GENOMIC DIFFERENCES BETWEEN HIGHLY FERTILE AND SUB-FERTILE HOLSTEIN DAIRY HEIFERS

A Thesis

by

ASHLEY ELIZABETH NAVARRETTE

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

MASTER OF SCIENCE

May 2012

Major Subject: Physiology of Reproduction

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	Thomas E. Spencer
Committee Members,	Charles R. Long
	Clare Gill
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ABSTRACT

Genomic Differences between Highly Fertile and Sub-Fertile Holstein Dairy Heifers.

(May 2012)

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Infertility in dairy cattle remains a major economic loss to dairy producers. Identifying dairy cattle with superior genetic potential for improved fertility would increase dairy farm profitability. Dairy heifers were classified into two groups based upon services per conception (SPC); those animals with a single SPC were determined to be highly fertile and animals with greater than or equal to 4 SPC were classified as sub-fertile. Whole genome association analysis was performed on 20 individual heifers from each group utilizing a 777K highly density (HD) single nucleotide polymorphism (SNP) chip. Genomic data were evaluated utilizing PLINK, a whole genome association analysis toolset, and 570,620 SNP were available for analysis with a total of 39 samples being analyzed. Forty-four SNP were determined to be associated with fertility classification ($P \leq 0.00001$) and were located on *Bos taurus* chromosome (BTA) 2, 4, 9, 19, and 26. The SNP and ranges between SNP were analyzed using BLAST-Like Alignment Tool (BLAT); SNP were associated with 5 candidate genes for reproduction. The SNP on BTA 2 were located within the region coding for the non-imprinted Prader-

Willi/Angelman syndrome 2 (NIPA2) gene, which is involved in gestational magnesium transport. Also on BTA 2, SNP were identified within the region encoding for cytoplasmic fragile X mental retardation 1 (FMR1) interaction protein 1 (CYFIP1). The CYFIP1 gene is involved with the functionality of FMR1 and has been linked to premature ovarian failure in humans. Additionally, 3 SNP on BTA 9 were located near monofunctional C1-tetrahydrofolate synthase (MTHFD1L), which has been linked to neural tube defects during gestation in humans A difference in allele frequency was observed between the two groups for SNP located on BTA19 in proximity to two genes, zinc finger 18 (ZNF18) and mitogen activated protein kinase 4 (MAP2K4). The ZNF18 motif and MAP2K4 were found to be involved in heart development of the early embryo and associated with toll-like receptors (TLR) involved in gonadotropin releasing hormone (GnRH) signaling, respectively. The involvement of one or all of these genes may further explain reduced fertility in dairy cattle.

DEDICATIONS

To my Mom, Janet Theaker,

Your sacrifices were not in vain…

To my grandparents, Waldo and Minnie Navarrette,

Thanks and Gig ‘em!

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NOMENCLATURE

AI	Artificial Insemination
AIS	Artificial Inseminations Per Conception
BLAT	BLAST-Like Alignment Tool
BTA	*Bos Taurus* Chromosome
CL	Corpus Luteum
CNV	Copy Number Variation
CYFIP1	Cytoplasmic FMR1 Interaction Protein 1
EDTA	Ethylenediaminetetraacetic acid
FMR1	Fragile X Mental Retardation 1
GHR	Growth Hormone Receptor
GnRH	Gonadotropin Releasing Hormone
IFN	Interferon
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
ISG	Interferon Stimulated Gene
MAP2K4	Mitogen Activated Protein Kinase 4
MTHFD1L	Monofunctional C1-Tetrahydrofolate Synthase
NEB	Negative Energy Balance
NIPA2	Non-imprinted Prader-Willi/Angelman Syndrome 2
PCI	Phenol-chloroform-isoamyl alcohol
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F 2 Alpha
PLINK	Whole Genome Association Analysis Toolset
POF	Premature Ovarian Failure
QTL	Quantitative Trait Locus
RefSeq	Reference Sequence
SNP	Single Nucleotide Polymorphism
SPC	Services Per Conception
TLR	Toll-like Receptor
WGA	Whole Genome Association
ZNF18	Zinc Finger 18

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CHAPTER I

INTRODUCTION

The dairy industry is affected by various cow and management factors that negatively impact production thereby reducing profitability. One of the major factors is reproductive failure, which continues to plague the dairy industry. According to a survey conducted in 1998 involving 27 New England dairy farms, reproductive failure is the number one reason cows involuntarily leave the dairy farm (20%) followed by mastitis (15%; Bascom et al., 1998). More recently, the National Animal Health Monitoring System (NAHMS, 2007) determined that beyond udder or mastitis problems (79.2%), reproductive problems (78.8%) are the major reason for cows permanently leaving the farm. The inability of dairy farms to achieve reproductive success has resulted in increased calving intervals, days to conception, days in milk and cull rate which are costly to dairy operations.

