, Ben, thank you for taking this crazy journey with me, without you, I would not have made it this far. Thank you for listening and having mountains of patience with me and my schedule, for that and many, many other reasons, I love you. Lily, you are the greatest gift that this time has given me, and I thank you for keeping a smile on my face and drive in my heart.

Thank you to my committee chair, Dr. Deborah Threadgill, for giving me this opportunity to learn and grow in her lab. Thank you to my committee members, Dr. David Threadgill, Dr. Penny Riggs, Dr. James Womack, and Dr. Clare Gill, for their guidance, support, and patience throughout the course of this research.

Thank you to Dr. Candace Hastings and Dr. Carolyn Sandoval for their guidance and encouragement of my teaching career choice. Thank you for helping to foster my love of teaching and develop my skills, I am forever grateful. To all of my past genetics' students, thank you for confirming and fostering my love of teaching and learning. I hope to have impacted your life, if only a little.

Thank you to all of my friends and colleagues in the genetics program and the GGSA. All of you have made my time here at Texas A&M University memorable and enjoyable. I hope I have made as much of an impression on you as everyone has on me.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a dissertation committee consisting of Dr. Deborah Threadgill of the Department of Veterinary Pathobiology, Dr. David Threadgill of the Department of Molecular and Cellular Medicine, Dr. Penny Riggs of the Department of Animal Science, Dr. James Womack of the Department of Veterinary Pathobiology (Retired), and Dr. Clare Gill of the Department of Animal Science.

The line cross analysis and resulting graphs were provided by Dr. Heath Blackmon of the Biology Department at Texas A&M University. Assistance in preparing the RNAseq data for analysis (trimming and demultiplexing) was provided by Kranti Konganti, Associate Director at Texas A&M Institute for Genome Sciences and Society. All other work for the dissertation was completed independently by the student.

Funding Sources

Graduate study was supported by start-up funds to Dr. Deborah Threadgill from Texas A&M University, as well as startup funds to Dr. David Threadgill from Texas A&M University. Support was also obtained through a teaching assistantship provided by the Department of Biochemistry and Biophysics through the Interdisciplinary Program of Genetics.

TABLE OF CONTENTS

Page

ABSTRACT……………………………………………………………...……………ii

DEDICATION……………………………………………………………...……………iv

ACKNOWLEDGEMENTS…………………………………………………..……………v

CONTRIBUTORS AND FUNDING SOURCES…………………………...…………...vi

TABLE OF CONTENTS………………………………………………………...….... vii

LIST OF FIGURES…………………………………………………………………...… ix

LIST OF TABLES …………………………………………………………………....x

CHAPTER I INTRODUCTION AND LITERATURE REVIEW ……………………… 1

Defining and Characterizing Embryo Spacing…………………………………...1
Conservation of Embryo Spacing……………..………………………………2
Explaining the Events of Embryo Spacing and Migration……………….……4
Tracking Embryo Migration and Spacing through Early Pregnancy…………….. 5
Migration Event………………………………...……………………..6
The Spacing Event ……………………………...………………..……7
Mechanics Related to Pre- and Peri-implantation…………………………….…..8
Timing ……..………………………………...………………..……8
Molecular Mechanisms of Myometrial Contractions…...……………….……9
Molecular Mechanisms of Embryo-Uterine Interaction…...……………..……10
Uneven Embryo Spacing and its Repercussions…………………….……………12
Human and Animal Impacts……………………..…...…………………..…..13
Inheritance Patterns……………………………………………………….……16
Mouse Models of Differential Embryonic Spacing ……………………………...16
C57BL/6J (B6) ……………………………...…...…………………..……16
C3H/HeJ and C3HeB/FeJ (C3H) …………………..…………………..……17
Recombinant Inbreds and the BXH Mice Series ………………..……..……18
Implications of this Research……………………………………………………. 20
In Vitro Fertilization …………...…………………..………………..……20
IVF & Spacing ………………...………………..…………………..……22
Rationale and Summary of Research……………………………………………. 22
Objectives ……………………...………………..…………………..……23

CHAPTER II GENETIC CONTRIBUTION & PHENOTYPIC VARIATION……….. 24

Introduction…………………………………………………………………24

Background……………………………………………………………………24
Materials and Methods………………………………………………………25
Mice Strains ……………………………………………………………25
Harvesting ………………………………………………………………27
Phenotype Characterization ……………………………………………28
Uterine Horn Measurement and Analysis ………………………………28
Blue Dye Injection ………………………………………………………30
Blastocyst Collection and Storage ………………………………………30
Embryo Collection and Storage …………………………………………32
DNA Extraction …………………………………………………………32
PCR and Gel Electrophoresis ……………………………………………33
Pedigree Generation and Analysis ………………………………………34
Recombinant Inbred Lines ………………………………………………35
QTL Mapping and Analysis ……………………………………………35
RNAseq …………………………………………………………………36
Results…………………………………………………………………………37
Parental Strain Embryo Spacing Confirmation …………………………37
Parity ……………………………………………………………………37
Litter Size and Horn Crowding …………………………………………38
Sex Bias …………………………………………………………………39
Implantation ……………………………………………………………41
Inheritance ………………………………………………………………43
Recombinant Inbred Lines ………………………………………………44
QTL Identification ………………………………………………………45
RNAseq …………………………………………………………………49

CHAPTER III DISCUSSION AND FUTURE DIRECTIONS………………………… 50

Discussion……………………………………………………………………. 50
Physiological Mechanisms ………………………………………………50
Contributing Genetic Effects ……………………………………………52
Candidate Regions/Genes of Interest ……………………………………54
Future Directions………………………………………………………… 55
Additional RNAseq ………………………………………………………55
Strain Testing ……………………………………………………………56
Testing Regions/Genes of Interest ………………………………………57
Physiological Mechanisms - Contractions ………………………………58
Implications ………………………………………………………………58

REFERENCES………………………………………………………………… 61

APPENDIX A FIGURES…………………………………………………… 66

APPENDIX B TABLES…………………………………………………… 82

LIST OF FIGURES

Page

Figure 1. Myometrial contraction pathway adapted (Ruan, 2011) ……………………...66

Figure 2. Visualization and measuring blue dye injections…………………………….. 67

Figure 3. Parental phenotype characterization …………………………………………68

Figure 4. JMP analysis of parity …………………………………………………….. 69

Figure 5. Uterine horn crowding analysis ……………………………………………70

Figure 6. General sex analysis ………………………………………………………. 71

Figure 7. Sex determination of embryos ……………………………………………..72

Figure 8. Mean comparisons of sex interactions ……………………………………..73

Figure 9. Blue dye injections …………………………………………………………74

Figure 10. Implantation timing ………………………………………………………75

Figure 11. Litter size distribution …………………………………………………… 76

Figure 12. Pedigree generated for C3H and B6 matings ……………………………… 77

Figure 13. Line-cross analysis utilizing pedigree data …………………………………78

Figure 14. CV comparison of recombinant inbred lines ……………………………… 79

Figure 15. QTL mapping of recombinant inbred lines ………………………………… 80

Figure 16. Top differentially expressed genes from RNAseq………………………….. 81

LIST OF TABLES

Page

Table 1. Parity testing…………………………………………………………………82

Table 2. C3H and B6 pedigree mating and phenotype results…………………………..83

Table 3. Phenotype results of the BXH series………………………………………….. 84

Table 4. Differentially expressed genes for embryo spacing……………………………89

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Defining and Characterizing Embryo Spacing

The process of embryo implantation in the uterus of mammalian species and the formation the decidual reaction have been extensively studied and the underlying molecular mechanisms have been defined [Yoshinaga, 2013]. However, the molecular mechanisms leading to equal spacing of embryos within the uterus of litter-bearing species remain largely unknown. Migration and spacing play a crucial role in the success of implantation and the embryo's ability to thrive. In conjunction with implantation, embryo migration and spacing are known to be important for the success and health of placental pregnancies of both litter-bearing and single offspring species. The disruption of these events, both natural and genetically, has been linked to early pregnancy failures [Carson et al. 2000, Hama 2007].

Embryo spacing was first characterized in the early half of the 20th century predominantly using rabbit, rat, and mouse models. This event, which is now characterized into two closely related steps (embryo spacing and embryo migration), is described as the movement of embryos within the uterine horn or uterine body in order to space them equidistantly prior to implantation. This event is highly regulated, but the genes and physiological mechanisms have not been wholly expounded for embryo spacing. However, Böving and his colleagues provided some enlightening data and compelling hypotheses that offer a foundation for understanding of embryo spacing and migration [Böving 1956, McLaren 1959, O'Grady 1969].

In addition to the traditional small animal models, the pig model contributed important information on embryo movement and implantation selection in the late 1970s. This research

gave insight into the conservation of regular spacing and its implications in embryo survival and health across species. The combined information from rabbit, rat, mice, and pig led to the discussion and possible extension of conserved regular embryonic migration and spacing to other species including equine, bovine, and even humans. Little is yet understood about the regulation and interactions influencing embryonic spacing, and this lack of understanding surrounding spacing is likely due to its propinquity to implantation and the interwoven and intricate mechanisms involved.

Conservation of Embryo Spacing

Through research using multiple animal models, the conservation of embryo migration and spacing across species has been confirmed. Böving, Michie & McLaren, and O'Grady and Heald characterized regular embryo spacing (through analysis of measurements) in a variety of species, including rabbit, rat, mouse, and pig [Böving 1956, O'Grady 1969, Anderson 1978, McLaren et al. 1959]. However, most of the early investigations on embryo spacing utilized the rabbit and mouse models. During this foundational research period on embryo spacing, mouse embryos were initially thought to randomly space within the uterine horns, and little regulation was thought to occur [Böving 1956]. However, per McLaren and Michie, it is now known that mouse embryos do not simply space randomly, but like rat and rabbit embryos, have regulated and deliberate embryo spacing, likely to prevent embryo crowding [McLaren et al. 1959].

The similarity among species in embryo spacing was primarily evaluated from the perspective of embryo migration and the uterine contractions associated with this event. These initial studies conflated migration and spacing, as these two events were assumed to be a single event. Although these steps occur in quick succession, the final spacing of embryos is more regulated and complex than can be attributed to myometrial contractions alone.

Böving, using a rabbit model, showed that the even spacing of embryos occurs regardless of the number of embryos present within a uterine horn [Böving 1956]. Similar findings from a rat model, confirmed the conservation of this phenomenon across multiple litter bearing species [O'Grady 1969]. Using a superovulation and single ovariectomy, McLaren and Michie reported that even when a single horn contains many embryos, the embryos tend to space as much as possible from one another. When the number of embryos exceeds a threshold, "embryo crowding" prevents sufficient spacing between embryos, and placentas of embryos in close proximity can actually fuse. This fusion typically leads to lower embryonic weights and, in turn, growth restriction [McLaren et al. 1959]. These discoveries imply the importance of spacing and migration to maintaining healthy embryos. Based on these previous studies, research in this dissertation investigated the effect of embryo number on spacing by utilizing a genetic mouse model system.

While species described above each exhibit embryo implantation within separate uterine horns, humans and other monotocous (single offspring per pregnancy) species exhibit implantation occurring within the uterine body [Böving 1956]. Therefore, determining the conservation and understanding the genetic and physiological mechanisms regulating embryo spacing and migration would reveal the translational capacity of this work. In species where, multiple embryos are rare events, migration is at the forefront of importance because spacing is non-existent in single embryo pregnancies. In these rare multiple-embryo instances, it is important to know how the uterus, and/or the embryos adjust, possibly by an underlying physiological or genetic mechanism, in order to preserve the health of the embryos. This is where embryo spacing, and its mechanisms become important to understand at a genetic level.

This type of translational research could be instrumental in improving reproductive technologies and understanding preimplantation abnormalities in humans, laboratory animals, and livestock.

Explaining the Events of Embryo Spacing and Migration

When Böving and his colleagues set out to determine whether spacing and migration was a random event or a controlled process, it was theorized that when the embryos are small and are not exerting pressure against the uterine lumen, agitated diffusion is occurring [Böving 1956]. This theory later became the accepted description for embryo migration. Simply put, the myometrium of the uterus contracts randomly and with minimal force, which disperses the embryos throughout the uterine horn in an arbitrary fashion. This led to the conclusion that the migration of embryos occurs indiscriminately [O'Grady 1969]. As embryos grow before implantation they exert increasing pressure on the uterine lumen/endometrium and in turn the myometrium. This pressure was thought to locally stimulate the uterus so that the myometrium shifts to a more regulated and deliberate contraction strategy which proceeds to evenly spaced embryos in relation to one another [Böving 1956, O'Grady 1969]. This evidence led to the suggestion of a stimulus-effector system being utilized. This system suggested that there was an endocrine stimulus, like progesterone, that causes the myometrial effect of contractions. Later research confirmed this system and elucidated, at least partially, the genetic pathways and mechanism responsible for the myometrial contractions that define embryo migration. However, the refined event of spacing is still largely not well understood and little is known about the contributing mechanisms, despite the many hypotheses.

In pigs, the hypothesis is very similar despite the differences in blastocyst morphology. Pig blastocysts change their shape from spherical to ovoid to tubular, and finally to filamentous. This change in shape corresponds with changes in migration and spacing of the embryos. It has

been noted that while in the spherical and ovoid forms, migration is slow and most of these embryos are found near the tubal end of the horn. This clustering can often result in touching of these embryos. However, between embryonic day 14 to day 18, elongation occurs, and filamentous blastocysts dominate the uterus environment and become regularly spaced [Anderson 1978]. With the domination of filamentous embryos during this time point and sudden regularity to the spacing of these embryos, a hypothesis emerged, that the blastocysts could be producing immunological or chemotactic factors that are manipulating the spacing of their embryo [Anderson 1978].

This comparison between mouse, rabbit, rat, and pigs illustrates phenotype conservation of spacing despite the variation seen in the progression, timing, and shape of the blastocyst and implantation. It also gives stock to the translational relevance of the findings in each of these different model organisms and in turn, gives a firm foundation for this dissertation research. The combination of foundational research, the phenotype conservation, and its translational nature emphasizes the need for genetic evidence of the mechanisms influencing the events of embryo spacing, for which little data exist.

Tracking Embryo Migration and Spacing through Early Pregnancy

The movement of embryos, once they have entered the uterine horn, has been broken down into two steps. The first step, "migration" describes how the embryos are loosely and randomly shuffled throughout a uterine horn, whereas the second step, "spacing" refers to the fine-tuned spacing of embryos that immediately follows the completion of migration. Migration, in comparison to spacing is considered crude with the driving force being hormones like progesterone and prostaglandin. Spacing is thought to be driven through the endocrine secretions and cytokines produced by both the embryo and the uterus.

Migration Event

Migration of embryos through the uterine horn is the first step of spacing, and occurs immediately after the embryos enter the horn, which is approximately embryonic day 3(E3) in the mouse. Early work in the rabbit and mouse attribute endocrine-driven myometrial contractions, likely by estrogen and progesterone, as the basis of embryo migration [Böving 1956]. This knowledge had been implicated in the movement of pig blastocysts [Anderson 1978]. Interestingly, these hormones are known to manipulate myometrial behavior, biochemical environment, and myometrial energetics. These data support the idea of a stimulus-effector model for migration [Böving 1956].

The stimulus-effector model is centered around progesterone in its role as an essential regulator, or stimulus. This in turn assigns the myometrial muscles to the role as effector, and the actual myogenic contractions as the resultant action. This hypothesis, first proposed by Dr. Böving in 1956, that states progesterone stimulates the myogenic contractions produced by the myometrium of the uterus, thus propelling the embryos throughout the uterine horns, has since been confirmed by genetics in 2007, which is explained in greater detail later [Böving 1956, Hama 2007].

In the rabbit and mouse, multiple types of myogenic myometrial contractions cause the embryos to migrate prior to regulated spacing. These peristaltic and anti-peristaltic (recoil)contractions propel the early embryos through the uterine horn. They are thought to occur randomly, and without the influence of the embryo, leading to the hypothesis that migration is maternally dependent and progesterone-driven [Böving 1956, Hama 2007]. Progesterone (P) drives the production of prostaglandin (PG), which then activates the myometrial smooth muscle. Local contractions recognized as having a role in the even spacing of embryos, which is also

controlled by the P>PG interaction [Böving 1956, Paria et al. 2002, Hama et al. 2007]. Phenotyping and physiological identification of the mechanisms controlling embryo migration have been elucidated since Böving's experiments, but until recently (early 2000s) the molecular mechanisms were not known.

The Spacing Event

During early research on embryo spacing, many researchers hypothesized that "cross talk" occurred between the uterus and the blastocysts through signaling interactions before implantation occurred. This "cross talk" was thought to refine the placement of embryos during the selection of their implantation sites. These researchers predicted that the blastocysts acted as individual local stimuli to the uterus and, in turn, cause an interaction between neighboring blastocysts [Böving 1956]. These same scientists suggested that progesterone was the dominating hormone for regulation during both migration and spacing, immediately before the initiation of implantation. As mentioned above, local contractions resulting in uterine luminal closure are ascribed to even spacing and are thought to be the result of this "cross talk" [Paria et al. 2002]. In rabbits, it was determined that as the blastocysts grew, they distended the uterine tissue/endometrium. This distention was thought to be a stimulus for those controlled local contractions. However, aside from progesterone contribution, the mechanism and its regulation that controls these local contractions, potentially occurring late on day 3, and any additional or alternative molecular mechanisms have yet to be revealed [Böving 1956, Paria 2002, Hama 2007]. Current research has begun to clarify the molecular mechanisms surrounding myometrial contractions as well as molecular mechanisms near the time of decidualization, which begins occurring late on embryonic day 3 [Paria 2002, Hama 2007, Chen 2011].

Mechanisms Related to Pre- and Peri-implantation

Extensive research has focused on implantation; the resulting discoveries while not complete have constructed a comprehensive representation of the roles of several growth factors, cytokines, and major pregnancy hormones [Carson 2000]. In contrast, embryo migration and spacing research has not progressed at the same speed, and much remains to be discovered about these intricate processes. The majority of the research that has been done on migration and spacing, has taken place in rabbits, rats, mice, pigs, and sheep. The intrauterine and interuterine migration experiments conducted in these models have begun to elucidate important genes, hormones, and other factors contributing to migration and spacing [McMillan et al. 1999, Nephew et al. 1992, Pope et al. 1982, Rahima et al. 1986, Böving et al. 1956].

Timing

Implantation can differ greatly among species based on their gestation length and embryo morphology. For rabbits, rats, and mice, implantation occurs relatively quickly once the embryos enter the uterine horn. For mice this occurs in a single day; initiation of implantation begins at the end of day 3 to the beginning of day 4 and is complete by the end of day 4 post coitum [Lee 2004]. However, the larger livestock species, like sheep, cattle, and pigs, have a longer and later implantation process. For example, implantation in the pig is spread out over multiple days and can range from day 10 through day 18 with most implantation completing between day 14 and day 18 post coitum [Anderson 1978]. This vast difference in timing of implantation among species seems to be independent of spacing and migration. This is important when designing experiments for this research and determination of this hypothesis's accuracy in the mouse. Additionally, verified overlap of implantation initiation and the conclusion of regulated spacing events occurs at the end of embryonic day 3 in the mouse.

Molecular Mechanisms of Myometrial Contractions

A set of genes has been identified that influence and control the myometrial contractions occurring during embryonic day 3 in the mouse. These include lysophosphatidic acid receptor 3 (*Lpar3*), cytosolic phospholipase A2 group IVA (*Pla2g4α*), and prostaglandin-endoperoxide synthase 2 (*Ptgs2*). These contractions are the direct effect of prostaglandin (PG) stimulation resultant from progesterone activation. This set of genes is known to be stimulated by progesterone and result in the myometrial stimulation of prostaglandin. The interworkings of these four genes and their respective roles in regulation of prostaglandin and, in turn, myometrial contractions have been elucidated via research focused on early implantation, mainly uterine decidualization [Hama et al. 2007, Dey et al. 2004]. These genes are known to influence and control various parts of the PG production pipeline, with *Lpar3* and *Pla2g4α* being the two most upstream effectors in this group. One of these genes, *Pla2g4α* , produces arachidonic acid, which is broken down to prostaglandin G that is converted by an enzyme produced by *Ptgs2* to prostaglandin H, which is then further broken down by several different synthases. These prostaglandin products are then used to contract and relax the myometrium and cause the myometrial contractions that crudely move the embryos throughout the horn during the first $2/3^{rds}$ of E3 in mice [Ruan et al. 2011]. *Lpar3* produces a membrane bound receptor in the endometrium which triggers G proteins. These proteins are then suggested to activate the enzyme produced by *Ptgs2* [Dey 2005]. Figure 1 provides a visual representation of this pathway that has been adapted from Ruan 2011.

These genes, when disrupted, are known to detrimentally affect the spacing of embryos. Mice null for *Lpar3* and *Pla2g4α* revealed detrimental effects on pregnancy viability of uneven spacing. Females of these null strains exhibited reduction of myometrial contractions

immediately prior to implantation. In addition, these females also displayed reduced litter sizes, a delay in implantation, and disruption of prostaglandin synthesis necessary for proper myometrial contractions [Chen et al. 2013, Hama et al. 2007]. Additionally, *Ptgs2* null mice exhibited a compensatory function, in which they utilized *Ptgs1* in place of the former. This adaptive ability to preserve pregnancy is one factor that makes explicating a complete schematic of events for spacing and migration so challenging [Paria et al. 2002].

Molecular Mechanisms of Embryo-Uterine Interaction

Progesterone is thought to condition the uterine horn tissue, so that it becomes reduced in its ability to conduct these stimuli, thus only allowing for localized myogenic contractions immediately prior to the decidual reaction late on embryonic day 3 in mouse [Böving et al. 1956]. The disruption, in a mouse model, of several genes implicated in the decidual reaction produced pregnant mice that exhibited signs of uneven spacing. This disruption affected a molecular pathway that includes the genes leukemia inhibitory factor (*Lif*), Heparin-binding-EGF-like growth factor (*Hbegf*), and Erb-B2 Receptor Tyrosine Kinase 4 (*Erbb4*). *Lif* has been identified to be upstream (in the pathway) of *Hbegf* and *Erbb4* has been tracked to be expressed in the blastocyst [Lim 2009]. Knockout mouse models have been generated for *Lif* and *Hbegf*, but both studies focused on implantation and decidualization, meaning no information was given on the effect, if any, the knockouts had on embryo spacing [Paria 2002].