Fertilization rates following natural service or artificial insemination in dairy cattle can approach 80-90% when male fertility is normal (Inskeep, 2004; Santos et al., 2004;); however, conception rates following a single insemination are closer to 35 to 45 % at days 27 to 31 of gestation (Diskin et al., 1980; Lucy, 2001; Santos et al., 2004). One of the possible reasons for reproductive inefficiencies is the increased genetic selection for higher milk production which has resulted in a loss of positive genetic traits for reproduction. Further research has found that due to the genetic inverse relationship

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between milk yield and reproductive performance, conjoint improvement in both areas can be achieved as long as emphasis on selection for both traits is a priority (Veerkamp et al., 2007).

Following parturition, nutritional requirements dramatically increase due to lactation and most cows enter a state of negative energy balance (NEB; Peter et al., 2009). During NEB, nutrients are diverted away from reproduction to insure adequate nutrition for metabolic activities. Whereas this may have a lengthening effect on the post-partum anestrous period, it does not explain the problem of repeat breeders in the dairy industry and increased incidence of embryonic death. Repeat breeders are defined as those animals that do not conceive following 3 inseminations and were absent of any metabolic or pathological issues that would affect reproduction (Yusuf et al., 2010). One of the explanations for repeat breeding is the elevated amounts of embryonic death observed in dairy cattle. Embryonic death occurring from days 0 to 24 of pregnancy is termed as early embryonic death, whereas embryonic death occurring between days 24 and 42 to 50 is termed as late embryonic death, followed by fetal abortions occurring post day 50 (Santos et al., 2004). Evaluations on embryonic viability illustrate that approximately 50 to 71.9% of embryos were viable 5 to 6 days post insemination (Santos et al., 2004). In addition, elevated rates (~40%) of embryonic loss tend to exist around the period of maternal recognition of pregnancy which occurs between approximately days 17 to 19 after insemination (Mann et al., 1999; Lucy, 2001; Thatcher et al., 1994). Also, the problem of embryo wastage is exemplified in those animals termed repeat breeders which experience appreciable embryonic death by d 7 of

pregnancy (Ayalon, 1978; Thatcher et al., 1994). The exact physiological differences between repeat breeders and those animals that achieve successful conception following one insemination appear to be multifactorial impediments such as uterine health, lack of progesterone, and possible genetic differences (Ayalon, 1978; Bulman et al., 1978; Casida, 1961). Therefore, with the complete sequencing of the bovine genome (Elsik et al., 2009), current research has focused on determining genetic differences between the two types of animals in hopes of gaining the ability to select, by either marker assisted selection or genomic selection, for animals with superior fertility (Pryce et al., 2010). A review of the scientific literature reveals that much emphasis has been placed on identifying markers for desirable fertility in the form of SNP or quantitative trait loci (QTL; Druet et al., 2008; Höglund et al., 2009; Schaeffer, 2006; Schrooten et al., 2004, Schulman et al., 2008).

Genomic testing has gained a great deal of attention in the dairy industry with the development of chips (arrays) with tens of thousands of SNP. A SNP occurs when one single nucleotide is different in an individual's genome compared to other individuals or when compared to that individual's paired chromosome. The SNP can occur in any region of the genome including coding regions, non-coding regions or intergenic regions. Single nucleotide polymorphisms may allow for the possible mapping of the entire genome with markers; with each contiguous pair of SNP there are four possible haplotypes that could be inherited (Schaeffer, 2006). Utilizing SNP markers, estimated effects of each haplotype, and an animal's genome, an estimated breeding value can be calculated based solely on genomics which potentially could eliminate the need for

traditional progeny testing (Schaeffer, 2006). The most important SNP are those that reside in the coding regions and lead to a functional change; however, those SNP that reside in non-coding regions should not be disregarded as they may play a role in gene transcription and gene splicing.

Quantitative traits involve many genes and their interactions that contribute to the overall expression with each gene eliciting a small to moderate effect and generally very few genes exert major effects (Braunschweig, 2010). Quantitative trait loci (QTL) may not be the genes for specific traits but are sections of DNA that are highly correlated to the trait desired. The use of QTL becomes greater in importance when fertility is observed as a complex trait rather than a single gene trait; fertility has a relatively low heritability compared to production traits (Braunschweig et al., 2010).