Embryo-uterine “cross talk” is known to occur immediately preceding implantation through the completion of implantation. A prime example of this phenomenon occurs immediately before the beginning of implantation, during the apposition of the blastocyst, through the HB-EGF >ErbB4 interaction, in which embryonic specific surface receptors, produced by the *Erbb4* gene, interact with maternal uterine cell specific ligands, produced by the

Hbegf gene [Paria 2002]. This molecular pathway starts with the *Lif* gene, which is required for the expression and functionality of the uterine Hb-EGF. However, the presence, or lack of, the receptor ErbB4 on the blastocyst drives the ability to detect the Hb-EGF in the uterus. Additionally, the pathway mentioned above, utilizing LPA, is known to enhance this Hb-EGF > ErbB4 response during embryo apposition [Lim 2009].

However, the gene *Bmp2* has been more directly implicated in the embryo spacing trait. One particular experiment that is important to embryo spacing is the bead experiment, which used beads filled with BMP2 to assess the response and interaction of the uterus [Dey et al. 2004]. When these beads were transferred with blastocysts there was significant uneven spacing detected. Interestingly, the same study also found that HB-EGF somehow regulated the expression and accumulation of BMP2 during this important time point for embryo spacing [Paria et al. 2001]. Unfortunately, very little else is known about this BMP2 – HB-EGF interaction, contributing to the ongoing lack of understanding surrounding embryo spacing and the molecular mechanisms involved.

Another interesting gene that has been suggested to influence embryo spacing is *Sfrp-2*. This gene is a WNT antagonist, which suggests that there are WNT genes also involved in embryo spacing. This gene is known to produce a protein that inhibits the ability for the decidualization reaction to occur, thus preventing implantation and likely preventing blastocyst apposition from initiating [Carson 2005]. Interestingly, the WNT signaling pathway and its expression is observed in distinct bands within the myometrium only on the side of the blastocyst apposition. It was also noted that the number of distinct bands were more numerous than the number of blastocysts that have the opportunity to implant [Carson 2005]. This suggests that the

interaction of SFRP2 and the WNT signaling pathway could play a significant role in embryo spacing, but additional research surrounding these molecular mechanisms is needed.

The foremost identification method of uneven migration and spacing has been the use of knockout models and expression assays for a group of genes, including *Lpar3, Pla2g4α*, several *Wnt* genes, Ptgs2, *Bmp2*, and *sFrp-2*, known to be important in implantation and consequently migration and spacing, through a variety of mechanisms including prostaglandin synthesis [Dey et al. 2004, Hama et al. 2007, Carson 2005]. The identification of spacing variation in these inbred strains of mice contributes to the discovery of a viable difference, either in a gene and/or mechanism that could prove useful to improving the success of IVF and ART. Strains like these, also have the potential to reveal the compensatory function of some genes important for spacing and implantation, given their viability and the comparison of gene expression profiles to an even spacing representative strain, like C57BL/6J. Although there have been several genes and molecular mechanisms and pathways implicated in embryo spacing, there are very little data to provide concrete evidence and elucidate a clear picture to the molecular mechanisms responsible for the embryo spacing trait.

Uneven Embryo Spacing and its Repercussions

Regular spacing of embryos, as mentioned above, is known to be conserved across many research animal models. The importance of this conservation, and the degree of preference for even embryo spacing in a large number of mammalian species is not well characterized. Research done in rats suggests that regular, even spacing of embryos lends protection against fetal growth restriction, reduction in placental weight, and improves general health of the fetus [Rahima et al. 1986]. The rat is not the only model to suggest the need for even spacing, mice

(and other laboratory animals), livestock and humans have also expressed similar importance of even embryo spacing to healthy pregnancies.

Human and Animal Impacts

When spacing/migration is disrupted in both single embryo and multi-embryo pregnancies, detrimental effects can occur. Humans are of particular interest here because of the increasing utilization of in vitro fertilization (IVF). IVF is highly dependent upon a narrow window of uterine receptivity for implantation. Therefore, any event that causes a delay in embryo implantation would cause a reduction in implantation success. When delayed implantation occurs, embryos may fail to implant due to blastocyst incompetency, failed signaling pathways, failed uterine receptivity, and uneven spacing [Wang et al. 2006]. A disruption in even embryo spacing can lead to crowding of embryos, and the possibility of competition for implantation sites and/or a delay in implantation.

Placenta previa, ectopic pregnancies, and twin-twin transfusion syndrome (TTTS) are thought to be associated with uneven migration and spacing of embryos prior to implantation. These conditions can be life threatening to both mother and embryo, depending on the severity of the case. Placenta previa occurs when an embryo implants too close to the cervical end of the uterine body. This causes the developing placenta to partially or completely cover the opening to the cervix, and dramatically increases the mortality risk of the embryo because of an increased risk of placental tearing and bleeding, particularly during birth [Mayo Clinic 2018]. Additionally, ectopic pregnancies are dangerous for both mother and embryo as the blastocyst implants somewhere other than the uterine body/horn. This most often occurs following tubal ligation, where an embryo will implant in the ovary, fallopian tubes, or even within the peritoneal cavity of the body. These pregnancies are often terminated early or caesarian section is ordered to avoid

significant danger to maternal health [Complications 2018]. These two conditions deal primarily with the migration event in humans, but spacing mechanisms are likely involved in litter-bearing species. The TTTS condition is anticipated to exhibit a disruption in embryo spacing due to the proximity of the two embryos, which results in the sharing of nutrients.

TTTS typically occurs in identical twins that share a placenta (monochorionic) and affects approximately 4,500 pregnancies each year in the United States. The sharing of a single placenta leads to the sharing of their blood supply and in turn nutrients and hormones. TTTS can be harmful, even fatal to one or both twins depending on the severity, and can lead to growth restriction, high (or low) blood pressure, and reduced or defective heart function [Chen 2017, TTTS Foundation 2018]. In an even rarer subset of cases in which twins are fraternal and also of differing gender, monochorionic diamniotic pregnancies with signs of TTTS can develop, and these twins usually show signs of chimeric blood postnatally. These rarer cases are most often seen to develop with the use of in vitro fertilization [Ekelund et al. 2008].

In addition to humans, other single offspring and litter bearing animals are also affected by uneven spacing events. Of particular interest are those species related to agriculture production, such as pigs, sheep, goats, rabbits, and cattle. These species can exhibit uneven migration and uneven spacing and have conditions of their own related to the ones described previously. In particular, one interesting condition seen in cattle is known as freemartin syndrome. A freemartin has been defined as a sterile female that is the result of a heterosexual twin pairing and the sharing of blood between the twins due to choriovascular anastomosis [Marcum 1974]. Like TTTS in humans, freemartin syndrome in cattle occurs in monochorionic pregnancies and, like the rarer cases in humans, differing gender twins display chimeric blood postnatally. Unknown in humans but known in cattle, is the infertility of the female twin, in

which 92% of the time in these heterosexual twin couplings, the female twin is unable to reproduce. This infertility is likely due to the exposure to high levels of male developmental hormones, which results in masculinity of the female [Marcum 1974]. The cattle industry relies heavily on the efficient reproductive performance of their heifers and cows. The expectation of heifers to breed, produce, and raise a calf in their first breeding season is paramount to the success and profit of the herd. Most large herds in the U.S are averaging 80 – 90% fertility in their herds [McDaneld et al. 2012]. While this number is large, having 10-20% of females that are not producing can remove much of the profit gained from the other producing females due to the expense of rearing and maintaining the females with fertility issues that result in lost production. The amount of this infertility due to freemartins has yet to be documented. This effect is even more detrimental in the dairy industry. Identifying low fertility, sterile females, or those females with an increased probability of producing these sub-fertile offspring as early as possible, would be invaluable to the success of the farm. A genetic screen could facilitate this identification prior to weaning.

Describing the pathways and genes responsible for the migration and spacing events in early pregnancy may lend to the development of genetic screens and treatments beneficial to preventing and/or treating these conditions. This could include the supplementation of arachidonic acid needed for proper prostaglandin synthesis, or potential supplementation of transcription factors, cytokines, or other hormones, similar to those responsible for upstream activation of genes like *Ptgs2* [Pope et al. 1982]. Genetic screening for maternal disruption of important genes could help to determine the need for supplementation in lieu of IVF or in conjunction with IVF. In addition, paternal genetic screening alongside maternal screening will

help to identify potential embryonic signaling defects leading to migration errors and/or infertility.

Inheritance Patterns

The implications of uneven embryo spacing are known to be determinantal and undesirable for many animals especially in litter bearing species such as pigs, rabbits, rats, and mice. Being able to identify this unfavorable trait once it occurs is not ideal due to its potential adverse health effects on the unevenly spaced embryos. Therefore, this dissertation study aimed to explore and explicate the inheritance pattern(s) of the embryo spacing trait. This elucidation, in conjunction with the results from the QTL mapping and RNAseq, would allow for the development of an assay that would detect the potential for uneven spacing and provide the foundation for a selective breeding program to reduce and/or remove this detrimental trait. This information could also provide valuable genetic information for human fertility with regards to improper implantation location. Through the inheritance part of this study, one mode of inheritance was implicated, maternal effect, and two gene actions were implicated, additive and dominance. The combination of these inheritance patterns insinuates the likelihood of more than one gene involved, suggesting that this embryo spacing trait is polymorphic.

Mouse Models of Differential Embryonic Spacing

C57BL/6J (B6)

This inbred strain of mice is one of the most commonly used genetic backgrounds, and the strain used to first sequence the *Mus musculus* genome. This strain originated as C57BL and was developed by Dr. C.C. Little in 1921 utilizing mice from Abbie Lathrop's collection. Before 1937, the original strain was separated into C57BL/6 and C57BL/10. This C57BL/6J is the 6 substrain that is maintained by The Jackson Laboratory [Tucker et al. 1992]. This B6 strain

produces an average litter size of 6.2 and is able to produce, on average, 2.5 offspring per female per month. They are also known to have a low sterility rate of around 8% [Nagasawa et al. 1973].

C3H/HeJ and C3HeB/FeJ (C3H)

The original C3H strain was developed in 1920 by Strong utilizing a Bagg albino and the DBA strain. From there, the C3H/He substrain was obtained by Heston in 1941 and a few years later, 1947, was transferred to The Jackson Laboratory and became known as C3H/HeJ. This C3H/HeJ strain is another very popular inbred strain [MGI, 1998]. The C3HeB/FeJ strain is also maintained by The Jackson Laboratory and was developed in 1948 by Fekete by transferring fertilized C3H/HeJ ova to C57BL/6 [MGI, 1998]. C3H are known to have ovarian tumors in 29 – 47% of females depending on reproductive stage and use [Heston, 1963]. In terms of reproduction, both of these C3H substrains are similar to B6 with the average litter size around 6.4 and sterility between 4-10% [Nagasawa et al., 1973].

These two closely related, but independently developed, wild-type inbred mouse strains both exhibit uneven embryo spacing to approximately the same degree. This uneven embryo spacing phenotype was first observed by a previous graduate student, Jennifer Dackor, in Dr. David Threadgill's lab as a secondary observation to the research she was studying. This led to the phenotypic verification of this uneven spacing phenotype through the research performed in this dissertation, which in turn led to the other experiments in this study to elucidate the underlying genetics of this trait. This information presented an opportunity for this dissertation to focus on embryo spacing and migration as seen and studied in the mouse. These strains greatly differ in embryonic spacing patterns that allow for visual detection of the uneven spacing phenotype.

Recombinant Inbreds and the BXH Mice Series

Recombinant inbred mouse lines are invaluable tools for genetic research. In particular, these recombinant inbred lines are useful for genetic linkage mapping and quantitative trait analysis (QTL). These lines provide mice with the same progenitor background but differing fixed alleles at multiple loci throughout the genome [Silver, 1995]. Recombinant inbred lines (RILs) allow for variability control since each individual within a line contains the same genotype. This allows for the direct comparison of variable phenotypes seen among the lines, such as uneven spacing, and in turn, genetic mapping of any phenotype with the help of QTL analysis. These lines also allow investigation of background effects with respect to the progenitor inbred strains [Broman, 2005]. For example, when looking at uneven spacing in these recombinant inbred lines, the percent uneven spacing can be compared to the two progenitor strains and through haplotype analysis, sections of genome can be traced back to either progenitor strain. This, in conjunction with quantitative trait loci analysis, allows for regions of interest to be located and background origin to be identified.

The construction of recombinant inbred lines is straight forward but is time-intensive due to the number of generations needed to obtain sufficiently differing lines and extinction rates due to incompatible alleles. Recombinant inbred lines are generated by first crossing the two progenitor inbred strains, in the case of the BXH series used here, C57BL/6J females bred to C3H/HeJ males [GeneNetwork, 2004]. The progenitors of this cross provide the identifying name of the resultant recombinant inbred line with the B representing the progenitor female, X denotes the breeding, and H represents the progenitor male [Silver, 1995; GeneNetwork, 2004]. It is important that the progenitor strains be inbred, so every mouse within a strain is homozygous for the same allele for each locus in the genome. Between the two inbred strains, it

is expected that some regions of the genome will be homozygous for different alleles. These differences between the progenitor strains is ultimately what allows the homozygous mosaic blocks from each progenitor characteristic of recombinant inbred lines that are so valuable during genetic mapping experiments. In a first generation cross between inbred strains, all the F1 individuals are identical in their genetic makeup, and they are heterozygous at every position in the genome that differed between the two lines. Beginning in the second generation, recombination events between chromosomes derived from the progenitor strains can be detected and contribute to phenotypic variation among the F2 mice. F2 males and females are selected to act as founders for each of the lines [Silver, 1995]. In the case of the first 12 lines in the BXH series, which was initially developed in 1969 by Benjamin Taylor at The Jackson Laboratory, the selection of the pairs of founder F2s was random and each pair was maintained discretely from other F2 founders. These F2 founders for each new line were bred together and the resulting progeny for each set of founders were kept separate and treated as genetically different lines. A single pair from those F3s were chosen and sibling mated and recombination again reduced the size of the haplotype blocks maintained. Because inbreeding leads to an accumulation of homozygosity across the genome, this process of single pair choice and sibling mating was repeated for approximately 20 generations, which provides ample time for alleles to become fixed (homozygous) for one or other allele from the progenitor strains (either C57BL/6J or C3H/HeJ for the BXH series) and for the mosaic pattern characteristic of different lines to be established [Silver, 1995].

In addition to the original 12 lines generated by Benjamin Taylor in 1969, Linda Siracusa developed another 8 lines during her time at the Kimmel Cancer Center in 1995. These 8 lines were generated in a similar manner as stated above. Linda Siracusa had the ability to use genetic

selection, and she chose to select these lines for a gene on chromosome 7, tyrosinase-negative albinos [GeneNetwork, 2004]. Of the 20 total generated lines, only 12 are currently available through The Jackson Laboratory. These lines include 10 of the original lines from Benjamin Taylor in 1969 (BXH2/TyJ, BXH4/TyJ, BXH6/TyJ, BXH7/TyJ, BXH8/TyJ, BXH9/TyJ, BXH10/TyJ, BXH11/TyJ, BXH14/TyJ, and BXH19/TyJ) and 2 lines generated by Linda Siracusa (BXH20/KccJ and BXH22/KccJ) [GeneNetwork, 2004; Williams, 2001]. This study utilizes all 12 available lines with varying degrees of success due to copulation and fertility rates. This BXH series currently has around 142 traits compiled in the BXH Phenotype database, however embryo spacing is not included as one of these phenotypes [GeneNetwork 2004]. Additionally, all 12 of the lines being used in this study have been genotyped utilizing the Wellcome-CTC-Illumina set of 13,377 single nucleotide polymorphisms (SNPs). Identification of microsatellites and additional markers was also accomplished. Of these markers identified, 8,311 are used by the GeneNetwork WebQTL for use in informative mapping [GeneNetwork, 2004]. This dissertation study utilizes the 12 BXH lines described above, their informative markers, and the GeneNetwork WebQTL interface to elucidate genomic regions of interest associated with the embryo spacing trait.

Implications of this Research

In Vitro Fertilization

In vitro fertilization (IVF) is the most commonly used form of assisted reproduction technology (ART) in humans, where >99% of cycles performed, are done solely by IVF and the remaining <1% includes at least a portion of the IVF protocol [CDC 2017, Sunderam et al. 2018]. A single cycle of IVF typically takes 2 weeks to complete. This cycle consists of ovarian stimulation by several drugs, which cause the ovaries to produce multiple eggs. These eggs are

collected surgically and fertilized in the laboratory. Many IVF procedures utilize intracytoplasmic sperm injection (ICSI) for the fertilization process. Once fertilized, embryos are cultured until the optimum age for implantation to occur (Day 3-5 post fertilization). Viable embryos are then transferred back to the maternal uterus, and ultrasound and blood tests are used days later to determine if implantation has successfully occurred [CDC 2017, Sunderam et al. 2018]. IVF success is dependent on the receptiveness of the uterus, competency of the embryo, and timing of the transfer. The uterus is receptive during a narrow time point, in which the uterus is amenable to the implantation, consequently this receptivity "window" encompasses the embryo migration/spacing time frame [Yoshinaga 1988].

In February of 2018, the 2015 Assisted Reproductive Technology Surveillance Summary was published by the Center for Disease Control (CDC). This surveillance summary demonstrates that for women 35 and under (the group most likely to get pregnant), only 41.3% of viable embryos transferred progress to implantation [CDC 2017, Sunderam et al. 2018]. This statistic is astonishing low, considering approximately 6 million women in the United States struggle with infertility and many will, or already do, see IVF as a viable solution [OWH 2018]. Another compelling statistic reported in this summary involved multiple embryo pregnancies. Of the successful pregnancies from ART and IVF, 35% were multiple embryo pregnancies [CDC 2017, Sunderam et al. 2018]. The high incidence of multiple embryo pregnancies, using ART & IVF, points to the need for understanding of how these embryos migrate and space and identification of the genes/mechanisms involved. This information could provide valuable screens, interventions, or new ART protocols that would increase the performance of ART/IVF and potentially reduce the increased risk of multi-embryo pregnancies. This is especially

important for women over 35, who are more likely to undergo transfer of more than one embryo, and in turn increase their chance of an already high-risk pregnancy.

IVF & Spacing

Implantation and pregnancy have been extensively studied in humans and model organisms, like the mouse, and the resultant discoveries have led to improvement of ART and IVF. However, minimal information is known with regards to embryo migration and spacing and the genes and mechanisms involved. The high incidence of multi-embryo pregnancies and the low implantation success rate using ART and IVF demonstrates the need for understanding and discovering the genes and mechanisms involved immediately prior to implantation, during the time of embryo migration and spacing. This information could lead to new genetic screens and protocols that would increase the success of embryo transfer and implantation during ART and IVF cycles.

Rationale and Summary of Research

The research described in this dissertation builds upon and enhances the knowledge base surrounding embryo migration and spacing. Using phenotypic data from the parental strains, C3H/HeJ, C3HeB/FeJ, and C57BL/6J and the recombinant inbred strains derived from those parental strains, additional genes relevant to spacing and migration have been identified through QTL analysis and differential expression analysis. This study provides a new avenue for future research to continue to elucidate the complete mechanisms surrounding embryo migration, spacing, and implantation. Additionally, this research sets the foundation for screening other mouse (also rabbit and rat) strains, both inbred and outbred, for uneven spacing to facilitate confirmation of these genes or finding additional genes that regulate embryo migration and spacing.

Objectives

The following research tests several hypotheses that could explain what is happening during embryo spacing and to a lesser extent, embryo migration. Parity, embryo number, embryo sex, implantation timing, inheritance, and molecular clues were all investigated to determine whether they influence embryo spacing. Using the aforementioned strains of mice, embryo spacing was characterized, measured, and quantified to determine the extent of variation between strains with even embryonic spacing compared to strains with uneven spacing. Additionally, other potential contributing factors were examined for their influence on the embryo spacing phenotype. A pedigree was generated to investigate mode of inheritance and gene action for this phenotype, keeping in mind previous information known, including embryo – maternal "cross talk." This pedigree data led to the query concerning the genes influencing the uneven embryo spacing phenotype and the utilization of recombinant inbred lines to investigate the chromosomal locations of involved genes. Through the phenotypic characterization of these lines, QTL analysis, and the RNAseq data on the parental embryos at multiple time points, genes of interest, including Poly ADP -ribose polymerase family member 8 (*Parp8*) and Embigin (*Emb*) were identified.

CHAPTER II

GENETIC CONTRIBUTION & PHENOTYPIC VARIATION

Introduction

Physiological mechanisms have long been implicated in embryo migration with the most prominent being myometrial contractions. Little information exists about the physiological mechanisms involved later in embryo spacing, but embryo "cross talk", the interaction of the embryo with the maternal uterus, has been implicated. This work provides uses an otherwise normal mouse strain that has abnormal embryo spacing to investigate potential mechanisms and to identify genes that contribute to embryo spacing and migration.

Several physiological factors including implantation, parity, embryo crowding, and sex bias were investigated. These factors were used to identify contributions to embryo spacing. Additionally, QTL analysis on recombinant inbred lines was utilized to identify candidate genes that may contribute to embryo spacing. The subsequent chapter will elaborate on the implications and interpretations of the data presented below.

Background

Böving and his colleagues developed protocols for accurate evaluation of embryo spacing. It was determined that there is an optimum condition (between 3-6) in which the number of embryos within a single uterine horn needs to be regulated so that the embryos have the opportunity to space evenly [Böving et al. 1956]. Large numbers of embryos were known cause crowding, and it was assumed by Böving and his colleagues, that this crowding prevented the ability to distinguish between regulated or random embryo distribution. The theory was that

there was only so much room within a uterine horn for embryos to move, and when an excessive number of embryos were present, regulated embryo spacing would look the same as randomly spaced embryos [Böving et al. 1956]. This early research provides support for the current work in that embryo crowding, due to embryo number in a horn, can prevent the distinction of even or uneven spacing due to the inability of the embryos to migrate any significant distance from one another.

The 1950 to 1960s set the precedent for embryo spacing evaluation utilizing rabbit, rat, and mouse models. Böving utilized the rabbit model and provided the procedures and analysis methods for evaluating embryo spacing and determining their degree of evenness. His research used a measurement system in which the uterine horns were laid out, measurements taken, and then converted into a percentage of uterine horn length. This method normalized all of the measurements so that all uterine horns were comparable irrespective of their actual length [Böving et al. 1956]. Standard deviations (SD) and coefficient of variation (CV) were used to estimate the evenness of spacing and predict the likelihood of embryo locations within uterine horn [Böving et al. 1956]. This work set the basis for how embryo spacing could be assessed and analyzed, and the research in this dissertation utilizes this method. While Böving and his colleagues set the foundation on how to accurately measure and assess the phenotype of embryo spacing, it is expected that the data herein will provide a foundation for further research on the molecular mechanisms surrounding embryo spacing and migration.

Materials and Methods

Mice Strains

All mouse procedures were approved by and performed according to the Texas A&M University IACUC. The strains utilized in this study include the parental strains: C57BL/6J (B6)

stock #000664, C3H/HeJ stock #000659, C3HeB/FeJ (C3H) stock #000658 and the recombinant inbred lines: BXH2/TyJ stock #000034, BXH4/TyJ stock #000011, BXH6/TyJ stock #000038, BXH7/TyJ stock #000014, BXH8/TyJ stock #000076, BXH9/TyJ stock #000008, BXH10/TyJ stock #000032, BXH11/TyJ stock #000039, BXH14/TyJ stock #000009, BXH19/TyJ stock #000033, BXH20/KccJ stock #003784, BXH22/KccJ stock #003786 [Williams et al. 2001]. These strains were obtained from the Jackson laboratory at 4 - 8 weeks of age and were allowed to acclimate for a minimum of one week before being put into the study. Each strain was either utilized in breeding trios to maintain lines, 1 male to 2 females, or in experimental cages with a maximum of 1 male to 3 females, which were checked for vaginal plugs daily. Mice were housed in standard ventilated cages and were fed a standard diet (Diet 2919, Envigo) and water ad libitum. Breeding cages were established with minimum 4-week-old mice and were allowed to breed and produce until at most one year of age. Study cages contained at minimum 4-week-old mice and at most 6-month-old females. Females used for the embryonic spacing assays were checked daily for presence of a copulation plug.

These strains were chosen based on unpublished results from research previously completed by Dr. Jennifer Dackor during her time as a graduate student in Dr. David Threadgill's laboratory. She found that B6 exhibited conjoined placentas suggesting even spacing, while C3HeB/FeJ exhibited conjoined placentas in a portion of mice tested suggesting uneven spacing. The other C3H strain, C3H/HeJ was chosen because of its use as a progenitor strain, along with B6, in the generation of the recombinant inbred lines. In addition to these reasons, there are additional benefits to using these three strains in the research described in this dissertation. In addition to their suggested, and eventual confirmed, even spacing the B6 strain has a vast amount of genomic resources available, due to its use in the development of the *Mus*

musculus genome sequence and its substantial usage as a background for many research endeavors. The two C3H are also popular inbred strains in research making them comparable, in terms of genomic resources, to B6. Together, these strains provided a robust foundation for this study.

BXH recombinant inbred lines were chosen because of the strains used in their development, C3H/HeJ and C57BL/6J. These recombinant inbred lines have also been genotyped and those data deposited into GeneNetwork, making analysis of the phenotype data quicker and more streamlined. Availability of these recombinant inbred lines provides important genomic resources and tools for identifying genes of interest implicated in the embryo spacing trait.

Harvesting

For most experiments, mice were euthanized using AVMA guidelines by CO_2 asphyxiation followed by cervical dislocation. For a small subset of experiments, mice were anesthetized using 1.5% tribromoethanol prior to euthanasia by CO_2 asphyxiation and cervical dislocation. Dosage was based on the weight of the mouse, with a typical dose for an adult mouse at 250mg/kg body weight.

Once humanely euthanized, female mice were placed on their dorsal side and isopropyl alcohol was used to smooth the fur before cutting. A small incision was made near the mammary glands the skin was separated from the subcutaneous layer. Lateral incisions were made to open the abdominal cavity in order to reach the reproductive tract. Each uterine horn and attached ovary were carefully located. Both uterine horns and their ovaries were excised together and removed in a single entity. Careful cutting was done in order to retrieve each ovary, uterine horn

and part of the cervix, and to separate the organs from the gonadal fat. Upon removal, the intact uterine horns and their respective parts were placed on bibulous paper for measuring.

Phenotype Characterization

For initial strain spacing assessment, females from the parental strains mentioned above, C3H/HeJ, C3HeB/FeJ (C3H), and C57BL/6J (B6), were mated to same strain males. Embryonic day 0.5 of gestation (E0.5) was designated as noon on the day vaginal copulatory plugs were observed. Females were harvested, using the aforementioned methods, on E6.5-E8.5 for phenotype characterization. Once the uterine horns were placed on bibulous paper, they were stretched gently so that the uterine horns were laid flat and straight, but not so excessively that the horns physically stretched and distorted. Each embryo was then marked with a single dot, counted, recorded, and the uterus was labeled with the strain, mouse number, gestation day, and date harvested. The uterus was then photographed for reference and later measurements. This protocol was also utilized for phenotypic characterization of females for the multigravida vs. primigravida pregnancy experiments, the pedigree generation harvests, and the recombinant inbred lines. However, while multigravida vs. primigravida pregnancy experiments and recombinant inbred line phenotypic characterization utilized B6, C3H, or BXH line females bred to same strain males, pedigree generation experiments involved different crosses to track differences in embryo spacing and migration. are

Uterine Horn Measurement and Analysis

Uterine horns were measured and analyzed to determine the extent to which embryo spacing significantly differed. Each horn was measured utilizing the free, online sourced, NIH program ImageJ [Schneider 2012]. When measuring, each horn was treated independently of the opposite horn, and measurements were taken in succession, in which, the end point of the first

measurement was the starting point of the second measurement. These measurements were started in the middle of the embryo closest to the ovary and finished in the middle of the embryo closest to the cervix. The unit of measurement was arbitrary because each measurement was converted into a percentage of the total distance. This total distance is described as the distance from the middle of the first embryo to the middle of the last embryo in that particular horn. It is important to note that in order for the measurements to be useable, each uterine horn must have contained a minimum of 3 embryos.

Measurements were recorded within a spreadsheet and were catalogued with their strain number, horn ID, mouse ID, date harvested, and number of embryos. Within this spreadsheet, the sample mean was calculated by taking the average of the actual distances between embryos (in % of the total distance). The SD was calculated by taking each measurement (in % of total distance) subtracting it from the sample mean, then squaring that result. Once squared, all of those numbers were summed, and that number was divided by n-1, to represent a sample population, and the resultant number was the variance. The square root of the variance was taken to give the SD for that horn. The CV was calculated by dividing SD by the sample mean. Once converted to percentages, a chi square test was performed, and a coefficient of variation was calculated to assess the variation of spacing within a horn and average CV for a strain. This average CV was calculated by first averaging all of the like litter size CVs for the horns in the strain. Then those CVs were averaged to find a final average strain CV. A CV of 0 was indicative of even spacing and a CV near 1 was indicative of random spacing. The chi square test was calculated using the actual measurements (in % of total distance) and expected distance for even spacing with regards to the number of embryos present (in % of total distance). A

significance cut off value of 0.05 was used to assess whether a single uterine horn was significantly unevenly spaced or not.

The SAS Institute Inc. (Cary, NC) program JMP was utilized to analyze the various measurements for all of the experiments comparing among and between strains. Oneway ANOVA was used to determine whether there were significant differences between means among strains. Oneway analysis graphs were used to visualize each strain's mean and its corresponding confidence intervals.

Blue Dye Injection

Fifteen B6 and fourteen C3H females in plug check cages (1 male to 2-3 females) were checked for copulation daily. Any female with a vaginal plug was removed and placed in a separate cage with other plug positive females from that day; ear punching was used for strain differentiation. The blue dye tail injection was performed on E4.25 and E4.5 to determine if any delay in implantation was observed, based on the presence or absence of blue dye in the uterine horns. Immediately before the procedure, mice were anesthetized as described above and then 0.1ml of 1% Chicago Blue Dye was administered via intravenous tail injection. The dye was allowed to effectively circulate throughout the body for 10 min and mark implantation sites by leaking from the vasculature due to increasing vascular permeability that is occurring at the site of implantation [Lim et al. 2002]. Figure 2 displays a successful dye injection on a B6 female. At the end of 10 minutes, the female was humanely euthanized, and her uterus excised utilizing the previous described methods.

Blastocyst Collection and Storage

Seven B6 and ten C3H females in plug check cages (1 male to 3 females) were checked for copulation daily. Any female with a vaginal plug was removed and placed in a separate cage

with other plug positive females from the same day; ear punching was used for differentiation. Time of vaginal plug was denoted as embryonic day 0.5 (E0.5) and blastocyst collection was done on E3.5 and E3.75. Using the protocol “Collection of Blastocysts” explained in *Manipulating the Mouse Embryo: A Laboratory Manual*, blastocysts were flushed from each uterine horn separately into M2 medium, collected, and transferred to a 0.6ml tube [Behringer et al. 2014]. This was done by euthanizing the E3.5 or E3.75 pregnant female via the methods described previously. Once euthanized, each uterine horn was carefully excised by snipping immediately below the uterotubal junction and immediately above the cervical junction. The horn was then held using a pair of thin forceps and a 30-gauge needle with approximately 0.5ml of M2 medium (Sigma-Aldrich) was inserted into the uterotubal cut end. The M2 medium was ejected from the needle, through the uterine horn, and into a small drop (approximately 0.2ml) of M2 medium resting in a small petri dish. Once both uterine horns were flushed into the petri dish, the dish was moved to a dissecting microscope for blastocyst identification and collection. Only embryos at the blastocyst stage were collected.

Collection was done via pipette with a filter, along with a pulled glass pipette. The glass pipette was pulled by heating the end of the pipette using a Bunsen burner and stretching the malleable heated glass to obtain a narrow tip. The narrow tip allowed for the uptake of the individual blastocysts without risk of losing them into the larger chamber of the glass pipette. Blastocysts were collected from the flushed M2 medium and placed into a new droplet of M2 medium. This allowed for the washing of the blastocyst and removal of any uterine cell contamination. The blastocysts were washed an additional time for further decontamination. Once sufficiently washed, blastocysts were again collected and placed in a 0.6ml tube and stored

in a -20˚C freezer until submission for RNA sequencing. E3.75 blastocysts were each placed in their own individual tube, while the E3.5 blastocysts were pooled for each mouse collected.

Embryo Collection and Storage

E6.5-E8.5 embryos from the uterine horns collected from 14 B6 and 16 C3H females in the multigravida vs. primigravida experiments were carefully excised from the uterine tissue and placed in a 1.7ml tube with 50uL of 1% sodium dodecyl sulfate (SDS) and immediately stored on ice prior to transfer, for long term storage, to a -20˚C freezer. Uterine horns were carefully laid out on bulbous paper, stretched until taunt, and the uterine horn data collected as previously described. Working one embryo at a time, and starting at the embryo closest to the ovary, incisions were made immediately around the embryo to sever it from surrounding uterine tissue. The embryo was then gently squeezed from the remaining uterine horn tissue and placed in the 1.7ml tube with 50uL SDS. Each embryo was immediately labeled with the embryo number designation, strain, mouse number, gestation day, and date. DNA was later extracted from these embryos for use in the gender influence experiment.

DNA Extraction

DNA was extracted from E6.5 – E8.5 embryos from 14 B6 and 16 C3H females that were bred to same strain males in the primigravida vs. multigravida experiment. This equated to 248 embryos collected for DNA extraction and sex identification. Embryos were collected and stored as aforementioned until DNA extraction could occur. Sodium hydroxide lysis was used to isolate the DNA for PCR. Briefly, the embryos were heated in 100ul of extraction buffer (25mM NaOH and 0.2mM EDTA) for 20 minutes at 95˚C. After briefly cooling the tubes, 5µL of 1M HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) was added to each tube, vortexed, and centrifuged for 5 minutes at maximum speed. Finally, 40µL from the top of the supernatant for

each sample was collected and placed into a new 1.7ml tube. DNA was then stored in a -20˚C freezer until used for PCR and gel electrophoresis.

PCR and Gel Electrophoresis

Once DNA was extracted from the E6.5 – E8.5 embryos mentioned as described above, Polymerase Chain Reaction (PCR) was performed on these samples using primers designed from the *Kdm5c/d* loci, which are homologues on the X and Y chromosomes. These two loci differ in size at a single intron, which is larger at *Kdm5c* on the X chromosome. The primers (SMCX-1 5'-CCGCTGCCAAATTCTTTGG-3' and SMC4-1 5'-TGAAGCTTTTGGCTTTGAG-3') for *Kdm5c* and *Kdm5d* are a common set of primers used for gender identification in *Mus musculus* [Bean et al. 2001]. Confirmed male and female samples, as well as a no-template control, were included in each PCR reaction. The following reagents and quantities per sample were utilized based on lab protocol: 12.7μL dH_2O, 2μL 10x red Qiagen buffer, 2μL 25mM $MgCl_2$, 0.4μL 2.5mM dNTPs, 0.4μL of each primer, 0.1μL of Taq polymerase, and 2μL of sample or control DNA (or nuclease free H_2O for negative control). Additionally, the following PCR parameters: 94˚C for 3 minutes, 35 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 1 minute, 72˚C for 7 minutes, and was held at 10˚C until removal from the Bio-Rad CFX96 thermocycler.

Once PCR was complete, products were separated by agarose gel electrophoresis through (3% agarose in 1 x TAE). A 100 base pair ladder (New England Biolabs) and bromophenol blue dye were utilized to verify the size of the PCR products. The PCR products produced two bands approximately 300bp in size that represented product for the X chromosome (larger fragment) and the Y chromosome (smaller fragment) [Bean et al. 2001]. Three percent agarose gels dyed with ethidium bromide (1%), incorporated for imaging purposes, were run in 1X TAE (Tris - acetate and EDTA – Ethylenediaminetetraacetic acid) buffer at 140V for 90 minutes. Once

complete, gels were imaged on a Bio-Rad Gel Doc XR+. The resultant images were black and white inverted and saved as publication quality files.

Pedigree Generation and Analysis

To uncover the inheritance pattern for embryo spacing phenotype, a pedigree was generated to investigate the genomic contribution effect on phenotype inheritance. Initially, 15 B6 females were mated to C3H males. These females were plug checked, harvested, and their phenotype characterized by the method described above. Simultaneously, additional B6 females were mated to C3H males to produce F1 offspring. These F1 males and females were interbred and 12 of these females were harvested and their phenotype characterized. The remaining females were used to generate the F2 population. Next, 16 F2 females were bred to F2 males, plug checked, harvested, and their phenotype characterized. The previously described breeding and harvesting were repeated for the reciprocal lineage of 12 C3H females bred to B6 males, 12 F1 females, and 13 F2 females respectively. In addition, several backcrosses were performed for each set of lineages. Table 2 shows both sets of crosses performed, and the number of females harvested in each cross. This table also displays the average CV and the degree of uneven spacing present within each cross. Additionally, Figure 12 shows these crosses in a standard pedigree format and summarizes the percentage of harvested horns by cross observed to have uneven spacing.

Once all of the pedigree phenotypes were collected and the CV calculated, the data were sent to a collaborator, Dr. Heath Blackmon, who had designed a package in R known as the Software for Analysis of Genetic Architecture (SAGA), which was used to analyze this study's pedigree data and allow for elucidation of the likely mode of transmission based on the genome present in each cross in relation to the coefficient of variation [Blackmon et al. 2016]. This

package allowed for rapid analysis of pedigree data and utilizes a weighted averaging system to precisely predict composite genetic effects. A C-matrix is developed using the means for each cross in addition to the standard error. This C-matrix, along with GLM and AICs are used to determine which of the composite genetic effects is most likely to be the mode of inheritance for a trait. A v_i score, variable importance, is used to provide validation of a composite genetic effect regardless of how large or small its contribution to the trait [Blackmon, 2016].

Recombinant Inbred Lines

Twelve BXH recombinant inbred lines obtained from The Jackson Laboratory and utilized to obtain phenotype data on the embryo spacing trait. BXH2/TyJ (n = 18), BXH4/TyJ (n = 5), BXH6/TyJ stock (n = 19), BXH7/TyJ (n = 14), BXH8/TyJ (n = 16), BXH9/TyJ (n = 16), BXH10/TyJ (n = 15), BXH11/TyJ (n = 2), BXH14/TyJ (n = 19), BXH19/TyJ (n = 19), BXH20/KccJ (n = 18), BXH22/KccJ (n = 10) females were harvested on E6.5 - 7.5 and their uterine horns excised, photographed, and measured for the embryo spacing phenotype, as previously described. The phenotype data was measured and analyzed as previously mentioned to obtain a CV that would be used in later analysis, including in the quantitative trait loci mapping experiments. The phenotype data obtained from these 12 lines and their progenitor strains were utilized for interval mapping and identification of regions of interest in the genome.

QTL Mapping and Analysis

Quantitative Trait Loci (QTL) mapping and analysis were performed using the web-based program, GeneNetwork. This program and database allow variation in the phenotypic data to be correlated with single nucleotide polymorphism (SNP) data compiled by strain in this program's database. Utilizing the embryo spacing phenotype data from the parental strains, C3H and B6, as well as the recombinant inbred lines, BXH series, we were able to construct a QTL

map. This was done by uploading each strain's phenotype data, in the form of a percentage of uneven spacing based on individual horn data. These data were then used to perform interval mapping with 1000 permutations and 2000 bootstraps [GeneNetwork, 2001]. Any chromosome that displayed a suggestive and/or significant LRS (Likelihood ratio statistics) peak and a corresponding bootstrap peak were further investigated. At each peak, haplotypes, collection of SNPs, were recorded, and local genes were documented and considered for likely influence on the uneven spacing phenotype.

RNAseq

Library preparation was done on whole and pooled blastocysts collected on E3.5 and E3.75 from both B6 and C3H females bred to same strain males. E3.5 blastocysts in M2 medium were pooled in groups of 10 embryos, while E3.75 embryos in M2 medium were collected and stored individually. Pooled embryos for both strains were utilized to check the effectiveness of the library preparation system, Ovation® SoLo RNA-seq System (part no. 0501 for mouse) but were not included in the sequencing [NuGEN, 2018]. Once the SoLo system was verified, the individual B6 (n = 8) and C3H (n = 8) E3.75 blastocysts were prepared, quantified, and checked for quality. The resultant RNA was sequenced on an Illumina NextSeq 500 machine, producing 75bp single-end reads. Once sequenced, the resultant data was demultiplexed and trimmed by Kranti Konganti, Associate Director at the Texas A&M Institute for Genome Sciences and Society. The remaining single-end reads will be analyzed on the Texas A&M HPRC ADA cluster using the Salmon program and the *Mus musculus* transcriptome, which was generated from the inbred strain C57BL/6J. Differential expression analysis will be performed on Rstudio using Dseq2.

Results

Parental Strain Embryo Spacing Confirmation

Nine female C3HeB/FeJ (C3H) and 8 female C57BL/6J (B6) were utilized to confirm the presence of uneven spacing. The B6 females were utilized to represent a population of embryos and displayed a mice with evenly spaced embryos, the average CV was 0.07901. All 8 B6 females were observed to have evenly spaced embryos in both uterine horns of every mouse. In contrast, all 9 of the C3H females displayed at least one significantly unevenly spaced horn, with 3 females having both horns significantly unevenly spaced. The average CV for C3H was 0.36155. Figure 3 shows a representation of the variation seen between the 2 main parental strains used (C3HeB/FeJ and B6). C3H/HeJ was also assessed using 7 females, and similar results to C3HeB/FeJ were found. Their CV was 0.33533, which is not statistically different from C3HeB/FeJ ($p = 0.7180$). These results gave validation and justification for all future experiments in that these results confirmed the presence of a significantly different phenotype ($p = <0.0001$) in C3H mice from the expected, conserved, even spacing phenotype.

Parity

After confirmation of the uneven spacing phenotype, several factors were tested in order to evaluate their influence and, determine their level of impact, if any. The first factor tested was parity, to determine the effect of number of pregnancies on embryo spacing. In this study, primigravida females were compared to multigravida females. Both parental strains, B6 and C3H, were tested. Seven B6 primigravida, seven B6 multigravida, eleven C3H primigravida, and five C3H multigravida females used in this study. The results of this study showed that parity does not significantly affect embryo spacing (Figure 4). This figure shows that based on the CV, which describes unevenly spaced horns, the parity is not significantly different between

primigravida females and multigravida females within the same strain. However, there was a significant difference between strains in both circumstances, which was expected and was used as a positive control. Figure 4a shows the parity analysis in three different scenarios. The first shows all of the data pooled based on whether it came from a multigravida female or a primigravida female, while the second and third photos exhibit parity separated by strain, either C3H or B6 (respective p values: 0.3365, 0.5328, and 0.9234). This triple analysis allowed for the distinction of any differences in spacing, as a result of parity, present within a strain and among the two parental strains. In conjunction with Figure 4, Table 1 shows the resultant average CV for each strain, the average p value for each strain, and their corresponding parity status.

Additionally, Figure 4b shows these same data by strain, in which the two left most images display individual parity status (primigravida or multigravida) compared between C3H and B6. The final image in Figure 4b shows the parity data pooled for each strain and compared (respective p values: 0.0001, 0.0008, and 0.0001). This was done to show that regardless of parity, there was still a significant difference in embryo spacing between the strains, acting as a positive control for the experiment. Although parity was ruled out, it was possible that other factors could still contribute to the embryo spacing phenotype.

Litter Size and Horn Crowding

The next factor that was tested was litter size and the impact of horn/embryo crowding. Previous research suggested that embryo crowding masks the ability to determine the spacing phenotype by not allowing sufficient room for embryo migration to be detectable. That particular research indicated that more than 6 embryos per horn makes measuring embryo spacing unreliable [Böving 1956]. Herein it was a rare event (3.94%) for a single horn to have more than 6 embryos, and the average number of embryos in a horn was 4.25. An average embryo number

of approximately 4 to 5 embryos per horn seemed to be the ideal and most informative for detecting abnormalities in spacing. It was also noticed that the average range of variation between 3 to 6 embryos in a horn did not vary much from one another (CV: 3Em = 25.61%, 4Em = 27.22%, 5Em = 26.48%, 6Em = 27.82%). It is important to note that the uterine horns utilized in this analysis came from the parental phenotype characterization, parity, and recombinant inbred phenotype characterization experiments. There was a difference in variation, based on CV, from the 3 to 6 embryo group and the 7 to 8 embryo group (CV: 7Em = 18.97%, 8Em = 17.26%). As seen in Figure 5, the CVs of 3 to 6 embryos were significantly different from the CVs of 7 to 8 embryos (p-value = 0.0006). However, based on the reduction in variation seen in the 7 to 8 embryos, as shown in Figure 5, this significant difference was likely due to uterine horn crowding, which inhibits reliable detection of variation in embryo spacing, and a very small sample size for the 7 to 8 embryos (11 of 279 horns). This information led to the question whether there was a gender bias contributing in lieu of embryo number bias.

Sex Bias

Sex bias was another factor to investigate as it would provide information into whether the sex chromosomes were influencing this phenotype, and if the developing sex hormones or pathways were contributing along with the previously known contributing maternal hormones. Firstly, oneway ANOVA was performed on C3H and B6 litter sizes, which were weaned from breeding cages, in order to detect any general sex bias unrelated to embryo spacing, that might have been present within either strain. Figure 6 shows the resultant graph and confirms that the normal and expected distribution of sex was occurring for both strains. This information rules out any general skewness for sex that could have biased any further studies related to sex and confirmed that our strains were directly comparable in terms of males and females per litter and

per horn. Further, the focus of the sex evaluation in regard to uneven embryo spacing was able to accurately inform about any preference for males or females to unevenly space over one another.