As previously mentioned, heritability for fertility traits still remains relatively low due in part to the large unexplainable residual variation in statistical models trying to predict traits such as calving interval and pregnancy rate, and the influence of environmental factors. The low percentage of variance in fertility traits is caused by a multitude of factors such as nutrition, environment and management (Veerkamp et al., 2007). Fertility rates generally have low heritability between 1 to 4% (Höglund et al., 2009; Lui et al., 2008) compared to that of production traits such as milk yield ranging from 30 to 40% (Sun et al., 2010).

Copy Number Variants (CNV) being copy number differences in DNA found between two or more comparable genomes may be one of the genetic differences between highly fertile and sub-fertile (repeat breeder) dairy cows. When compared to

SNP, CNVs captured 17.7% of the total detected genetic variation versus SNP detection of 83.6%, but the two types have very little overlap which provides evidence that CNVs may be useful in marker determination especially if paired with SNP detection to more completely detect the entire genome (Stranger et al., 2007).

Reproductive failure is a major economic loss in the dairy industry due to various factors such as environment, nutrition, and management. Although many factors affect fertility, increased incidence of early embryonic mortality occurring between d 8 and 17 of pregnancy due to failure of the embryo to promote maintenance of the corpus luteum (CL) is one of the most prevalent reasons for reduced fertility. Over the past few decades dairy cow selection has focused primarily on production traits, in turn, selecting away from fertility traits. With genetic advances and successful completion of assembly of the Bos taurus genome sequence it may be possible to utilize QTL, SNP, and CNVs to locate genetic markers to determine the genotypic differences between those highly fertile animals and those termed repeat breeder animals.

The objectives of this study were to identify a population of highly fertile and sub-fertile dairy heifers and identify genetic differences for fertility to understand the maternal contribution to early pregnancy leading to a possible explanation of early embryonic death and an explanation for the decline in dairy cow fertility.

CHAPTER II

LITERATURE REVIEW

Causes and Consequences of Low Fertility in Lactating Dairy Cattle

Various factors can be linked to the cause of the continued decrease in fertility seen in U.S. dairy operations such as disease status, nutritional effects, high production, inbreeding, and management. The presence of diseases can have detrimental effects on fertility and achieving successful conception (Fourichon et al., 2000). A meta-analysis was conducted between 1987 and 1998, and found that clinical ketosis, dystocia, and retained placenta resulted in an increase of 2-3 more days until first service and a 4 to 10% drop in conception rate (Fourichon et al., 2000; Hertl et al., 2010). Diseases such as mastitis also have been observed to have a negative effect upon reproductive performance in dairy cattle (Pinedo et al., 2009; Hertl et al., 2010). A retrospective data analysis was conducted in Chile to determine the effect of high linear somatic cell count (LNSCC) on reproductive performance (Pinedo et al., 2009). It was determined that animals with an elevated LNSCC had a 44% decrease in the pregnancy risk and those animals that experienced an elevated LNSCC during the first 90 days of gestation experienced a higher incidence of abortion (Pinedo et al., 2009). Another data analysis was performed utilizing Holstein dairies located in New York to determine the effects of clinical mastitis caused by either gram positive or gram negative bacteria on reproduction (Hertl et al., 2010). Those animals that experienced clinical mastitis 14 to 35 days post artificial insemination service had a decrease in the probability of

conception (Hertl et al., 2010). Also, those animals that developed clinical mastitis as a result of a gram negative infection occurring 1 week post artificial insemination service had an 80% decrease in probability of conception (Hertl et al., 2010).

Lameness can affect reproduction by increasing days to conception by 12 days (Fourichon et al., 2000). It has also been found that lameness can result in a shortened period of mounting by other animals possibly resulting in poor estrus detection by observers (Walker et al., 2008). Reproductive disorders such as metritis, cystic ovaries, and abortion have resulted in a 20% decrease in conception rates, 20 to 30 more days until conception, and 70 to 80 days until conception, respectively (Fourichon et al., 2000). Other causes of infertility can be improper nutrition and the presence of a prolonged NEB following the onset of lactation (Walsh et al., 2011).

Over the years, the dairy industry has progressed towards cows with exceptional milk yield. This increase in production also leads to an increase in dietary intake and altered patterns of metabolism (Gutierrez et al., 2006), which may have led to the sub-fertility experienced on dairy farms (Chagas et al., 2007; Peter et al., 2009). Following parturition, dairy cows enter into a state of NEB due to the increased nutritional demand for milk production. A large proportion of nutrients are diverted away from other areas such as body reserves and reproduction. When attempting to increase nutrient intake to overcome NEB, an increase in milk production is seen without any improvement in reproductive performance (Horan et al., 2004). Cows appear to have a physiological target for body reserves early in lactation so cows with an increased amount of body reserves prior to parturition tend to lose more body condition than cows that are thinner

prior to parturition. In turn, energy stores in late gestation, calving, and early lactation all affect the length of the postpartum anestrous period and the probability of a successful pregnancy (Chagas et al., 2007).

High milk yield has been associated with declining fertility levels since the 1950's with conception rates near 66% in 1951 compared to 40 to 50% since 1975, and 35 to 45% in the last decade (Butler et al., 2004; Loeffler et al., 1999; Yamaguchi et al., 2010). Within the United States there has been a consistent decline in conception rates by 0.45% per year (Lopez-Gatius, 2003). Based upon a trial conducted in Spain, cows that were artificially inseminated or naturally bred resulted in the same conception rates, 37.1% and 37.2%, respectively, (Lopez-Gatius, 2003) illustrating the maternal not paternal contribution to fertility is compromised. Research previously conducted demonstrated the negative effects of high milk yield causing a 0.15% lower rate for first service conception and an increase of 0.32% more services per conception when compared to lower producing cows within their respective herds (Bagnato et al., 1994). Inbreeding also plays a role in decreasing fertility among dairy cattle in the United States and worldwide (Gonzalez-Recio et al., 2007; Lucy et al., 2001; Mc Parland et al., 2007; Sewalem et al., 2006; Weigal et al., 2001). Research conducted in Spain demonstrated that animals with an inbreeding depression of 6.25 to 12.5% resulted in a 1.68% decrease in pregnancy rate; those animals with an inbreeding depression greater than 25% had a 6.37% decrease in pregnancy rates (Gonzalez-recio et al., 2007). Similar numbers have been seen in research conducted in other countries as well (Mc Parland et al., 2007; Sewalam et al., 2006; Weigal et al., 2001). Present levels of inbreeding in the

United States are approximately 5% and are expected to be near 10% by the year 2020 (Lucy et al., 2001). Negative effects of inbreeding have been characterized within the United States and concluded that an increase of 1% in inbreeding leads to 0.17 increase in services per conception, a 2 day increase in days open, and a 3.3% decrease in conception rate (Lucy et al., 2001).

Lastly, a lack of proper farm management and personnel knowledge can influence reproductive success, with the greatest influence usually being the variable of insemination technician (Jamrozik et al., 2005). All of these factors have contributed to the drastic decline in fertility over the past few decades with the inherent rise in high producing dairy cattle (Fourichon et al., 2000). The decrease in fertility not only makes cows less productive and extends the time required to achieve successful conception; it has a negative effect on the economics of the individual farm with a decrease in profitability. The average dairy cow survives only 3 lactations, hence decreasing the amount of available replacement heifers leading to outside purchase of animals (Wathes et al., 2007). Those animals that are the most profitable are those that can combine both high milk production and high fertility (Wathes et al., 2007). Decreased fertility in heifers is linked to heifers that are poorly growing leading to an increase in number of services per conception. In addition, it was found that heifers between 24 and 25 months of age at calving had higher fertility rates, higher maximum total milk yield for the first lactation, and had overall better herd survival over the next 5 years (Wathes et al., 2007). However, the decreasing fertility in cows following onset of lactation is far greater and of much more a concern to the dairy industry than the slight decrease in heifer fertility

(Olsen et al., 2011). The onset of lactation is a major energy requirement leading to improper energy balances which has been found to have a detrimental effect on fertility among other aspects of cow health (Olsen et al., 2011). The incidence of repeat breeders in U.S. dairy herds can range from 17 to 28% (Moss et al., 2002). A cost analysis performed by Bartlett et al. (1986) demonstrates the economic impact of repeat breeder cows over the past 2 decades has increased by as much as 158% if only one insemination is required following onset of lactation in a repeat breeder that was previously classified as a repeat breeder during a previous lactation (Bartlett et al., 1986; Lafi et al., 1992). Costs associated with multiple inseminations include delayed conception costs such as feed costs, extra inseminations, extra veterinary service, and losses due to culling. Lactations with repeat breeders were estimated to experience a loss of $385 (Bartlett et al., 1986). It has also been estimated that there is a loss of $140 per cow with a second insemination, $279 with a third insemination, $429 with 4 inseminations, and $612 with 5 inseminations (Bartlett et al., 1986). Another economic analysis resulted in a mean cost of $994 for repeat breeder syndrome (Lafi et al., 1992). Brooks (1998) conducted a similar study involving repeat breeders and determined that 73% of repeat breeders achieved successful conception with three or less AI services in the next lactation. Taking into account the above figures it becomes apparent that repeat breeders pose a threat to the economic success of dairy farms in the United States. Research yet to be conducted involves analyzing those animals that displayed high or low fertility (repeat breeders) as a heifer and determining if said fertility carries over following the onset of