One hundred and forty-nine embryos from 9 C3H and 9 B6 pregnant females were utilized to test if adjacent embryo sex influenced spacing. The sex of each embryo was identified through the use of PCR and gel electrophoresis. Figure 7 shows XY PCR products used to sex individual embryos. Females have a single band from the homozygous XX chromosomes, while males as two bands due differences in the Kdm5c/d regions on the X & Y chromosomes. It was seen that the DNA extraction method worked more efficiently on the control female and male ear punches than the sample embryos. However, the DNA extraction worked efficiently enough to obtain reliable sex results.

It was noted that the average ratio of females to males for each strain was 1 to 1.03 (C3H) and 1.17 to 1 (B6). These numbers fall in the normal expected ratios of 50% for each sex in each strain. The more informative piece of information is the locations, in respect to one another, of the males and females within a horn. Figure 8 displays representative horns from B6 (left) and C3H (right) with each embryo labeled with its respective sex. This figure also shows the results for the statistical analysis in a graph, which shows that there is a lack of any potential sex bias from local sex interactions of the embryos (male to male, male to female, and female to female). This information is consistent with the previous experiments, in which there was a significant difference between the strains (C3H and B6) for CV in embryo spacing. However, when looking at the interaction data, separately within each strain, the interactions show no significant difference for either strain (B6 p-value = 0.5531 and C3H p-value = 0.8729). This led to examining the timing of implantation in both C3H and B6 as a factor for embryo spacing prior to implantation.

Implantation

Previous research using several mice knockout models revealed that under controlled circumstances embryo crowding does not directly cause a disruption in the timing of implantation, and the utilization of this information led to the question of whether our test model (C3H) would show that differential implantation timing may lead to abnormal spacing or if C3H and B6 both exhibit normal implantation timing despite a difference in embryo spacing [Chen et al. 2013]. To test if implantation timing impacts spacing, four time points were utilized (E3.5, E3.75, E4.25 and E4.5). A disruption in the timing of implantation would have been observed if, on E4.25, no prominent blue sections within either uterine horn, which would have occurred if the vasculature of the uterine tissues was leaky near the site of an implanting embryo, were observed after 10 minutes, once the uterus was excised. The E4.5 time point was tested first in order to detect any delay in implantation, or lack thereof. Only after the E4.5 time point was finished, and no delay detected was the E4.25 time point utilized to determine if a less severe delay in implantation was present. Figure 9 displays representations of both B6 and C3H uteri after the Chicago Blue Bye injection and uterus excision. After the observation of implantation sites at the E4.25 time point, earlier time points were looked at to determine if implantation was disrupted between Day 3.5 and E4.25 of gestation.

Based on the E4.25 and E4.5 data and the data resulting from the parental characterization and parity experiments, it was predicted that this study would not reveal a disruption in implantation. The two time points previously described (E3.5 and E 3.75) were used to determine if there was a disruption in the timing of implantation observed in the C3H strain but not in B6 strain. On average, more embryos implanted for the E4.25 and E4.5 blue dye injection uterine horns than were flushed when utilizing the E3.5 and E3.75 uteri (Figure 10).

This is likely due to the initiation of implantation starting to occur at the correct time, which resulted in fewer blastocysts being flushed, because some blastocysts would no longer be in suspension in the uterine fluids located in the uterine lumen. After this analysis, it was concluded that in our model the uneven embryo spacing seen in C3H does not affect the timing of implantation. Additionally, from those blastocysts recorded, 22 E3.75 B6 blastocysts, 17 E3.75 C3H blastocysts, 15 E3.5 B6 blastocysts, and 15 E3.5 C3H blastocysts were collected for later use in a RNAseq experiment.

Despite the absence of a disruption in the timing of implantation, there was an interesting trend that can be tracked starting with the uterine horns of the E4.25 and E4.5 mice. The average number of embryos/mice gradually decreases from E4 to E6-8 and again decreases through weaning (Figure 11). From E4 to E8, C3H mice consistently had a slightly larger number of embryos present compared to B6 mice, however at the time of weaning, C3H weaned slightly fewer mice than B6. Additional time points would be needed to determine the rate of decrease and at which point this embryo loss stabilizes. However, this decrease has the potential to be associated with embryo spacing, which according to previous research, the closer embryos implant to one another the more likely those embryos are to experience conjoined placentas. This fusing of placentas could be directly related to embryo spacing due to proximity issues during implantation. Additionally, conjoined placentas are known to cause fetal growth restriction, and if severe enough, fetal death of one or both embryos [Twin-Twin 2016]. Furthermore, repeating the E3.5 and/or E3.75 experiments with the additional use of the ovaries, in order to count the corpora lutea, which would provide further validation on the lack of any minor disruption in implantation timing, while giving an accurate count of ovulation rates. Using corpora lutea will allow for the comparison of ovulation rates to implantation rates.

Inheritance

Inheritance patterns were evaluated with the generation of a pedigree that includes reciprocal crosses and backcrosses of B6 and C3H backgrounds. Figure 12 exhibits the completed pedigree including mouse numbers, CV and percent uneven spacing. This information was used to evaluate differences among the crosses. This resulted in the observation of a significant difference in the spacing of the parental reciprocal crosses (25% vs 50%). This difference implicates a parent-of-origin effect but does not distinguish between the embryo imprinting and maternal environment. Further sibling crosses in the F1 and F2 generations showed a conserved decrease in embryo spacing variation in the F1 crosses and then an increase in variation at the F2 generation, but not restored to the parental variation. The data do not fit Mendelian expectations but the input of a maternal effect by dominance or additive gene action would provide an explanation of the degree of unevenness seen in the Figure 12 pedigree.

All of the crosses' CVs were taken and analyzed using the previously described R package by a collaborator, Dr. Heath Blackmon. This analysis resulted in the identification of a maternal effect by dominance gene action and, to a lesser extent, additive gene action. Figure 13 displays the output from the R package and the distribution of the various crosses in the pedigree. This figure shows that while additive gene action is suggested (Mea), it is not the only mode occurring (gray line), hence the suggested confidence of the maternal effect, either within the mother or via the embryo. Dominance gene action is also implicated, however the points on the lower graph of Figure 13 do not follow a strictly stairstep dominance pattern as would be expected. This, again, is likely explained by the influence of the maternal effect. This line cross analysis is informative regarding mode of inheritance; however, it does not suggest the number of gene(s) that are involved in the embryo spacing trait. QTL analysis implicates that

information, but the line cross analysis aids in illuminating the mechanisms at work from the genes involved. Although this line cross analysis suggests a mode of inheritance, it will require further study to confirm that mode.

Recombinant Inbred Lines

Knowing the broad source of the phenotypic anomaly and how the trait is inherited is not enough to be useful for developing measures and potential treatment options. Identifying the genetic components and factors influencing the uneven phenotype will lend to the clarification of a complete schematic defining the migration and embryonic spacing events. Utilizing recombinant inbred lines will provide the phenotypic characterization, and SNP/QTL analysis to help uncover these genetic contributions. Twelve lines of BXH recombinant inbred lines were utilized, and their phenotype characterized as mentioned previously. Measurements were done using ImageJ, a publicly available program developed from NIH [Rasband, 2016]

Figure 14 displays the average CV for each of the 12 recombinant inbred lines, the two parental strains, and the reciprocal F1 crosses. One-way ANOVA analysis was performed on the CV of these lines and strains to determine if there was a significant difference in embryo spacing from either parental strain, particularly B6, as this would have indicated typical uneven spacing for the recombinant inbred lines. The results showed that all of the recombinant inbred lines and the reciprocal crosses were significantly different from the parental strain B6. Additionally, the BXH11 line was identified as an outlier, which is likely due to the low number of uteri that were able to be harvested during uterine horn collection. BXH11 was identified as a low fertility, despite the presence of vaginal plugs and young animals. These CV data and the percentage of uneven spacing for each line or strain was further used in the identification of Quantitative Trait Loci (QTL).

QTL Identification

Using the web-based QTL analysis tool, GeneNetwork, the parental and recombinant inbred phenotypic data was assessed with previously generated SNP data in order to determine a correlation between the two tools. SNPs for the BXH lines were identified from dbSNP and Sanger sequencing performed by UCLA [GeneNetwork, 2001]. Haplotypes for the BXH series were also analyzed alongside the QTL mapping. This haplotype analysis occurred once a chromosomal region of interest was located and was used as an indicator of accuracy of the QTL mapping. If the region of interest, based on the QTL mapping results, was accurate then the haplotypes of the BXH series should match up, at least with the majority of the BXH series, with the C3H parental strain. These QTL and haplotype analyses allowed for the elucidation of regions of interest and consequently genes of interest that could later be tested and/or further validated with the use of RNAseq.

Figure 15 shows the results of QTL mapping using the percent uneven spacing for each line or strain utilized. All 12 BXH lines were included in the first analysis (Figure 15a) along with the parental strains and the F1 reciprocal crosses. Chromosome 15 had a significant likelihood ratio statistic (LRS value) and was the only region in this mapping round to do so. Chromosome 4 showed to be suggestive based on its LRS value, however the frequency of the LRS peak for this region was lower than other non-suggestive regions, indicating that the confidence of this region to be applicable to embryo spacing is low based on the bootstrap test, and is not likely to be extensively involved in embryo spacing. The region of significance on Chromosome 15 was further investigated for markers and to analyze the haplotypes at this region to determine credibility. Figure 15b shows a zoomed in representation of Chromosome 15 at the region of significant LRS. The red and green bars along the top represent the haplotype for each

strain or line and its origin in regard to the parental strain. The red bars represent a homozygous haplotype originating from B6, while a green bar represents a homozygous haplotype of C3H origin. Additionally, the grey bars represent unknown origin, and any blue bars would represent heterozygosity from B6 and C3H origin. What was seen from this haplotype analysis was that the BXH series, with the exception of the outlier BXH11, had a haplotype of B6 origin. The only two strains/lines that had C3H origin were the parental strain C3H and BXH11, the outlier. This indicated bias with respect to the outlier. Therefore, two further analyses/mappings were done to account for this outlier's influence.

The first remapping was done using marker regression, to find the most significant locus on chromosome 15, and composite interval mapping, including the outlier in the mapping group. Composite interval mapping allowed for the masking of the marker closest to the QTL on chromosome 15, which allowed for the detection of other loci that were not seen because of the QTL seen on chromosome 15 due to the outlier, BXH11. This composite interval mapping was, again, done with 1000 permutations and 2000 bootstraps and included the parental data. The marker that was regressed upon was chosen based on the closest marker in the Chromosome 15 region of significant LRS and was denoted as rs3023429, which can be visualized in Figure 15b. After the composite mapping, the region on chromosome 15 no longer had a significant LRS value, as expected, however there was no longer any significant peaks. There was a suggestive peak on chromosome 13, therefore composite interval mapping using the marker mentioned above was again run, but this time the outlier line BXH11, was excluded. The resultant QTL map showed the suggestive region on Chromosome 13 now had a significant LRS value as well a corresponding LRS frequency bar based on the bootstrapping tests. Once zoomed in on this peak, it is revealed that the significant LRS value covers approximately 1.2MB, however the use of the

confidence bars generated from bootstrapping narrows the area to two regions of interest approximately 20kb and 40kb in size. This peak is also located near another region on the same chromosome that had a suggestive LRS value and was next to the corresponding LRS frequency bootstrapping bar (yellow). However, this region is considerably larger at approximately 15 Mb in size and gene rich.

Additionally, a suggestive peak on Chromosome 9 was also observed, but the confidence of this peak is only suggestive and not as strong as the peak on Chromosome 13 based on bootstrapping as can be seen by the low yellow bar of LRS frequency. Besides Chromosome 13 and 9, an interesting anomaly occurred on Chromosome 19. Figure 15d shows that the bootstrapping confidence estimation suggests a QTL region on Chromosome 19, but the data is not as suggestive. This chromosome remained of interest when performing the second interval mapping without the inclusion of the outlier BXH11 to see if this exclusion reveals suggestive or significant association with Chromosome 19. The haplotype analysis for Chromosome 13 revealed that in this region of interest the majority of strains, excluding BXH4 and C57BL/6J, contain the C3H/HeJ haplotype. BXH4, which has an average CV closest to that of C57BL/6J, was the only recombinant inbred line to have the same haplotype as C57BL/6J for this region of interest.

The second remapping effort was utilizing interval mapping with 1000 permutations, 2000 bootstraps, and including the parents, but excluding the outlier BXH11. The results were similar, but less robust and clean in comparison the previously described results of the composite interval mapping. Whereas the two regions on Chromosome 13 are still seen, now only the taller peak is suggestive, leaving the lesser peak below the threshold. Despite the change in significance, the location for the peaks are the same and the orientation of the bootstrapping LRS

frequency bar is the same. Additionally, the same Chromosome 9 peak is present but not significant or suggestive, in fact there was no significant peak, and only a single suggestive peak on Chromosome 13. Interestingly, the LRS frequency bar (yellow) generated from bootstrapping is still present, but the data does not reach the suggestive threshold, but did have an increased LRS value in comparison to the composite interval map. This comparison can be seen by looking at Figure 15d and 15e. Although not suggestive, there was an increase in LRS values, from the composite interval map, for peaks on Chromosome 4 and 6, and stable non-suggestive peaks on Chromosome 8 and 11. These may not be significant or suggestive, but it may lead to the prospect that there are many genes involved in embryo spacing, that might require the use of knockout models to explain the mechanisms involved and potentially controlled by those genes. Differential expression through RNAseq could also illuminate the influence, if any, of these lesser peaks, as well as, the more notable suggestive and significant peaks such as the ones on Chromosome 13.

Investigation of the genes revealed two long intergenic noncoding RNA genes the of Chromosome 13 (Gm6416 and 4930435F18Rik), as well as 3 unclassified genes (A430106F12Rik, A930041H05Rik, and 5930433N17Rik). These findings provided little information to explain mechanisms behind embryo spacing; two other genes were found in this region that may have greater likelihood of contributing to embryo spacing. Embigin (Emb) and poly (ADP-ribose) polymerase family member 8 (Parp8) were identified in the region of significant LRS values on Chromosome 13 [GeneNetwork, 2001; Finger, 2018; Smith 2017]. Embigin (*Emb*) has been identified as a transmembrane glycoprotein that resides in immunoglobulin superfamily. Additionally, this gene is known to be expressed both in the embryo and in the mother. For the embryo, this gene has been implicated in cell-substratum

adhesion that is facilitated by integrins, however little else is known about this gene with regards to pregnancy [Tachikui 1999]. Interestingly, this gene was found to be increasingly expressed in the adult ovary, mammary gland, genital fat pad, and the placenta [Yue 2014]. Whether this supplies evidence for its influence on embryo spacing has yet to be determined.

The other interesting gene identified is poly ADP – ribose polymerase family, member 8 (*Parp8*). The expression of this gene does not seem to be involved directly with pregnancy, but there is known increased expression in the embryo's neurological system during late pregnancy [Yue 2014]. Unfortunately, there are no data on *Parp8* individually, as it has only been studied in massive expression and genomics screenings. Therefore, additional research is needed to determine if this gene is truly influencing embryo spacing.

RNAseq

The RNAseq data, when assembled and analyzed, gives insight into differentially expressed genes originating from the embryo. This information in conjunction with the QTL analysis results provides data and confidence for identification of interesting genes that have the potential to play a role in embryo spacing and/or migration that occurs during this early time period in pregnancy. Figure 16 shows a graph that displays the top 10 up-regulated and the top 10 down-regulated genes in C3H compared to B6. While this information is important, further experimentation is needed to verify these genes' roles, if any, in embryo spacing. This is because this RNAseq looked at whole embryos, which could show up-regulated genes involved in embryo development and not in embryo spacing. Table 4 shows up-regulated genes and down regulated genes, along with the standard errors and p-values for those genes.

CHAPTER III

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The research completed and described in this dissertation helps to further the understanding of the events within the uterus preluding implantation. This research adds data to the direct research was done on embryo spacing and migration, in which the vast majority of that research occurred several decades ago surrounding myometrial contractions and their role in embryo spacing [Böving, 1956, McLaren 1959, O'Grady 1969, Anderson 1978]. More recent studies only mention embryo spacing and migration in passing and often as an afterthought to various stages of implantation, upon which the research is focused [Bibhash 2001, Carson 2005, Carson 2000, Dey 2004, Chen 2013, Celik 2017]. This research provides the evidence towards the advance in the genetic understanding at this stage of pregnancy and provides a foundation for further genetic research around embryo spacing and migration.

Physiological Mechanisms

The objective of this work was to enhance the understanding of the physiological mechanisms of embryo spacing and the genetics regulating this event. Although all possible physiological mechanisms could not be tested, four important factors were examined. Conduction included sex, implantation timing, embryo number, and parity were examined for their potential role or influence on embryo spacing. Implantation and embryo number had been previously examined in rabbits, however it was found that these factors and mechanisms did not affect embryo spacing with regard to the myometrial contractions [Böving, 1956]. Later research by implantation, however this information was done in knockout models where litter size was

reduced due to the gene removed [Chen 2011]. This lack of information on a model that separated litter size and embryo spacing served as the hypothesis to be tested using the C3H inbred strains, which were chosen due to the identification of uneven spacing by a previous graduate student in Dr. David Threadgill's lab. Ultimately, the current work showed found that embryo spacing in the C3H mouse model results in normal and expected litter sizes in comparison to evenly spaced strains, and that no disruption in the timing of implantation occurs. This result showed that embryo spacing and the disruption that may occur in the mouse occurs independently of implantation, and the embryo number per horn is not affected by the degree of spacing, for both even or uneven spacing phenotypes.

In the case of embryo crowding, when 7 or more embryos are present in a uterine horn, the degree of evenness of a uterine horn cannot be assessed due to compact spacing. While, that many embryos in a horn is rare, it does happen in enough frequency to mention as a curious phenomenon that could provide another line of research that would translate into animal agriculture. This could be especially important to the swine industry, where litter size and births per litter are increasingly important and of interest to farmers and producers, with the prime example being the incorporation of the Meishan breed and interbreeding them with other leaner body breeds. Additionally, variation in spacing in uterine horns with a low number of embryos, for example 3, could also be biased in an opposite effect than embryo crowding. Refractory zones between embryos have been suggested, like that of the data and information surrounding the gene *Sfrp2* [Carson 2005]. So far, these suggestions have not been validated, but based on the hypotheses it would be possible that embryo horns with a low number of embryos, 3 or less, are scattered far enough apart during the myometrial contractions that they are not close enough for a refractory zone to be established.

Sex and parity were two physiological mechanisms that had not been examined with regards to embryo spacing, however they are two factors that would have given important insight into how embryo spacing is facilitated. This dissertation examined these two factors and found that neither affected the degree of evenness of embryo spacing. This information showed that the sex chromosomes were unlikely to contribute to the embryo spacing trait and that the sex of the embryo does not affect the spacing of neighboring embryos. With regards to parity, data presented in this dissertation showed that the evenness of spacing is unchanged in either C3H or B6 regardless of the number of pregnancies a female may have. The lack of change in embryo spacing seen with parity suggested that there were no defects in the uterine repair between pregnancies and no fluctuation in the uterine environment between pregnancies during the embryo spacing time point. Ruling out these physiological mechanisms provides valuable insight into the direction of hypotheses to pursue both in genetic inquiry and other physiological mechanisms.

Contributing Genetic Effects

Determining the transmission of the embryo spacing trait from one generation to another can be a bit complex when multiple genes are suspected of contributing to this trait. The generation of a pedigree and observing the phenotypes of each of the crosses provided insight into the lack of mendelian genetics and the likely presence of multiple genes at work for this trait. The additional use of the line cross analysis provided a suggested mode of inheritance coupled with the effect that was likely occurring through the suggested mode. The mode that was suggested based on a significant difference was a maternal effect. However, the line cross analysis is unable to discern between a maternal effect that is having an influence on embryo spacing through the maternal uterus from a maternal effect that is having an influence on embryo

spacing through the maternal genome present in the embryo. Embryo transfer experiments would be able to distinguish between these two hypotheses. There is also the undiscernible possibility that both of these maternal effects are present and interacting or a paternal embryonic effect could be possible. Additionally, there is the suggested presence of dominance gene action, and with lesser certainty additive gene action.

The maternal effect in conjunction with dominance gene action was the likeliest contributing genetic effect. However, the line cross analysis is not able to differentiate between single gene and polygenic mechanism. Hence the need and utilization of QTL analysis, which suggested the likelihood of multiple genes, at least 2 maybe 3, influencing the embryo spacing trait. That information along with the suggested mode of inheritance begins to explicate this trait in a way that previous research has failed to demonstrate. This suggests that the maternal genome plays an important role, as expected, in the embryo spacing trait, and that there is likely at least one gene exhibiting a dominance gene action. This gene action functions in which one allele masks the effect of another allele, therefore, giving differing phenotypic values to the homozygous state of one allele in comparison to the homozygous state of the other allele and the heterozygous state. For example, AA and Aa may exhibit even spacing whereas aa could exhibit uneven spacing, or the reverse could also be true. However, with the variation in the degree and frequency of embryo spacing seen in the crosses shown in this dissertation's pedigree, a dominance gene action is not the only influence likely occurring. There is the suggestion of additive gene action as well, in which the addition of an allele either enhances or attenuates a particular phenotype, in this case it would be embryo spacing. Combine either or both of these with the presence of maternal effects, either though the uterus or through the maternal genome present in the embryos through imprinting and it begins to provide insight into the inheritance of

embryo spacing. Knowing how this trait is inherited will aid in future research uncovering the genes and/or other genetic elements responsible for this trait.

Candidate Regions/Genes of Interest

Identifying regions of interest and the genes included in those regions helped to establish a foundation for further genetic research into the embryo spacing. The QTL analysis in this dissertation identified several regions of interest on three different chromosomes. In order for both regions on Chromosome 15 and Chromosome 13 to be identified as statistically significant, composite mapping, in which the locus on Chr. 15 was controlled for, was utilized. This could indicate a masking effect that the region on Chromosome 15 has over the region on Chromosome 13 or it could also mean that the region of Chromosome 15 is not real. These hypotheses could be tested, potentially, by phenotyping additional BXH11 individuals. This could prove to be difficult since that particular line had reduced fertility, which decreased the number of litters obtained and females actually pregnant. Therefore, additional and alternative methods could be used to investigate these regions without phenotyping this line, including haplotype analysis of other inbred strains, RNAseq, and developing other genetic mouse models.