lactation. Further research is needed in this area to determine any potential patterns or genetic differences between repeat breeders and those animals deemed high fertility.

Early Pregnancy and Maternal Recognition in Cattle

Approximately 5 to 6 days post fertilization, embryos enter the uterus in the morula stage. The next developmental stage is the blastocyst which consists of the differentiation of 2 specific cells types, the inner cell mass and the trophectoderm cells (Cross et al., 1994; Senger, 1999). The cells of the inner cell mass containing gap junctions will eventually comprise the three embryonic germ layers: ectoderm, mesoderm, and endoderm. The trophectoderm cells containing tight junctions can be found around the inner perimeter of the blastocyst and will inevitably form the chorion that serves as part of the fetal-maternal placental interface (Cross et al., 1994; Senger, 1999). Between days 9 to 10 the blastocyst hatches from the zona pellucida and it is at this point that it begins to transform from a spherical shape to that of ovoid. Roughly between days 12 and 14 the now ovoid embryo begins to elongate to what is referred to as a filamentous conceptus (Betteridge et al., 1988; Thatcher et al., 1986). The ovoid embryo starts at a size of about 2 mm and by day 19 will have elongated to approximately 200 mm in length (Thatcher et al., 1986). After day 19 is when the conceptus begins to perform implantation by apposition and adherence of the trophectoderm to the endometrium and more specifically the luminal epithelium. Placentomes have been characterized at first being present at day 30 (King et al., 1979) so prior to their formation the conceptus relies solely on uterine histotroph for survival.

However, prior to the completion of conceptus elongation, maternal recognition must occur which is mediated by bovine interferon tau (bINFτ) secreted from the trophectoderm cells of the conceptus and the critical period for this to occur is between days 15 and 18 (Green et al., 2010; Hansen et al., 1988; Mann et al., 1999; Thatcher et al., 1986; Thatcher et al., 2001). Early pregnancy can be determined based upon the presence of IFNτ in blood serum at approximately day 18 of pregnancy; however detection of IFNτ is difficult due to extremely low circulating levels so detection of the presence of interferon stimulated genes (ISG) on leukocytes is utilized instead (Green et al., 2010). Interferon-tau also plays a role in prostaglandin biosynthesis and signaling during this crucial time (Thatcher et al., 1995). At the time of pregnancy recognition, prostaglandin F2α (PGF2α) serves as the luteolytic factor whereas prostaglandin E2 (PGE2) serves as a luteotrophic factor. The relationship between IFNτ and prostaglandins is not well known. Earlier research conducted involved giving injections of bovine conceptus secretory proteins to cows in diestrus resulting in overall lower plasma levels of PGF2α at the time of normal luteolysis; bovine conceptus secretory proteins was the early name given to substances preventing luteolysis secreted from the conceptus prior to the discovery of IFNτ (Knickerbocker et al., 1986). However, it has been found that IFNτ influences the cell specific expression of cyclooxygenase-2, PG synthases (specifically PGFS and PGE2) and more specifically the varieties of EP2 and EP3 synthases in the endometrium, myometrium and the CL (Arosh et al., 2004). In addition, IFNτ does not cause an increase in receptor expression of PGF2α or those enzymes and transporters necessary for PGF2α action. It has been shown that IFNτ may