The use of RNAseq and studying differential expression between C3H and B6 at the blastocyst level aids in the understanding of the differences between the two strains during the embryo spacing time point. Sequencing the RNA of the two parental strains is useful, but it does not explain the entire picture, and all of the genes that are differentially expressed during this timepoint in the blastocysts may not be influencing the embryo spacing trait but may be involved in embryo development. Therefore, additional RNA sequencing on blastocysts from mixed C3H and B6 background and uterine tissue from C3H & B6 would assist in narrowing the region of interest when compared to the original RNA sequencing data from this dissertation study.

Future Directions

This research provides a promising genetic foundation for elucidation of additional mechanisms surrounding embryo spacing and migration. However, it is only a foundation and additional research will be necessary in order to clarify and validate the proposed genes identified in this dissertation. Additional experiments and studies will now be able to be designed and executed to identify genes and mechanisms important to both human and agricultural pregnancies. These experiments might include phenotyping additional inbred strains, performing additional RNAseq, testing genes of interest, and testing other potential contributing physiological mechanisms.

Additional RNAseq

This dissertation utilized RNA sequencing to determine whether differentially expressed genes between C3H/HeJ and C57BL/6J within the embryo during the time period could be associated with embryo spacing. While this information is useful, it does not show the complete picture. There would be additional benefit with sequencing the RNA from pregnant maternal uterine tissue of C3H/HeJ and C57BL/6J true breedings, as well as, RNA from pregnant maternal uterine tissue and embryos from C3H/HeJ females crossed with C57BL/6J males and the reciprocal cross. This would give insight and distinction into whether the maternal uterine environment or maternal imprinting is the predominant motivator of the embryo spacing mechanism. Additionally, the intercross of the 2 parental strains and its reciprocal cross will further illuminate significant differences in expression attributed to embryo spacing. This F1 expression data can be compared to this dissertation's true breeding parental embryo RNAseq data to identify the most likely expression patterns that are associated with embryo spacing and

not just differences in genetic background. These additional expression assays will benefit not only elucidation of the mode of inheritance but also lend further evidence for testing genes of interest.

Strain Testing

The easiest way to determine the frequency of uneven spacing in mice, and potentially other organisms, is to test other strains of mice. The inbred strains of mice would be the simplest to start with, as there are many molecular tools already available and they are expected to be fixed at all loci in their genome. Additionally, with these inbred strains of mice, it would be straight forward to identify the inbred strains that share the same haplotype as C3H in the regions of interest identified in this dissertation's QTL mapping. Due to the vast molecular tools and sequencing data available for inbred mice strains, this identification could be done without the need to genotype mice. Once strains with the same haplotype have been identified, those inbred strains can be phenotyped for the embryo spacing trait. This will give insight into whether the regions if interest identified in this dissertation's QTL mapping are informative or an artifact of limited data points. If the regions of interest prove to be informative, an outbred strain of mice like CD-1 or the use of the inbred collaborative cross mice could be tested for the C3H haplotype, and potentially minimize the region of interest.

The inclusion of the collaborative cross mice and the CD-1 outbred strain will further narrow the region including the genetic mechanisms driving embryo spacing. The increased variation seen in the collaborative cross strains and the CD-1 strain will potentially narrow the region of interest through the use of the C3H haplotype. It will be likely that the collaborative cross strains and/or the CD-1 strain will not contain the entire C3H haplotype of interest, therefore identifying groups of CC strains with the same parts of the C3H haplotype that may

also match the CD-1 outbred strains haplotype. Then testing for the uneven embryo spacing trait in both the CC strains and the CD-1 strain may help to narrow the region of interest, genes of interest, and/or the genetic mechanisms involved in embryo spacing.

Testing Regions/Genes of Interest

This dissertation identified two potential regions of interest, one on Chromosome 13 and another, under different circumstances, on Chromosome 15. The previously proposed experiments will aid in the validation, exclusion, and/or narrowing of one or both of these regions. Once a potential genetic target, either protein encoding or possibly regulatory, a modified mouse model could be developed to verify any targets. This could be achieved using either a knockout model, knock-in model, and/or an overexpression model. Each of these models provide insight into what the target's role, if any, is in embryo spacing. The knockout mouse model is the most common and straight forward of the options. Remove the genetic target and determine if embryo spacing is affected. This is best done in a strain with even spacing, like C57BL/6J. This would ensure that any uneven spacing could be attributed to the modified region of the genome and not something already present. Additionally, using a knock-in model to potentially correct the uneven spacing on a strain like C3H/HeJ would provide similar but reciprocal validation to the target region/gene. Finally, developing an overexpression model of a target region/gene of interest on a strain like C3H/HeJ, would provide insight into the ability of this region to manipulate the degree of evenness of embryo spacing. Overexpressing this same region in a strain like C57BL/6J would also provide insight into this region's ability to disrupt the evenness of spacing with a difference in expression. All of this model observation would provide validation, or lack of, for the proposed regions identified as potentially significant in this dissertation.

Physiological Mechanisms - Contractions

Another interesting and important factor in embryo spacing and migration is myometrial contractions. This physiological mechanism was studied early in the observation of embryo spacing, and to date the general mechanism and the major genes and hormones involved have been explicated. The regions of interest on Chromosome 13 and 15 previously mentioned do not contain the genes already identified in the function and control of those early myometrial contractions. However, there is a possibility that there are additional genes and or regulatory elements that have not been identified and attributed to migration through myometrial contractions. Unfortunately, the ability to reliably and easily measure myometrial contractions in vivo has not been identified. A new protocol would need to be developed that allows for the *in vivo* manipulation and/or measurement of myometrial contractions. Being able to observe and measure myometrial contractions will allow for the identification of the exact timepoint in which the migration and crude spacing of embryos occurs which will then indicate the time point at which embryo spacing switches from crude myometrial contractions to more refined, tightly regulated, endocrine driven embryo spacing. While the endocrine activity has not been validated, there has been previous research that suggests the presence of endocrine activity facilitating embryo spacing and the concept of refractory zones. However, it is important to understand the myometrial contractions and their role, if any, in this dissertation's observations of uneven spacing in C3H/HeJ in order to determine the need to explore if any endocrine activity is also impacting the degree of evenness with regards to embryo spacing.

Implications

This dissertation's research set the foundation for additional studies on embryo spacing and the implications of uncovering the genes and/or regulatory elements could provide valuable

data and eventual treatments for both animal agriculture and human pregnancy. The spacing of embryos has been linked to embryo health in both animals and humans. In animal agriculture the genetic information on embryo spacing would be important for litter bearing species like pigs, especially for those breeds that produce large litter sizes like the Chinese meishan. This breed of pig can produce 15 to 16 piglets per litter and are known for their high rate of embryo survival, making this breed of pig an ideal candidate for further study with regards to embryo spacing [Rothschild, 1996]. Additionally, this data could prove to be valuable with regards to the condition in cattle known as Freemartin Syndrome which involves a twin heterosexual pregnancy in which the fertility of the female twin is lost. This loss of fertility is known to affect the dairy industry in which the heifer/cow is the most important asset and also the costliest. The cause of this infertility is the sharing of nutrients and in turn hormones between the male and female twin. This happens because of a proximity issue of the two twins and their placentas [Twin-twin 2016]. Based on this information, it can be inferred that embryo spacing may play an important role in these twinning events, and understanding the genes involved could provide a genetic screen to identify heifers/cows that are likely to produce these types of detrimental pregnancies.

In addition to its application in animal agriculture, there is potential for this data to become useful for multiple embryo and abnormal human pregnancies. Ectopic pregnancies, placenta previa, and twin-twin transfusion syndrome all display either embryo proximity issues or improper implantation location. Understanding how embryo spacing occurs and the genetic mechanisms at work both during myometrial contractions and closer to implantation will allow for the development of genetic screens that could predict the potential for a woman to experience an ectopic pregnancy, placenta previa, or twin-twin transfusion syndrome. Additionally,

depending on the role of these genes in the physiological mechanisms surrounding embryo spacing there also is potential for supplement and drug development to provide correction during early pregnancy at the time of embryo spacing and migration. This development would have the potential to enhance the efficiency and accuracy of in vitro fertilization and other fertility treatments in not only humans but the animal agriculture as well.

REFERENCES

Anderson, L. L. (1978), **Growth, protein content and distribution of early pig embryos**. Anat. Rec., 190: 143-153. doi:10.1002/ar.1091900112

Bibhash C. Paria, H.S., Sudhansu K. Dey, (2001) **Implantation: molecular basis of embryo-uterine dialogue**. International Journal of Developmental Biology, 45: p. 597-605.

Bean CJ, Hunt PA, Millie EA, Hassold TJ (2001) **Analysis of a malsegregating mouse Y chromosome: evidence that the earliest cleavage divisions of the mammalian embryo are non-disjunction-prone**. Hum Mol Genet 10:963–972

Blackmon, H. and J. P. Demuth (2016). "**An information-theoretic approach to estimating the composite genetic effects contributing to variation among generation means: Moving beyond the joint-scaling test for line cross analysis**." Evolution **70**(2): 420-432.

Böving, B.G. (1956), **Rabbit blastocyst distribution. American Journal of Anatomy**, 98(3): p. 403-434.

Broman, K. W. (2005). **The Genomes of Recombinant Inbred Lines**. Genetics, 169(2), 1133–1146. http://doi.org/10.1534/genetics.104.035212

Carson, D.D., et al. (2000) **Embryo Implantation. Developmental Biology**. 223(2): p. 217-237.

Carson, D.D. (2005) **Uterine sensing of the embryo.** Proceedings of the National Academy of Sciences of the United States of America. 102(24): p. 8397-8398.

Celik O., Acet M., Celik S., Sahin L., Koc O., Celik N. (2017) **Hypothesis: Co-transfer of genuine embryos and implantation-promoting compounds via artificial containers improve endometrium receptivity.** Medical Hypotheses, 103 p. 65-70

Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. (2017) **2015 Assisted Reproductive Technology National Summary Report**. Atlanta (GA): US Dept of Health and Human Services;

Chen Peter J. (2017) **Twin-to-twin transfusion syndrome**. Available from: https://www.nlm.nih.gov/medlineplus/ency/article/001595.htm.

Chen Q, Zhang Y, Peng H, Lei L, Kuang H, Zhang L, Ning L, Cao Y, Duan E (2011) **Transient {beta}2-adrenoceptor activation confers pregnancy loss by disrupting embryo spacing at implantation**. J Biol Chem 286: 4349 – 4356

Chen, Q., et al.(2013) **Navigating the site for embryo implantation: Biomechanical and molecular regulation of intrauterine embryo distribution**. Molecular Aspects of Medicine. 34(5): p. 1024-104

Complications of Pregnancy. (2018). John Hopkins Medicine. Accessed May 8th 2018. https://www.hopkinsmedicine.org/healthlibrary/conditions/pregnancy_and_childbirth/complicati com_of_pregnancy_85,p01198.

Dey, S.K., et al.(2004) **Molecular Cues to Implantation**. Endocrine Reviews. 25(3): p. 341-373.

Dey, S.K. (2005) **Reproductive Biology: Fatty Link to Fertility**. Nature 435, p. 34-35.
Ekelund, C.K., et al., **Dizygotic monochorionic twin pregnancy conceived following intracytoplasmic sperm injection treatment and complicated by twin-twin transfusion syndrome and blood chimerism**. Ultrasound in Obstetrics and Gynecology. 32(6): p. 832-834.

Envigo. (2015). **Teklad Global 19% Protein Extruded Rodent Diet** (pp. 1). Madison, WI: Teklad Diets - Envigo. https://www.envigo.com/resources/data-sheets/2019-datasheet-0915.pdf

Finger JH, Smith CM, Hayamizu TF, McCright IJ, Xu J, Law M, Shaw DR, Baldarelli RM, Beal JS, Blodgett O, Campbell JW, Corbani LE, Lewis JR, Forthofer KL, Frost PJ, Giannatto SC, Hutchins LN, Miers DB, Motenko H, Stone KR, Eppig JT, Kadin JA, Richardson JE, Ringwald M. (2017). **The mouse Gene Expression Database** (GXD): Nucleic Acids Res. 4;45 (D1): D730-D736.

GeneNetwork WebQTL (2001) **RRID:SCR_002388** Date Accessed: April 3rd, 2018 www.genenetwork.org.

GeneNetwork WebQTL (2004) **Information on Groups of Mice** Date Accessed: June 12th, 2018. http://www.genenetwork.org/mouseCross.html.

Hama, K., et al.(2007) **Embryo Spacing and Implantation Timing Are Differentially Regulated by LPA3-Mediated Lysophosphatidic Acid Signaling in Mice**. Biology of Reproduction. 77(6): p. 954-959.

Heston W. E. (1963) **Genetics of neoplasia**, in Methodology in mammalian genetics (Burdette W. J., ed), pp. 247-268. Holden-Day, San Francisco.

Lee KY & DeMayo FJ (2004) **Animal models of implantation**. Reproduction 128 679–695.

Lim, H., et al. (2002) **Molecules in blastcyst implantation: Uterine and embryonic perspectives, in Vitamins & Hormones**. 2002, Academic Press. p. 43-76.

Lim, H. J., & Dey, S. K. (2009). **HB-EGF: a unique mediator of embryo-uterine interactions during implantation.** Experimental Cell Research, 315(4), 619–626. http://doi.org/10.1016/j.yexcr.2008.07.025

Marcum, J.B. (1974) **The freemartin syndrome**. Animal Breeding Abstracts. 42(6): p. 227-242.

Mayo Clinic Staff. (2018) **Placenta Previa**. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/placenta-previa/symptoms-causes/syc-20352768.

McDaneld, T.G., et al. (2012) **Y are you not pregnant: Identification of Y chromosome segments in female cattle with decreased reproductive efficiency1234**. Journal of Animal Science. 90(7).

McLaren, A. and Michie, D. (1959) **The spacing of implantation in the mouse uterus**. Mem. Soc. Endocr. 6,65.

McMillan, W. H. and A. J. Peterson (1999). "**Transuterine embryo migration in recipient cattle**." Theriogenology 51(8): 1577-1586.

MGI (1998, April 9th, 1998). "**Inbred Strains of Mice**." from http://www.informatics.jax.org/inbred_strains/mouse/docs/C57BL.shtml http://www.informatics.jax.org/inbred_strains/mouse/docs/C3H.shtml

Nagasawa H., Miyamoto M., and Fujimoto M. (1973) **Reproductivity in inbred strains of mice and project for their efficient production**. Exp. Animals (Japan) 22, 119-126.

Nephew, K.P., et al. (1992) **Influence of the embryo on intrauterine migration in sheep**. Journal of animal science. 70(6).

NuGEN, (2018). **Ovation® SoLo RNA-Seq Systems.** https://www.nugen.com/sites/default/files/M01406_v3.1_User_Guide%3A_Ovation_SoLo_RNA-Seq_System_1287.pdf

Office on Women's Health, U.S. Department of Health and Human Services, womenshealth.gov, (Feb. 9th, 2018). **https://www.womenshealth.gov/a-z-topics/infertility**

O'Grady, J. E. and P. J. Heald (1969). "**The position and spacing of implantation sites in the uterus of the rat during early pregnancy**." J Reprod Fertil 20(3): 407-412.

Paria, B. C., Ma, W., Tan, J., Raja, S., Das, S. K., Dey, S. K., & Hogan, B. L. M. (2001). **Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors**. Proceedings of the National Academy of Sciences of the United States of America, 98(3), 1047–1052.

Paria, B.C., et al. (2002) **Deciphering the Cross-Talk of Implantation: Advances and Challenges**. Science. 296(5576): p. 2185-2188.

Pope, W.F., R.R. Maurer, and F. Stormshak, (1982) **Intrauterine migration of the porcine embryo: influence of estradiol-17 beta and histamine.** Biology of Reproduction. 27(3): p. 575-579.

Rahima, A. and N.W. Bruce, (1986) **Spacing of conceptuses in the uterine horn and local effects on fetal and placental weights throughout gestation in the rat**. Journal of Reproduction and Fertility. 78(2): p. 741-747.

Rasband, W.S. (2016) **ImageJ**, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016

Richard Behringer, M.G., Kristina Vintersten Nagy, Andras Nagy, (2014) **Manipulating the Mouse Embryo: A Laboratory Manual**, Fourth Edition. 4th ed: Cold Spring Harbor Laboratory Press.

Rothschild, Max F. (1996) **Breeds of Livestock – Meishan Swine**, Iowa State University, Ames. http://afs.okstate.edu/breeds/swine/meishan/index.html/

Ruan, Ye Chun, Zhou, Wenliang, Chan, Hsiao Chang. (2011) **Regulation of Smooth Muscle Contraction by the Epithelium: Role of Prostaglandins**. Physiology, 26(3): 156-170.

Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W. (2012), **"NIH Image to ImageJ: 25 years of image analysis"**, Nature methods 9(7): 671-675, PMID 22930834

Silver, Lee M. (1995) **Mouse Genetics: Concepts and Applications**, 1st ed: Oxford University Press.

Smith CL, Blake JA, Kadin JA, Richardson JE, Bult CJ, the Mouse Genome Database Group. (2018). **Mouse Genome Database** (MGD)-2018: knowledgebase for the laboratory mouse. Nucleic Acids Res. 4;46 (D1): D836–D842.

Sunderam S, Kissin DM, Crawford SB, et al. (2018) **Assisted Reproductive Technology Surveillance — United States, 2015.** MMWR Surveill Summ; 67(No. SS-3):1–28. DOI: http://dx.doi.org/10.15585/mmwr.ss6703a1

Tachikui H., Kurosawa N., Kadomatsu K., Muramatsu T. (1999) **Genomic organization and promoter activity of embigin, a member of the immunoglobulin superfamily** Gene, 240, pp. 325-332

TTTS Foundation (2016) **How Often Does TTTS Occur?** Available from: http://tttsfoundation.org/medical_professionals/what_is_ttts.php

Tucker P. K., Lee B. K., Lundrigan B. L., and Eicher E. M. (1992) **Geographic origin of the Y chromosomes in "old" inbred strains of mice**. Mamm. Genome 3, 254-261.

Wang, H. and S.K. Dey, (2006) **Roadmap to embryo implantation: clues from mouse models**. Nat Rev Genet. 7(3): p. 185-199.

Willams, R. W., et al. (2001). "**The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis**." Genome Biol 2(11): Research0046.

Yue, F., Cheng, Y., Breschi, A., Vierstra, J., Wu, W., Ryba, T., … Ren, B. (2014). **A Comparative Encyclopedia of DNA Elements in the Mouse Genome**. Nature, 515(7527), 355–364. http://doi.org/10.1038/nature13992

Yoshinaga K. (1988) **Uterine Receptivity for Blastocyst Implantation.** Annals of the New York Academy of Sciences 541(1) p. 424-431 https://doi.org/10.1111/j.1749-6632.1988.tb22279.x

Yoshinaga K. (2013). **A sequence of events in the uterus prior to implantation in the mouse.** Journal of assisted reproduction and genetics, 30(8), 1017-22.

APPENDIX A

FIGURES

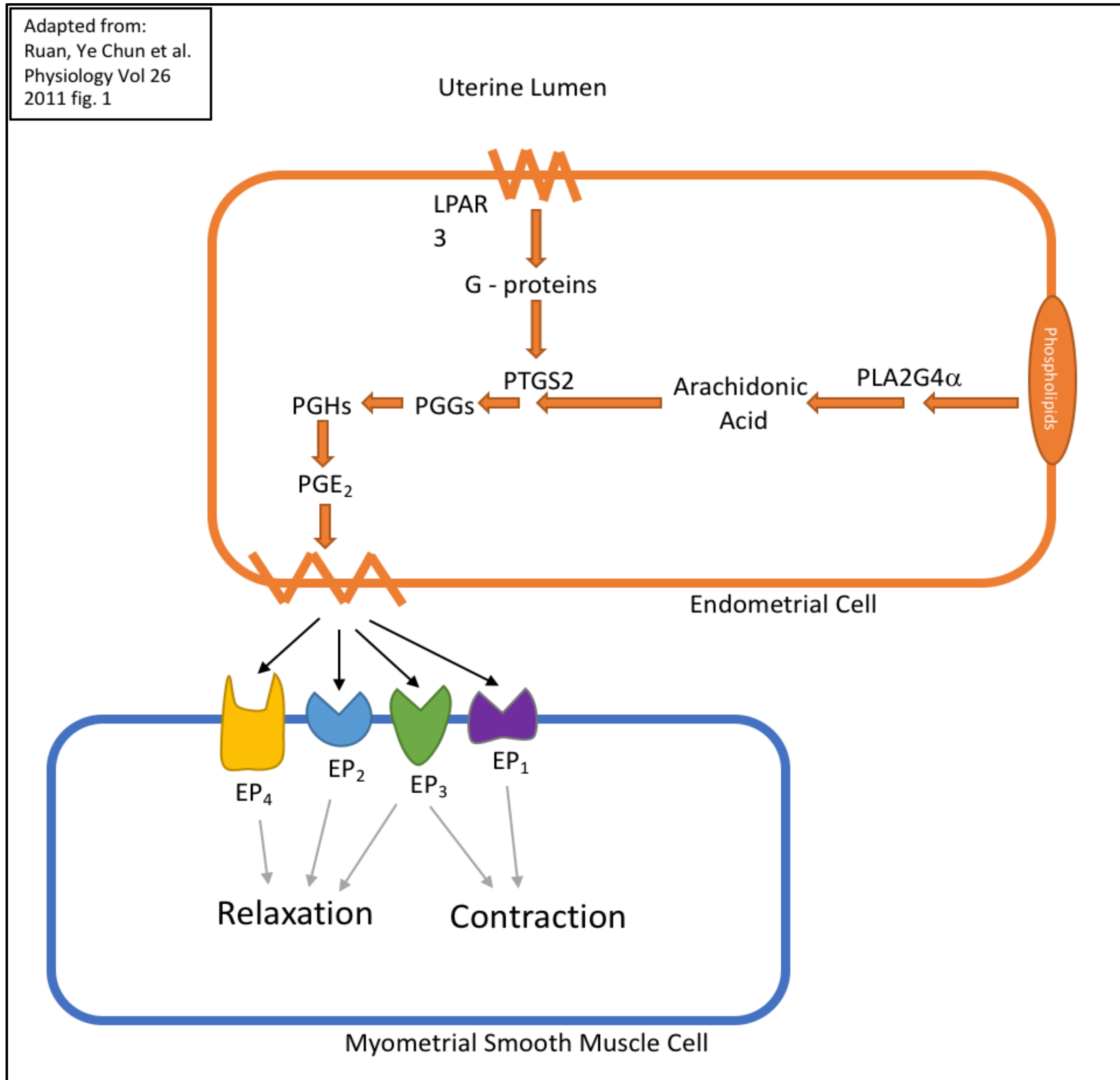


Figure 1. Myometrial contraction pathway adapted (Ruan, 2011) LPAR3 binds LPA from the uterine lumen and produces G-proteins which activate PTGS2. PLA2Galpha converts phospholipids to arachidonic acid, which is utilized by the activated PTGS2 to produce prostaglandin G (PGG). PGG is converted to prostaglandin H and then to prostaglandin E (PGE). PGE activates different prostaglandin receptors on myometrial smooth muscle cell causing the myometrium to either contract or relax.

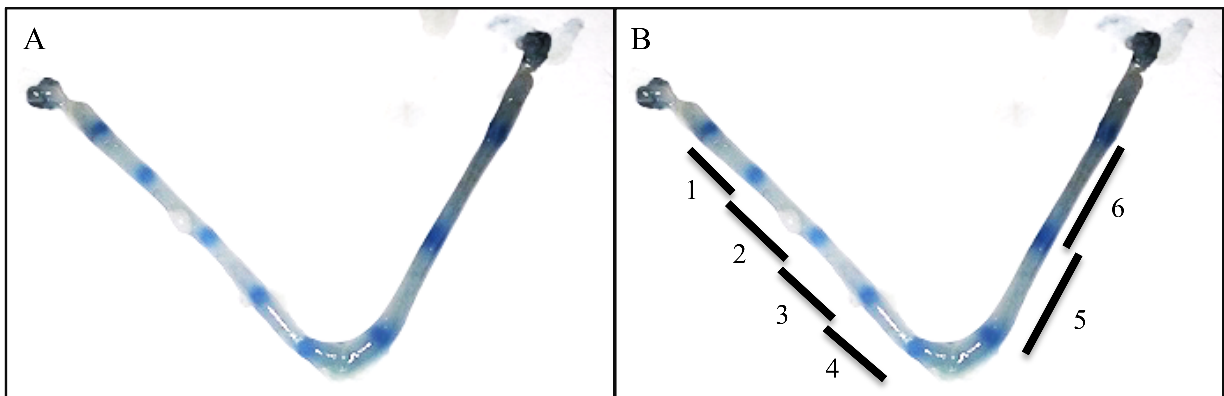


Figure 2. Visualization and measuring blue dye injections A) 1% Chicago Blue Dye injection on a B6 E4.25 uterus. B) Same uterus as A but representing distances (1-6) measured to determine spacing phenotype in each horn. These measurements are taken utilizing ImageJ. Depiction of measurements are shown on the side for example, but were done directly over each horn.

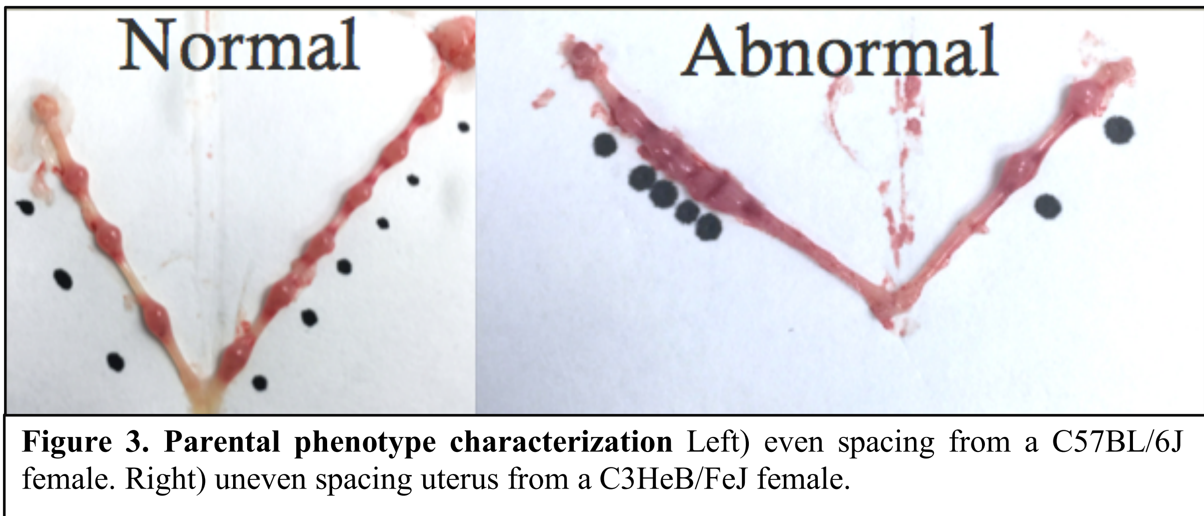


Figure 3. Parental phenotype characterization Left) even spacing from a C57BL/6J female. Right) uneven spacing uterus from a C3HeB/FeJ female.

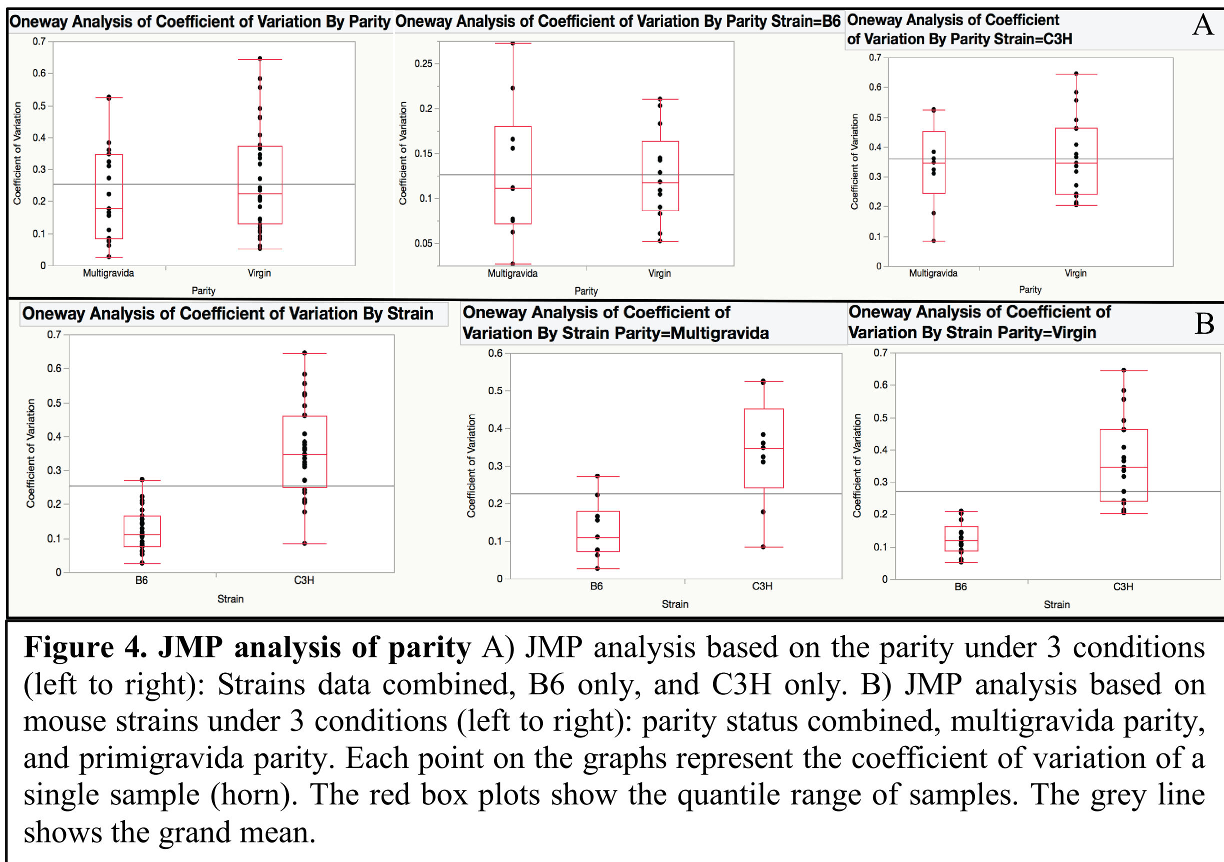
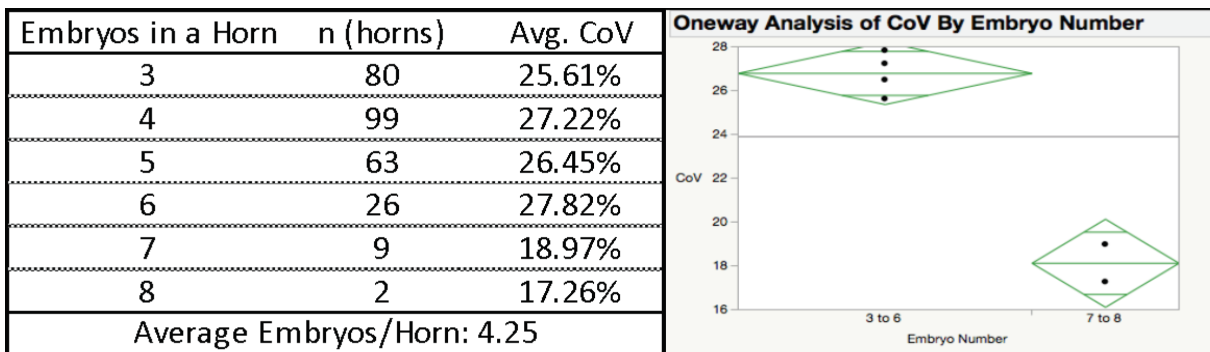


Figure 4. JMP analysis of parity A) JMP analysis based on the parity under 3 conditions (left to right): Strains data combined, B6 only, and C3H only. B) JMP analysis based on mouse strains under 3 conditions (left to right): parity status combined, multigravida parity, and primigravida parity. Each point on the graphs represent the coefficient of variation of a single sample (horn). The red box plots show the quantile range of samples. The grey line shows the grand mean.



Embryos in a Horn	n (horns)	Avg. CoV
3	80	25.61%
4	99	27.22%
5	63	26.45%
6	26	27.82%
7	9	18.97%
8	2	17.26%
Average Embryos/Horn: 4.25		

Figure 5. Uterine horn crowding analysis The table on the left displays the number of horns and the average coefficient of variation for each level of horn occupancy. The JMP image on the right displays the statistical Oneway analysis graph for comparing the average coefficient of variation between the 3 to 6 embryo group and the 7 to 8 embryo group. Each point represents one of the embryo occupancy group's average CV (3,4,5,6,7, or 8). The green diamond shows the mean and the 95% confidence interval. The grey line shows the grand mean. This graph shows a difference in difference between the two groups, however uterine horns with 7 or 8 embryos exhibit embryo crowding, which biases the ability to accurately remark and record embryo spacing degree in these horns.

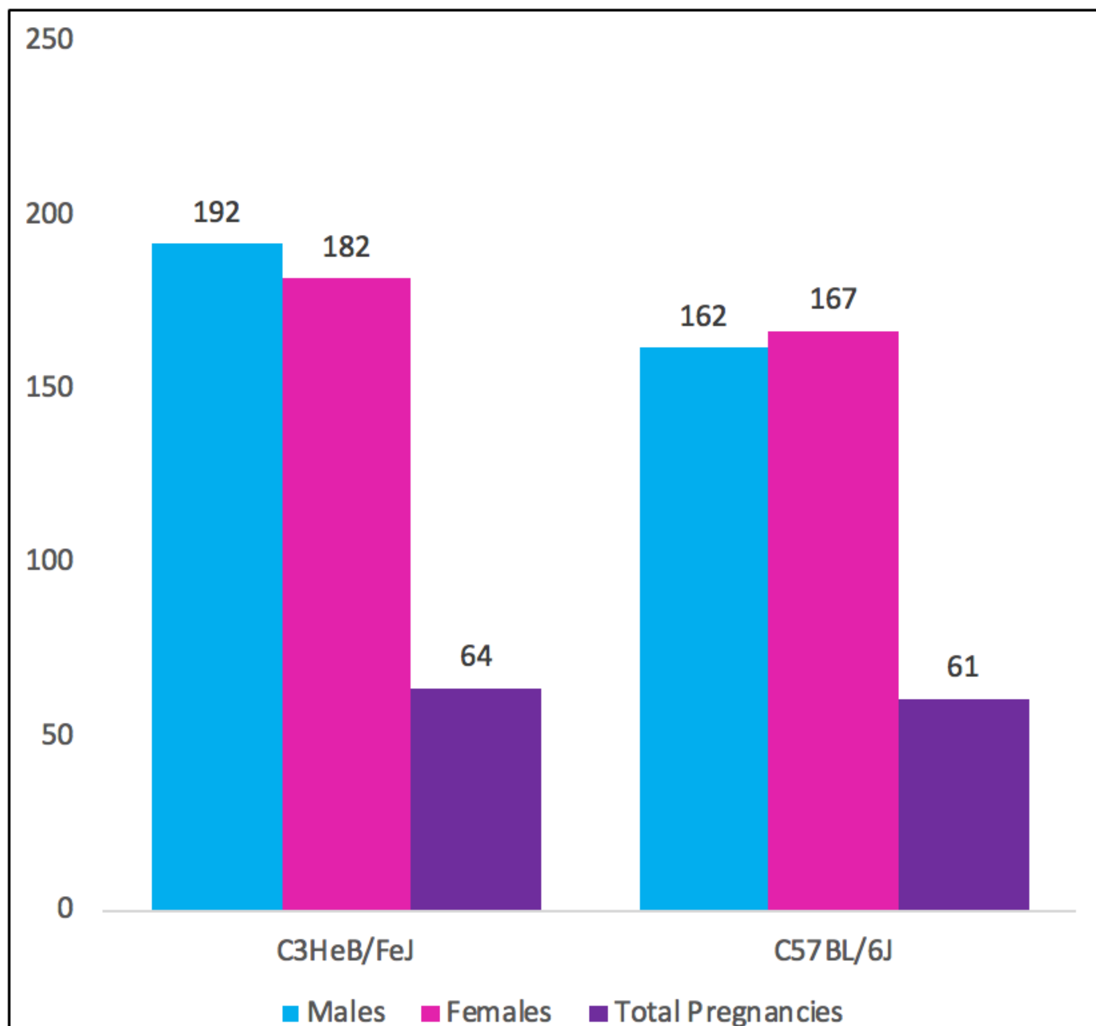


Figure 6. General sex analysis This graph displays the gender distribution for each strain in relation to the total number of pregnancies. The average litter size was 5.84 for C3H and 5.39 for B6. Additionally, there were 51.34% males and 48.66% females per litter for C3H, and 49.24% males and 50.76% females for B6.

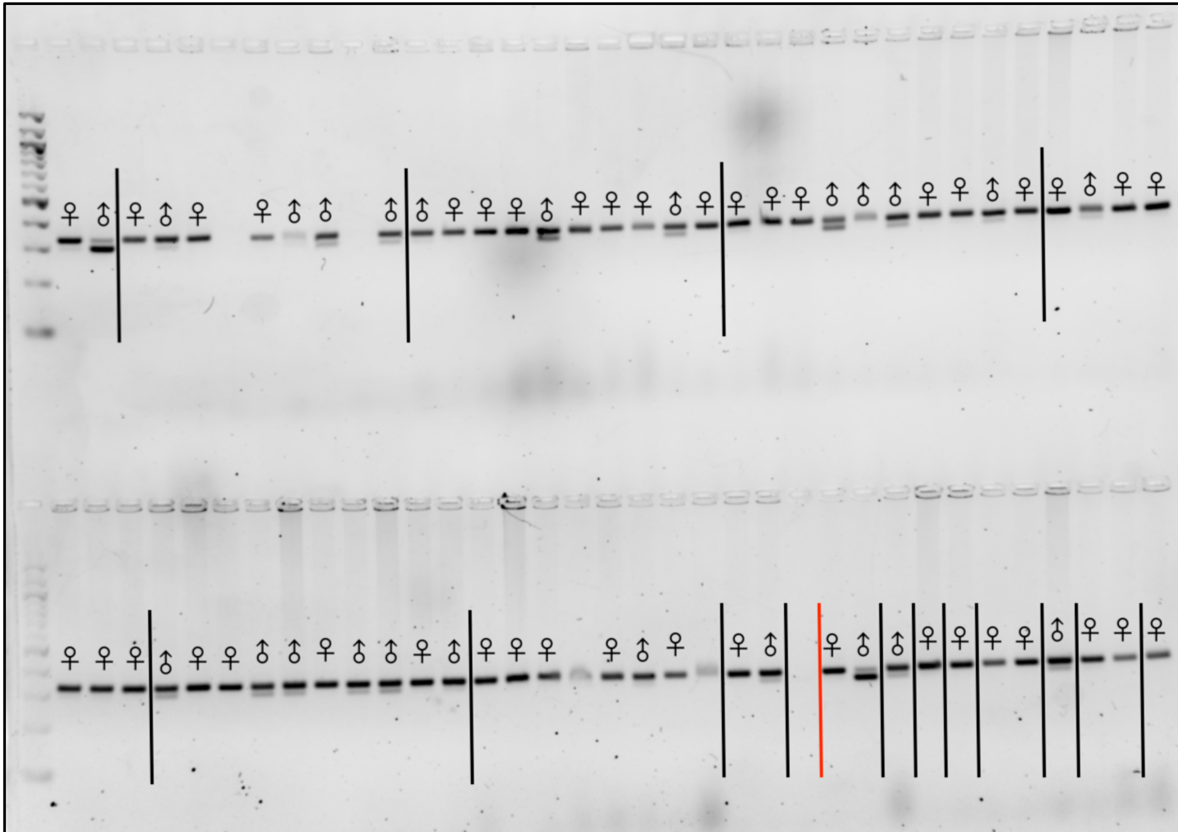


Figure 7. Sex determination of embryos A 3% agarose gel was used to run 149 PCR samples containing embryo DNA collected from the parity study. Additionally, 100bp ladder, 2 positive controls, and 1 negative control were used for each 96 well plate of PCR product generated. Each band or set of bands was assigned a sex based on whether they exhibited a single band (female) or a double band (male). The black vertical lines separate where a litter of embryos ends, and a new litter set begins. A red vertical line indicates the end of the 96 well plate and is always adjacent to the negative control. Additionally, then end of this gel shows samples that were rerun for clarity and verification of sex.

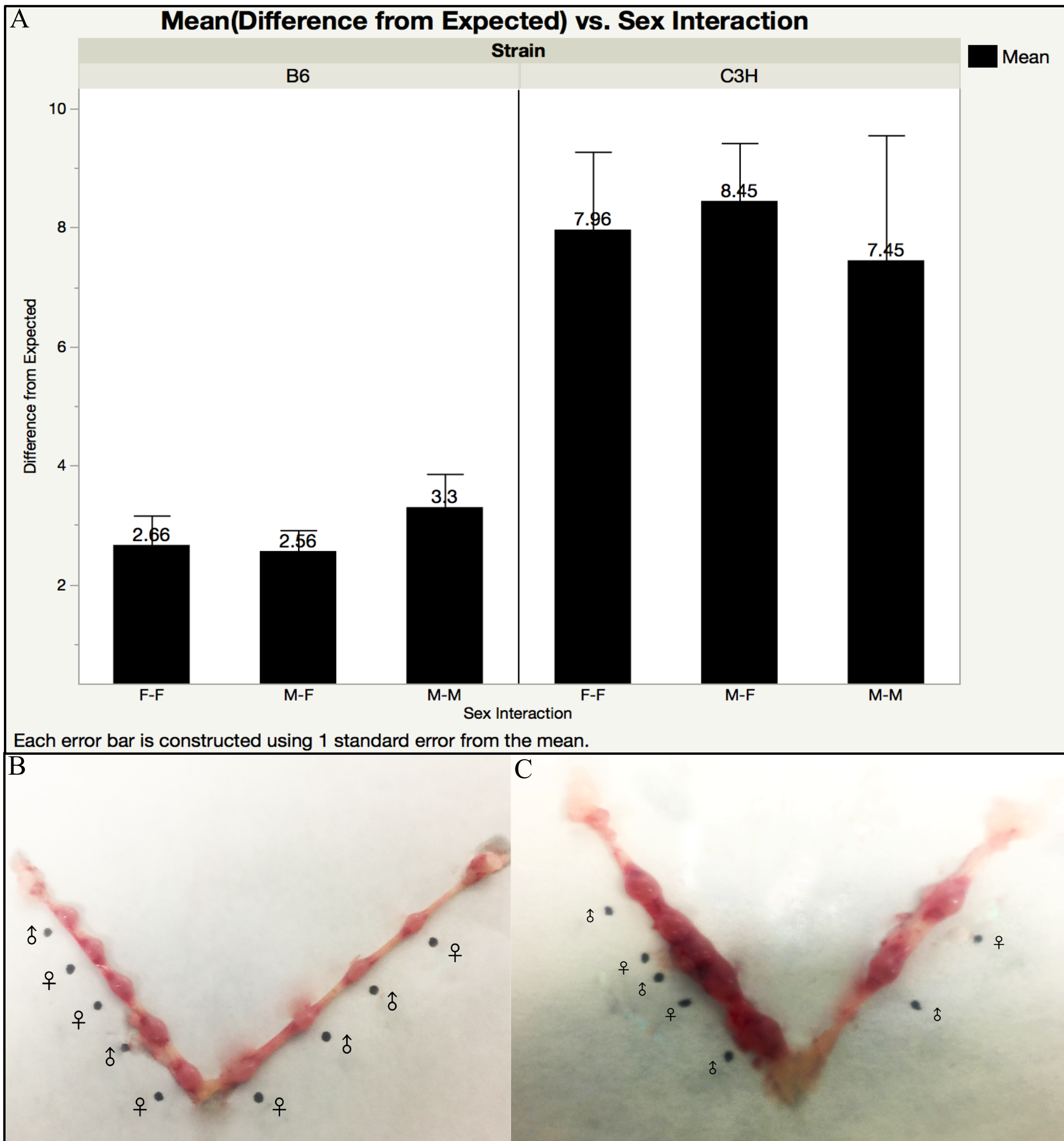


Figure 8. Mean comparisons of sex interactions A) Graph of analysis of sex interactions. Measurements (in % of total length of the horn) were subtracted from their expected measurement (in % of total length of the horn) and then averaged for each sex interaction. Sex interaction is represented as a female embryo adjacent to another female (F-F), female embryo adjacent to a male embryo (M-F), and male embryo adjacent to another male embryo (M-M). Based on the graph, there is no indication of sex interaction contributing to embryo spacing, as no interaction is favored over another. B) B6 multigravida female, whose embryos were collected on E7.5 and sexed via PCR and gel electrophoresis. C) C3H primigravida female, whose embryos were collected on E7.5 and sexed via PCR and gel electrophoresis.

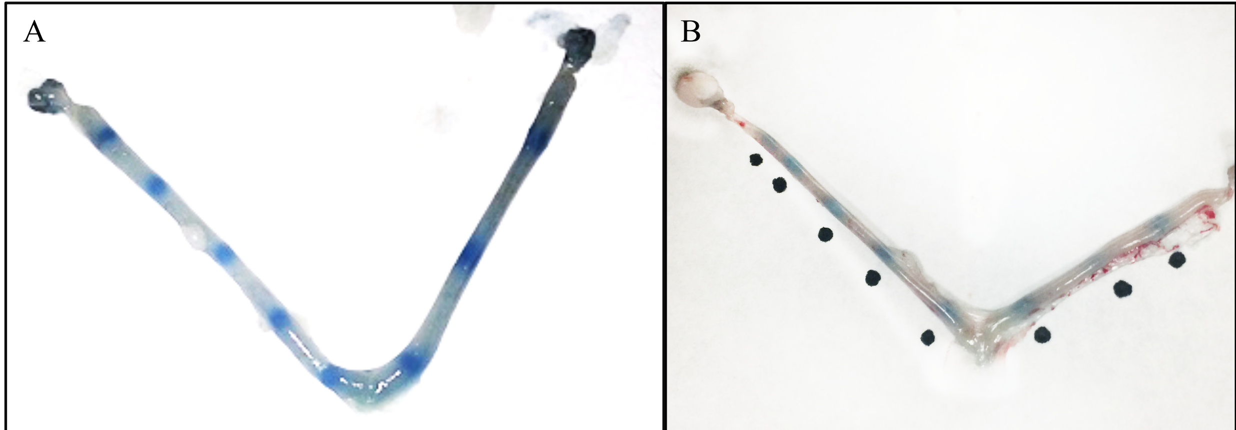


Figure 9. Blue dye injections A) 1% Chicago Blue Dye injection on a B6 E4.25 uterus. B) 1% Chicago Blue Dye Injection on a C3H E4.25 uterus.

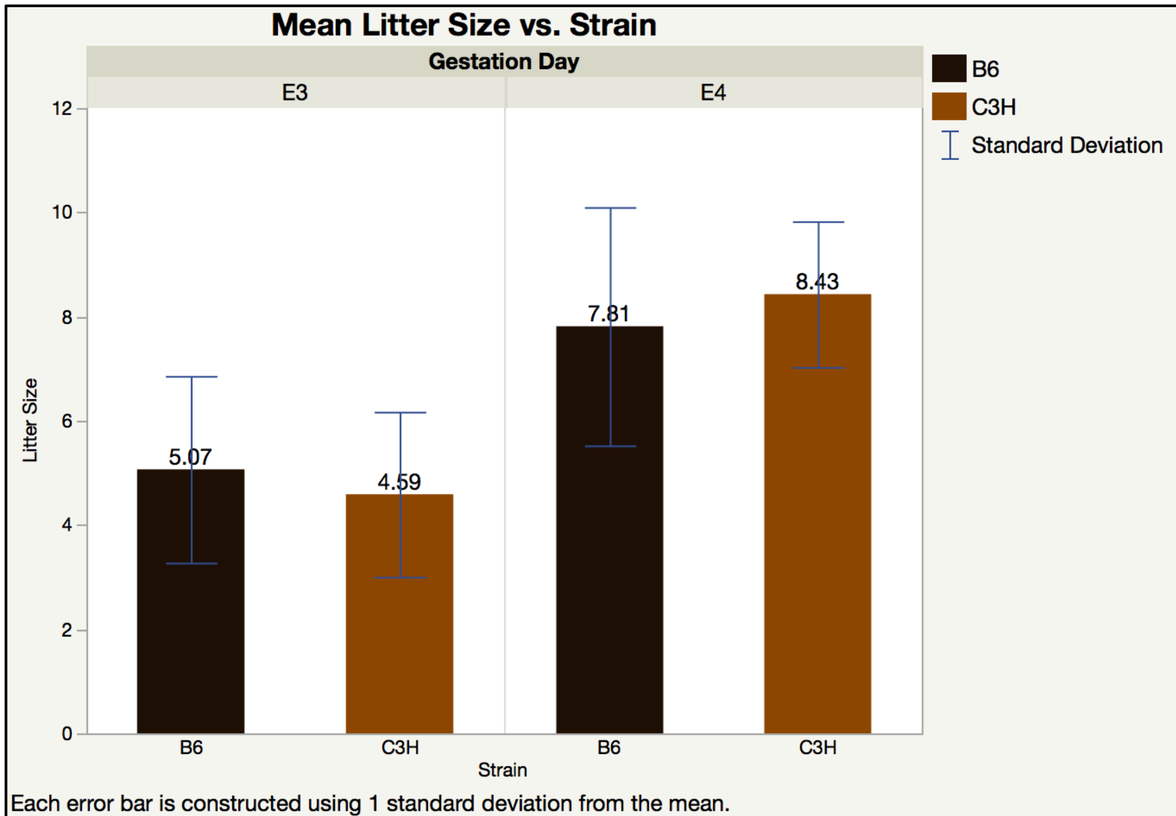


Figure 10. Implantation timing This graph presents a comparison of mean litter sizes for E3 (E3.5 and E3.75) and E4 (E4.25 and E4.5) collected (E3) or blue dyed embryos (E4) with respect to parental strain (B6 or C3H). The results show that litter size did not significantly differ between the two strains for either gestational age. However, there is a difference between the mean litter sizes for both strains between the gestational ages.

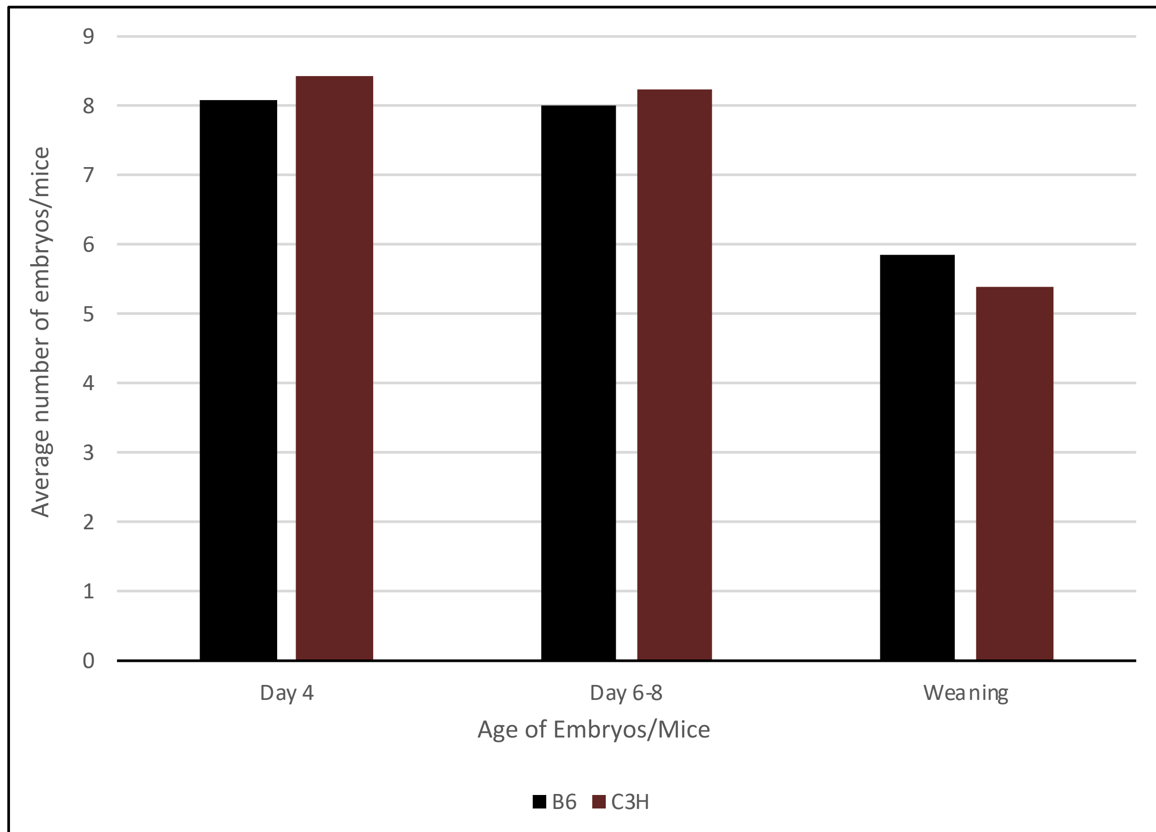


Figure 11. Litter size distribution This graph displays the average number of embryos (or mice in regard to weanlings) per litter for each parental strain of mice. It shows that between E4 and E6-E8 there is no discernable difference in the number of embryos per litter, however at weaning there is approximately a 2 embryo decrease from E8, with a larger decrease seen in C3H.

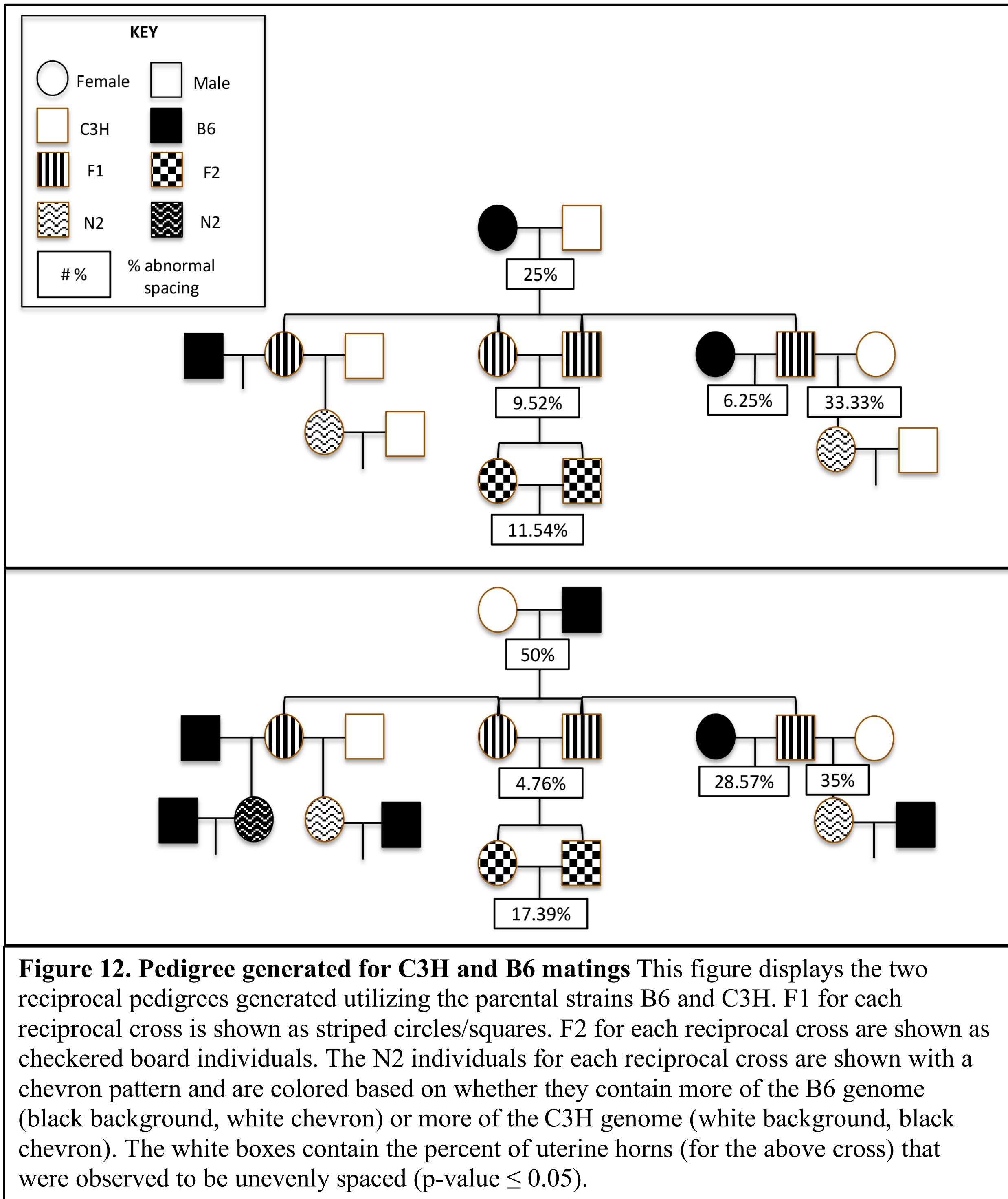


Figure 12. Pedigree generated for C3H and B6 matings This figure displays the two reciprocal pedigrees generated utilizing the parental strains B6 and C3H. F1 for each reciprocal cross is shown as striped circles/squares. F2 for each reciprocal cross are shown as checkered board individuals. The N2 individuals for each reciprocal cross are shown with a chevron pattern and are colored based on whether they contain more of the B6 genome (black background, white chevron) or more of the C3H genome (white background, black chevron). The white boxes contain the percent of uterine horns (for the above cross) that were observed to be unevenly spaced (p-value ≤ 0.05).

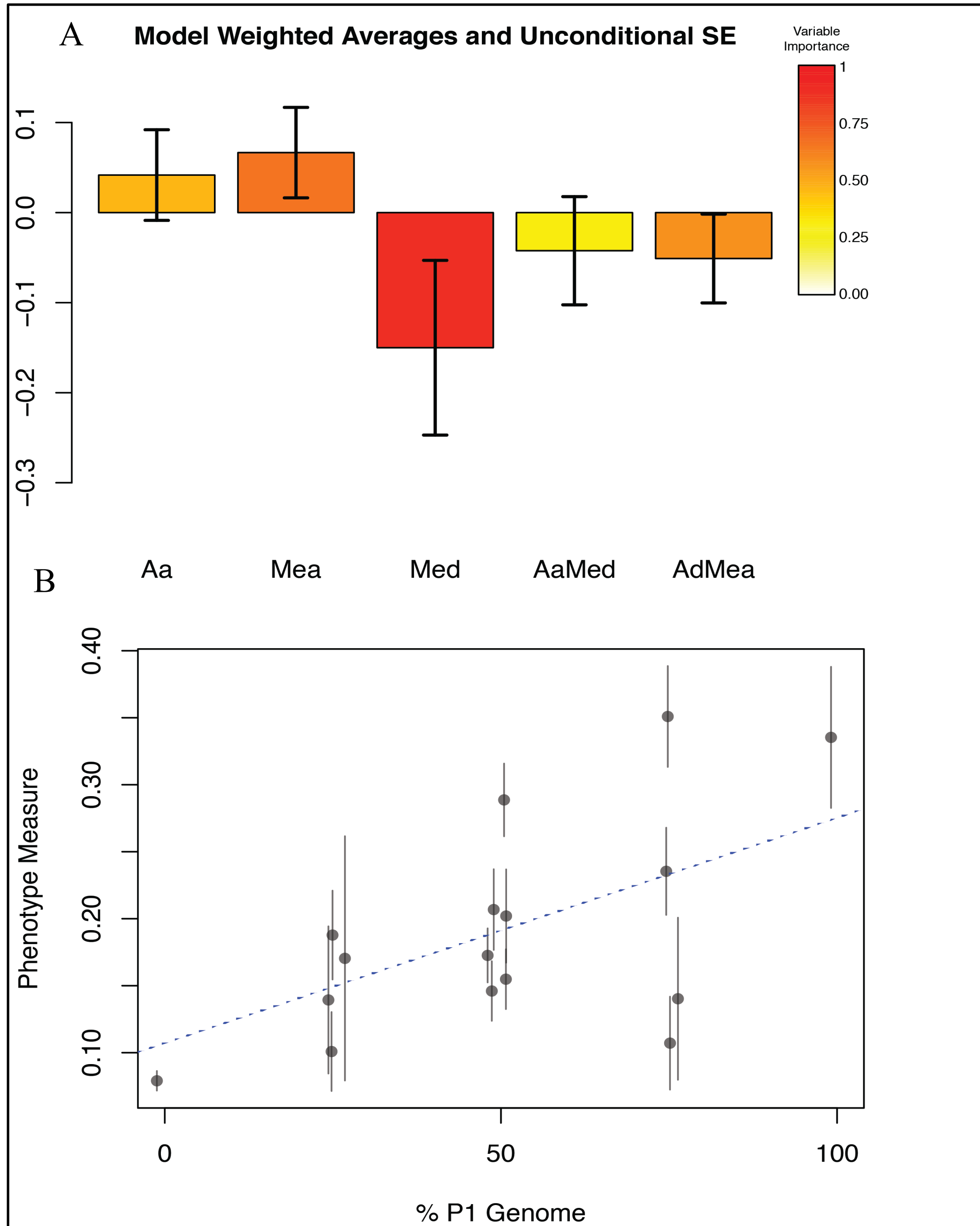


Figure 13. Line-cross analysis utilizing pedigree data A) Inferred composite genetic effects responsible for divergence in embryo spacing. The most likely, which is significantly different from 0, is maternal effect - dominance (Med). Although variable importance of maternal effect - additive (Mea) is lower the confidence interval does exclude zero. B) Comparison of spacing phenotype and the amount (in %) of C3H genome present in the resultant embryos of each cross included in the line cross analysis. The upper most point and the lowest point are the parental strains, C3H and B6 respectively. The blue line represents the expected value if a strictly additive model is used.

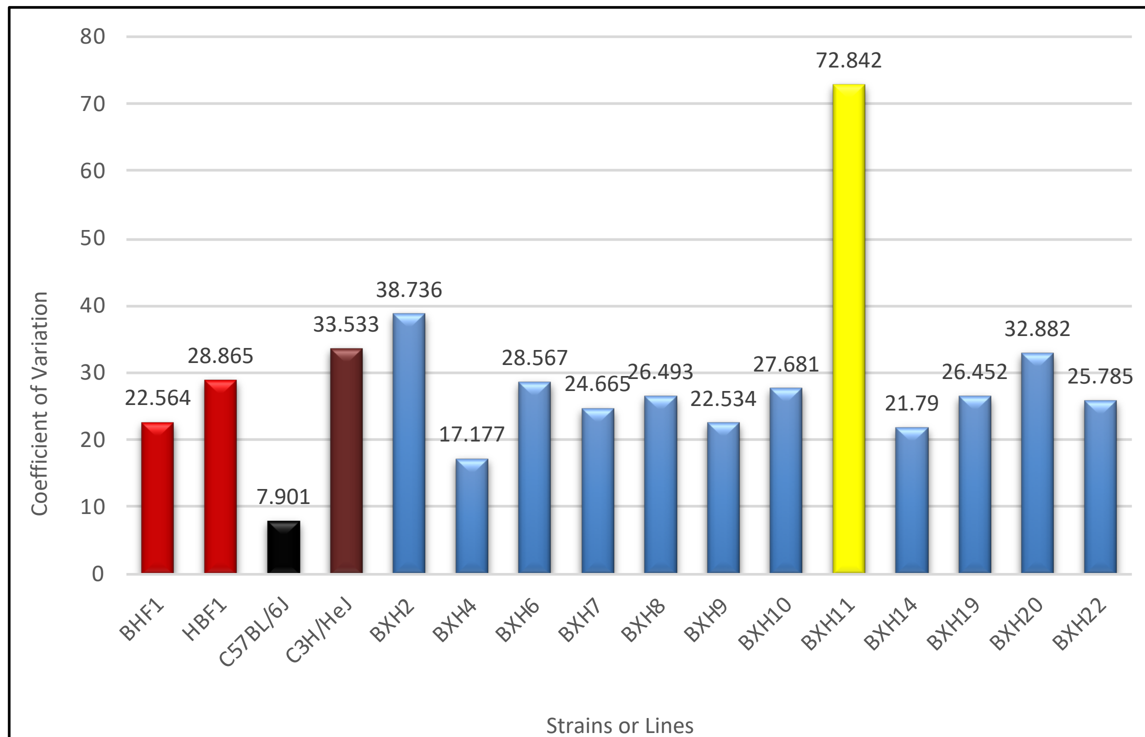


Figure 14. CV comparison of recombinant inbred lines This graph shows the average coefficient of variation for each BXH line, the two parental strains (black-B6, brown-C3H), and the reciprocal F1 crosses of the parental strains (red). The BXH11 (yellow) is an outlier, and likely due to the low number of animals harvested. The remaining lines fall approximately within a range between the two parental strains.

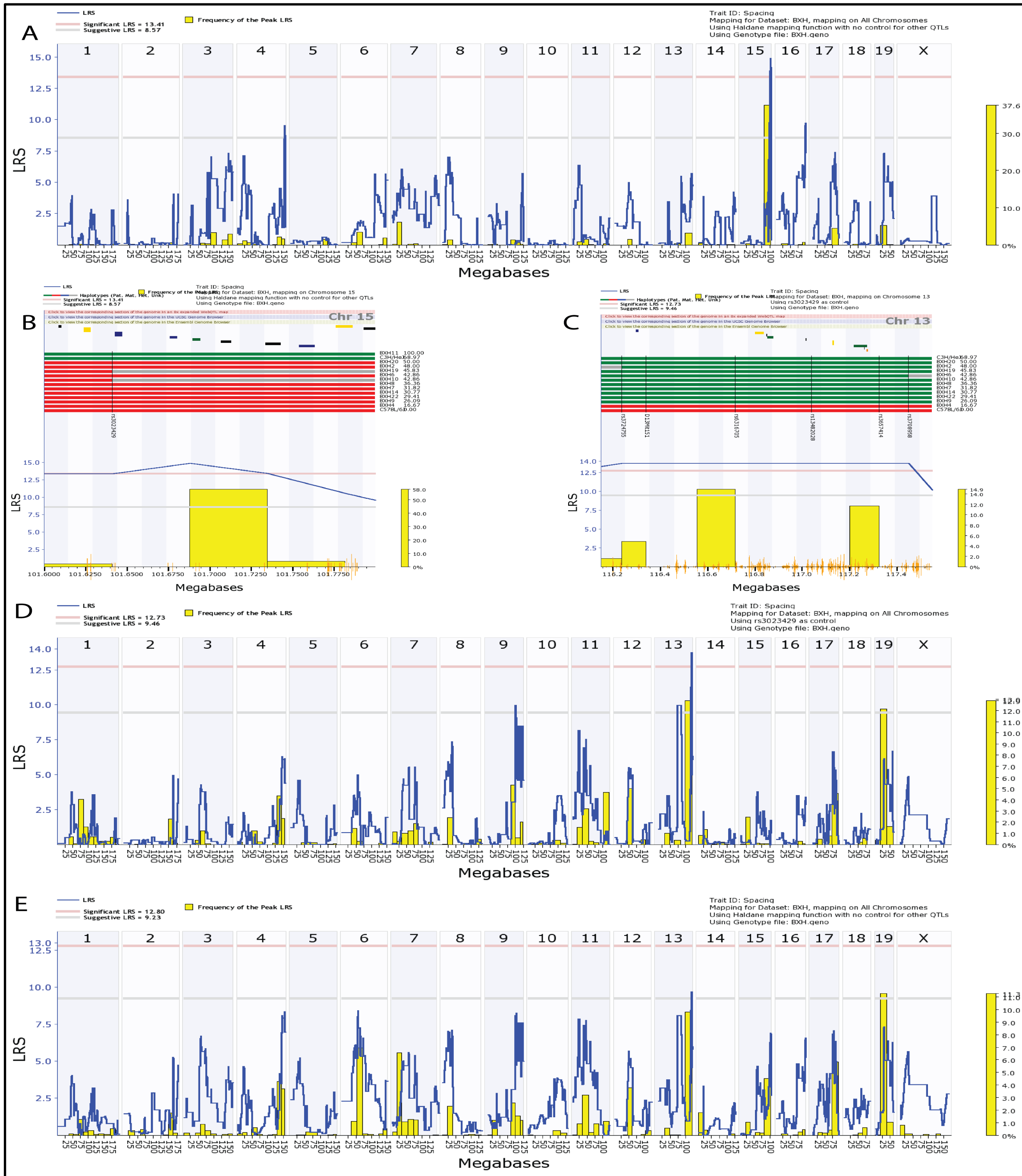


Figure 15. QTL mapping of recombinant inbred lines A) Interval mapping including all 12 recombinant inbred lines, parental strains, and reciprocal F1 crosses. B) Zoomed in view of Chromosome 15 from the interval mapping of A. Haplotypes for each included strain is also seen in red (B6 origin), green (C3H origin), and grey (unknown). C) Zoomed in view of Chromosome 13 from composite interval mapping of D. Haplotypes were again included. D) Composite interval mapping including 11 of the 12 (removed the outlier, BXH11) recombinant inbred lines, parental strains, and reciprocal F1 crosses. E) Interval mapping including 11 of the 12 recombinant inbred lines, parental strains, and reciprocal F1 crosses.

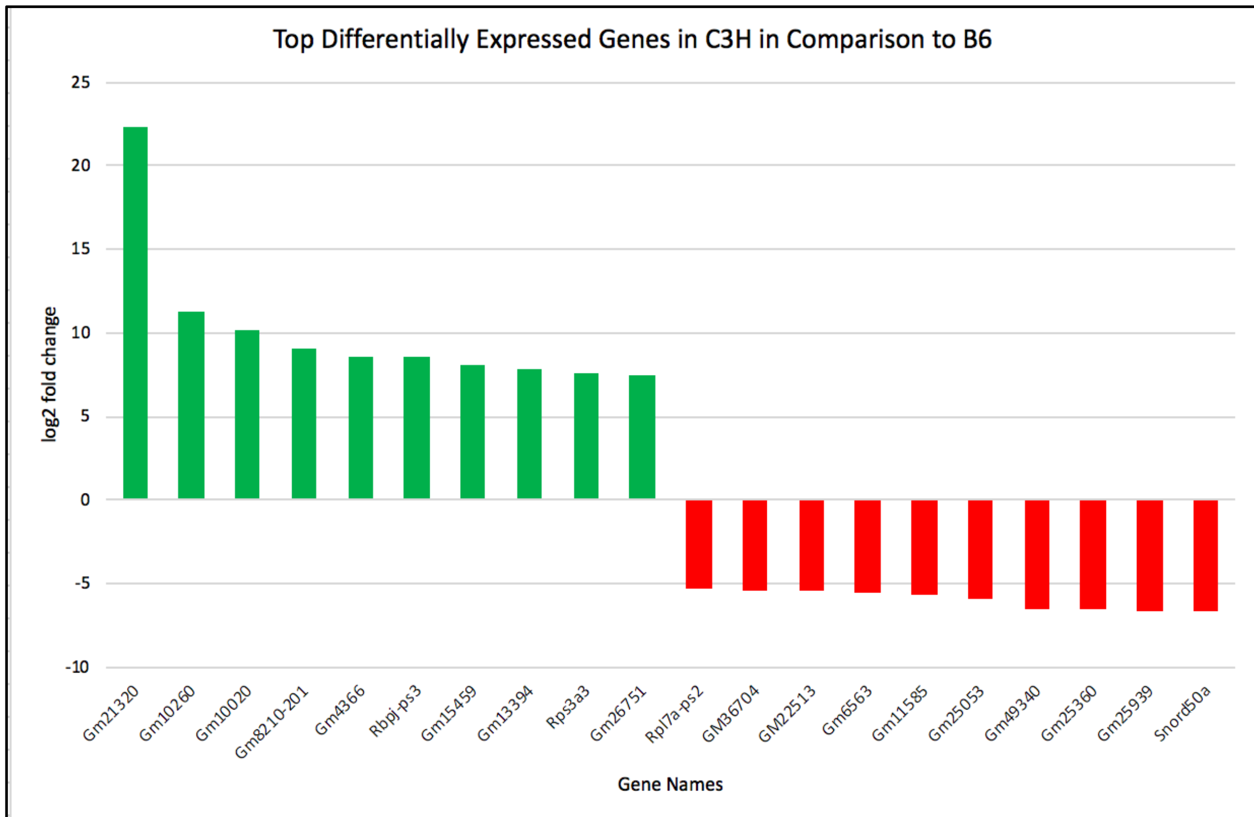


Figure 16. Top differentially expressed genes from RNAseq This figure shows the top 10 up-regulated genes (green) and the top 10 down-regulated genes (red).

APPENDIX B

TABLES

Strains	n (horns)	Avg. p-value	Avg. CV	Avg. # of embryos/horn	Avg. Litter size
B6 primigravida	13	0.6315	0.1253	4.23	7.857
C3H primigravida	19	0.01657	0.3717	4.68	8.091
B6 multigravida	11	0.7032	0.1961	5.18	8.143
C3H multigravida	9	0.0230	0.3370	4.66	8.4

Table 1. Parity testing This table shows the results of the analysis on the phenotype testing primigravida females and multiple pregnancy females to determine if any significant difference is present. The n number represents the number of horns sampled, and the average p value shows the average significance level for each strain and their corresponding parity. Additionally, the average coefficient of variation for each strain and their parity is listed. The average number of embryos per horn and the average litter size is also displayed.

Crosses	**n (# of horns)**	**Avg. CV**	**% uneven spacing****	**% trending uneven spacing***
B6♀ X **C3H**♂ [*BC*]	24	0.2256	25%	0%
F1♀ [*BC*] x **F1**♂ [*BC*]	21	0.1460	9.52%	14.29%
F2♀ (F1♀[*BC*] x F1♂[*BC*]) x **F2**♂ (F1♀[*BC*] x F1♂[*BC*])	26	0.1726	11.54%	3.85%
F1♀ [*BC*] x **B6**♂	6	0.1418	16.66%	0%
F1♀ [*BC*] x **C3H**♂	10	0.1155	0%	10%
B6♀ x **F1**♂ [*BC*]	16	0.1534	6.25%	12.5%
C3H♀ x **F1**♂ [*BC*]	24	0.2479	33.33%	12.5%
N2♀ (F1♀[*BC*] x C3H♂) x **C3H**♂	9	0.1428	11.11%	0%
N2♀ (C3H♀ x F1♂[*BC*]) x **C3H**♂	8	0.3183	25%	25%
C3H♀ x **B6**♂ [*CB*]	20	0.2887	50%	15%
F1♀ [*CB*] x **F1**♂ [*CB*]	21	0.1548	4.76%	9.52%
F2♀ (F1♀[*CB*] x F1♂[*CB*]) x **F2**♂ (F1♀[*CB*] x F1♂[*CB*])	23	0.2019	17.39%	0%
F1♀ [*CB*] x **B6**♂	7	0.1099	0%	0%
F1♀ [*CB*] x **C3H**♂	3	0.1018	0%	0%
B6♀ x **F1**♂ [*CB*]	21	0.2095	28.57%	0%
C3H♀ x **F1**♂ [*CB*]	20	0.2630	35%	10%
N2♀ (F1♀[*CB*] x B6♂) x **B6**♂	4	0.1953	25%	0%
N2♀ (F1♀[*CB*] x C3H♂) x **B6**♂	5	0.1674	20%	0%
N2 ♀ (C3H♀ x F1♂[*CB*]) x **B6**♂	5	0.2784	60%	0%

Table 2. C3H and B6 pedigree mating and phenotype results The first column represents the various matings performed. The two sections display reciprocal crosses utilizing the parental C57BL/6J(B6) and C3HeB/FeJ(C3H) strains. The second column shows the n number of horns harvested. The third column shows the average coefficient of variation for each cross. The last two columns display the frequency of horns containing unevenly spaced embryos and its degree of significance.

* trending is defined for each horn having a p-value between 0.06 and 0.15.

** uneven spacing is confirmed for each horn with a p-value of 0.05 or less.

Strain / Line	n (# of horns)	Avg. CV	% Uneven spacing*	% trending uneven spacing**
B6	12	0.0790	0%	0%
C3H/HeJ	13	0.3353	61.54%	7.69%
BXH2/TyJ	25	0.3874	48%	0%
BXH4/TyJ	6	0.1718	16.67%	16.67%
BXH6/TyJ	21	0.2855	42.86%	4.76%
BXH7/TyJ	22	0.2467	31.82%	4.55%
BXH8/TyJ	22	0.2649	36.36%	13.64%
BXH9/TyJ	23	0.2253	26.09%	4.35%
BXH10/TyJ	21	0.2768	42.86%	14.29%
BXH11/TyJ	3	0.7284	100%	0%
BXH14/TyJ	26	0.2179	30.77%	11.54%
BXH19/TyJ	24	0.2645	45.83%	0%
BXH20/KccJ	16	0.3288	50%	0%
BXH22/KccJ	17	0.2578	29.41%	5.88%

Table 3. Phenotype results of the BXH series This table displays each parental strain and the BXH recombinant inbred series and the results obtained for these strains from the embryo spacing phenotyping experiments. The second column shows the number of horns harvested and used for phenotyping each strain. The third column shows the average coefficient of variation for each strain. The last two columns show the % of total horns phenotyped that showed significantly uneven spacing (p-value ≤0.05*) or close to significance and different from B6 (p-value between 0.05 and 0.15**)

Gene	log2FoldChange	lfcSE	p-value
ENSMUSG00000110439.1	22.28767243	2.975358778	6.85E-14
ENSMUSG00000069117.4	11.27918331	0.816586048	2.14E-43
ENSMUSG00000057262.2	10.19242607	0.915903626	9.14E-29
ENSMUSG00000094497.1	9.095305049	0.784879888	4.73E-31
ENSMUSG00000107383.1	8.579496826	1.290513618	2.97E-11
ENSMUSG00000079575.3	8.535743663	0.9065264	4.69E-21
ENSMUSG00000100801.1	8.01766119	0.994386282	7.45E-16
ENSMUSG00000083773.4	7.816643314	1.086070952	6.15E-13
ENSMUSG00000059751.7	7.621733506	0.556467378	1.06E-42
ENSMUSG00000097542.1	7.429816909	1.659698753	7.58E-06
ENSMUSG00000084383.1	7.421162642	1.466389013	4.17E-07
ENSMUSG00000062353.6	7.192714141	0.547738123	2.17E-39
ENSMUSG00000091957.3	7.154632159	0.454210778	6.68E-56
ENSMUSG00000066878.4	7.143038479	1.323806577	6.82E-08
ENSMUSG00000102500.1	7.132632391	2.975745237	0.016533395
ENSMUSG00000088273.1	6.970012166	0.962600076	4.46E-13
ENSMUSG00000094344.1	6.807624908	0.648385649	8.70E-26
ENSMUSG00000103653.1	6.699455219	1.384588883	1.31E-06
ENSMUSG00000080242.5	6.622954704	0.721442552	4.30E-20
ENSMUSG00000084349.3	6.400279912	0.880936258	3.72E-13
ENSMUSG00000085666.3	6.268236642	0.684637732	5.41E-20
ENSMUSG00000096403.2	6.171563765	0.69452934	6.34E-19
ENSMUSG00000097554.1	6.043838461	0.416060363	8.25E-48
ENSMUSG00000067608.4	5.994979256	0.937182119	1.59E-10
ENSMUSG00000040808.4	5.818289507	0.69732131	7.20E-17
ENSMUSG00000050533.3	5.809975682	2.978123818	0.051070783
ENSMUSG00000062611.4	5.745070581	0.817718716	2.13E-12
ENSMUSG00000098915.1	5.697120561	0.631083292	1.76E-19
ENSMUSG00000061897.5	5.678407245	1.165950767	1.11E-06
ENSMUSG00000066478.3	5.580928357	0.976643818	1.10E-08
ENSMUSG00000115379.1	5.394096562	2.440657446	0.027098236
ENSMUSG00000069083.4	5.299095405	2.97980504	0.075348641
ENSMUSG00000072968.4	5.278760406	1.060022097	6.36E-07
ENSMUSG00000101111.1	5.214382724	1.72138226	0.002452157
ENSMUSG00000083458.1	5.208611663	0.924123521	1.74E-08
ENSMUSG00000025193.14	5.20589934	0.316180523	6.55E-61
ENSMUSG00000101939.1	5.10038243	0.487790622	1.37E-25
ENSMUSG00000059040.4	5.028368128	0.61844851	4.27E-16

ENSMUSG00000093456.7	4.992241941	1.420634521	0.000441258
ENSMUSG00000071772.13	4.947677693	1.318636757	0.000175348
ENSMUSG00000038859.7	4.929903201	0.499002896	5.11E-23
ENSMUSG00000103880.1	4.903723237	0.986307579	6.63E-07
ENSMUSG00000078480.3	4.871745808	0.881707071	3.29E-08
ENSMUSG00000115293.1	4.808320616	2.155172576	0.025677115
ENSMUSG00000081455.1	4.744698151	1.424074218	0.000862933
ENSMUSG00000082645.3	4.704862047	2.447638114	0.054579977
ENSMUSG00000078611.3	4.683495289	1.071984241	1.25E-05
ENSMUSG00000085342.1	4.598541644	1.501758371	0.002197864
ENSMUSG00000072324.4	4.574791384	0.504942346	1.30E-19
ENSMUSG00000113482.1	4.540607622	1.473991507	0.002066643
ENSMUSG00000023046.6	4.462734568	0.68020934	5.35E-11
ENSMUSG00000075391.6	4.446204765	0.620761318	7.92E-13
ENSMUSG00000098170.1	4.371098458	1.553819087	0.004906148
ENSMUSG00000084335.1	4.368412221	2.984900242	0.143329632
ENSMUSG00000080330.1	4.33264749	2.985168699	0.146670967
ENSMUSG00000095596.1	4.310738377	2.644585092	0.103096328
ENSMUSG00000113625.1	4.296203064	1.253056808	0.000606752
ENSMUSG00000104217.1	4.295894523	2.985451535	0.150166679
ENSMUSG00000099762.3	4.292266633	1.211723551	0.000396681
ENSMUSG00000092534.8	4.29213146	2.985480899	0.150528172
ENSMUSG00000058135.12	4.291455743	0.598397182	7.41E-13
ENSMUSG00000110720.1	4.288834828	1.006807614	2.05E-05
ENSMUSG00000113258.1	4.288500069	2.985509308	0.150877647
ENSMUSG00000080893.1	4.282680696	0.671759589	1.83E-10
ENSMUSG00000082804.3	4.27873685	1.145276987	0.000186982
ENSMUSG00000080994.2	4.270751971	1.545086303	0.005708243
ENSMUSG00000081684.4	4.21867966	1.535029041	0.005991001
ENSMUSG00000074280.6	4.215348355	1.141952872	0.000223066
ENSMUSG00000072731.6	4.200804542	2.986217328	0.159507164
ENSMUSG00000085791.4	4.197130752	2.986247929	0.159876701
ENSMUSG00000024437.6	4.193403913	0.558501281	5.99E-14
ENSMUSG00000026185.8	4.18761555	0.530371331	2.89E-15
ENSMUSG00000091952.3	4.184846335	0.661695551	2.54E-10
ENSMUSG00000097891.1	4.136305683	1.471018459	0.004925513
ENSMUSG00000025784.5	4.130282813	0.674147422	8.97E-10
ENSMUSG00000082319.3	4.115415751	0.423776686	2.70E-22
ENSMUSG00000096049.1	4.089036868	1.707138682	0.016608693

ENSMUSG00000082480.1	4.088538367	0.907791011	6.67E-06
ENSMUSG00000044065.3	4.084385687	0.70700617	7.60E-09
ENSMUSG00000094404.1	4.082742216	1.580676984	0.009797163
ENSMUSG00000074485.1	4.056903475	0.849121852	1.77E-06
ENSMUSG00000107470.1	4.047286891	0.63988861	2.53E-10
ENSMUSG00000029368.10	4.011408526	0.927737047	1.53E-05
ENSMUSG00000112559.1	-4.001307279	1.257393257	0.001461486
ENSMUSG00000091905.3	-4.002253534	2.213947773	0.070646161
ENSMUSG00000094762.1	-4.014806475	1.734965566	0.020664682
ENSMUSG00000086668.1	-4.016593975	1.740500828	0.021014542
ENSMUSG00000100930.1	-4.032851813	1.797985138	0.024897849
ENSMUSG00000097853.1	-4.040722017	2.583702781	0.11783476
ENSMUSG00000110190.1	-4.050072441	0.957128112	2.32E-05
ENSMUSG00000100699.1	-4.061862059	2.991642426	0.174547317
ENSMUSG00000094021.7	-4.06398034	1.461728108	0.005431583
ENSMUSG00000093961.1	-4.079614362	2.991430269	0.172640859
ENSMUSG00000099257.1	-4.092362625	1.154714674	0.000394036
ENSMUSG00000081791.1	-4.095282703	1.598187147	0.010393504
ENSMUSG00000100388.1	-4.115012281	2.988719532	0.168559271
ENSMUSG00000101771.6	-4.117163803	2.199401034	0.061213833
ENSMUSG00000102054.6	-4.127029959	2.87834715	0.15162381
ENSMUSG00000094668.1	-4.130798811	1.339420155	0.002042237
ENSMUSG00000090351.1	-4.135622661	2.990777524	0.166728711
ENSMUSG00000093950.2	-4.14188377	2.990706094	0.166077433
ENSMUSG00000088922.1	-4.14419617	2.99067979	0.165837386
ENSMUSG00000101444.6	-4.14573317	2.536659959	0.102190099
ENSMUSG00000079273.2	-4.148932415	1.419726745	0.003474057
ENSMUSG00000095935.1	-4.212079857	2.989925832	0.158906944
ENSMUSG00000107510.1	-4.218471851	1.214988838	0.000516548
ENSMUSG00000100984.1	-4.246362261	1.3211268	0.001308097
ENSMUSG00000100649.1	-4.254271908	1.16332579	0.000255193
ENSMUSG00000110585.1	-4.269202733	1.046217945	4.49E-05
ENSMUSG00000074447.2	-4.297010477	1.621576009	0.008051616
ENSMUSG00000113365.1	-4.315546654	1.345719311	0.001341877
ENSMUSG00000094044.1	-4.340277847	2.988593992	0.146423528
ENSMUSG00000091281.1	-4.353573867	2.988462428	0.145173277
ENSMUSG00000095528.8	-4.37632164	2.988241748	0.143053582
ENSMUSG00000096363.1	-4.380066719	2.988203825	0.142706652
ENSMUSG00000081620.1	-4.397069141	1.661685737	0.008141379

ENSMUSG00000091165.1	-4.405488586	1.088151401	5.15E-05
ENSMUSG00000081587.1	-4.423081704	1.123571361	8.26E-05
ENSMUSG00000090603.1	-4.44958477	1.216670625	0.000255004
ENSMUSG00000093072.1	-4.464777329	1.476412301	0.002493969
ENSMUSG00000099894.1	-4.493565164	2.987147532	0.132504214
ENSMUSG00000061724.5	-4.560317792	1.477854471	0.002030264
ENSMUSG00000114611.1	-4.563253531	1.290031618	0.000404211
ENSMUSG00000094346.1	-4.594306785	1.650042473	0.005363402
ENSMUSG00000094643.1	-4.616869357	1.381297131	0.000830525
ENSMUSG00000083170.1	-4.628326157	2.985995498	0.121138853
ENSMUSG00000096481.1	-4.669694277	1.503730962	0.001900184
ENSMUSG00000043870.9	-4.670032992	2.985659998	0.117781331
ENSMUSG00000087007.1	-4.674810631	1.385383341	0.00073982
ENSMUSG00000078154.3	-4.713480762	2.985320553	0.114362213
ENSMUSG00000072421.4	-4.714519238	1.332193462	0.000401775
ENSMUSG00000077562.1	-4.718454664	1.650990427	0.004263822
ENSMUSG00000100848.1	-4.722071036	2.985254629	0.113695597
ENSMUSG00000099908.1	-4.794453552	1.652486812	0.003715508
ENSMUSG00000101815.1	-4.808725983	2.984610915	0.107141914
ENSMUSG00000112268.1	-4.809997834	1.397058401	0.000575412
ENSMUSG00000101907.1	-4.821551089	1.630631612	0.003107881
ENSMUSG00000095229.1	-4.840567114	2.984383841	0.104810772
ENSMUSG00000061684.5	-4.879790243	1.298891885	0.00017204
ENSMUSG00000100693.1	-4.887609615	2.375326867	0.039623004
ENSMUSG00000081781.1	-4.894124651	1.410091864	0.000518941
ENSMUSG00000071769.11	-4.926518625	0.985934098	5.83E-07
ENSMUSG00000064801.1	-4.932616671	2.427513619	0.042157451
ENSMUSG00000112781.1	-4.959144254	2.983580585	0.096483657
ENSMUSG00000109138.1	-4.984822147	2.272810809	0.028290011
ENSMUSG00000065368.2	-5.223882221	2.982006862	0.079808054
ENSMUSG00000082334.1	-5.301499918	1.357665801	9.43E-05
ENSMUSG00000106283.1	-5.317204137	1.348654937	8.06E-05
ENSMUSG00000114272.1	-5.39786455	1.197101871	6.51E-06
ENSMUSG00000096349.1	-5.494549437	0.51592124	1.74E-26
ENSMUSG00000051255.5	-5.563961611	1.379415824	5.49E-05
ENSMUSG00000082045.4	-5.680142087	2.979884926	0.056629248
ENSMUSG00000095395.2	-5.933960679	2.978963369	0.046376007
ENSMUSG00000069825.12	-6.520005752	2.551305599	0.010601813
ENSMUSG00000094655.1	-6.563614717	0.510220341	7.15E-38

ENSMUSG00000093843.1	-6.624379231	0.437394439	8.16E-52
ENSMUSG00000094263.1	-6.64292734	0.941349338	1.70E-12

Table 4. Differentially expressed genes for embryo spacing This table shows 157 genes that are either up-regulated (log2 fold change 4 and up) or down-regulated (log2 fold change -4 and down). Gene names are expressed in ensemble gene IDs. Additionally, each gene has a corresponding log2 fold change, standard error, and p-value.