directly or indirectly increase PGE2 biosynthesis and PGE2 may play a role in endometrial receptivity (Arosh et al., 2004; Hansen et al., 1988; Mann et al., 1999; Thatcher et al., 2001). Research conducted in sheep by Hansen et al. (1988) determined that ovine trophoblast protein-1 (oTP-1), another name given to what is now referred to as IFNτ, was detected between days 13 to 21 of gestation from the total cellular RNA of embryos. This time is during the critical period of maternal recognition and rescue of the CL (Spencer et al., 2004). Specific prostaglandins have also been shown to have a role in myometrial quiescence and immune function at the fetal-maternal interface. The inadequate production and actions of PGF2α and PGE2 may be a major result of the inability of the endometrium to respond to IFNτ which would inevitably lead to embryonic loss and loss of pregnancy (Arosh et al., 2004; Thatcher et al., 2001). Failures in development during the peri-implantation period account for almost 80% of embryonic losses that occur in farm animals (Cross et al., 1994). As with all placental mammals, conceptus survival also depends on proper apposition and adhesion to the luminal epithelium of the endometrium; immobilization of the conceptus by the endometrium is accomplished by papillae from the conceptus lodging into endometrial crypts (Spencer et al., 2004). In ruminants, implantation is of the synepitheliochorial nature with the formation of placentomes. Placentomes are comprised of 2 parts: the cotyledon contributed by the fetus and the caruncle which is contributed by the uterine epithelium (Senger, 1999). Placentomes are necessary for absorption of the hematotroph nutrition which becomes vital as gestation progresses. As previously stated placentomes first become present about day 30 of pregnancy and continue to develop throughout

gestation reaching nearly 100 in number by term in cattle, but the attachment phase begins between days 21 to 30 with the attachment of the trophoblast to the intercaruncular and caruncular surfaces (Boshier, 1969). Within the interplacentomal region the placenta is that of a diffuse type quite similar to that of swine with the interdigitation of the chorionic villi and the endometrial crypts; these sites are where histotrophic nutrition is absorbed for conceptus utilization.

Implantation in ruminant species is characterized by the formation of a multinucleate synctium, which results in the death of some of the maternal epithelium cells (Boshier, 1969; Spencer et al., 2004). Binucleate giant cells are present in the trophectoderm by days 17 to 19 but they are rarely seen in the luminal epithelium at this time (Spencer et al., 2004). Binucleate cells will eventually migrate and fuse with the uterine epithelium to form trinucleate cells. These trinucleate cells can then fuse with other invading binucleate giant cells to form a synctium (Spencer et al., 2004). This method of implantation allows for a decrease in the number of tissue layers between the fetus and maternal blood supply allowing for intermittent exposure of the maternal capillaries to the fetal epithelium.

In dairy cattle, early embryonic death is a major problem that occurs between days 0 and 24 of pregnancy (Santos et al., 2004). Even higher rates (~40%) of embryonic death occur between days 17 and 19 during the period of maternal recognition and initiation of placentation (Mann et al., 1999; Thatcher et al., 1994). In regards to repeat breeders the greatest amount of death occurs by d 7 of pregnancy with nearly 30% loss (Ayalon, 1978; Thatcher et al., 1994).

Maternal Contributions to Early Pregnancy

During the metestrus and diestrus stages of the estrous cycle the CL on the ovary secretes progesterone and maximum progesterone secretion is achieved during the diestrus stage when the CL is fully mature. Progesterone is a necessary steroid hormone for the maintenance of pregnancy and is supplied predominantly by the CL until day 90 of gestation when the caruncle gains a great ability to synthesize progesterone along with continued progesterone synthesis from the cotelydon (Izhar et al., 1992). High systemic progesterone levels during the post-conception period have been associated with an increase in embryonic growth rate, IFNτ production, and pregnancy rates in cattle (Carter et al., 2008). Insertion of a progesterone releasing device has been found by Carter, et al. (2008) in beef heifers, to result in an increase in the number of viable embryos flushed from a donor as well as an increase in embryonic size seen at days 13 and 16. However, there was no increase in size seen from embryos recovered on days 5 or 7. Observations involving dairy cattle have determined an association between low progesterone levels and low fertility, especially during the early embryonic stage (Stronge et al., 2005). Research observed in ewes suggests that progesterone administration early in the estrous cycle allows for specific changes in the endometrium that accommodate conceptuses at a more advanced stage. This may reflect early synthesis and release of polypeptides stimulated by progesterone that are associated with maintenance of pregnancy (Garret et al., 1988). Furthermore, progesterone supplementation in ewes has resulted in advanced development in blastocysts and it may play a role in the activation of certain genes in the endometrial epithelia such as galectin

15 and components of uterine histotroph (Satterfield et al., 2006). The expression of progesterone receptor within the uterus could play an important role in survival of the peri-implantation embryo. Kimmins et al. (2001) determined through immunoperoxidase staining of bovine uteri that progesterone receptor expression decreased in the caruncular region in pregnant animals, however, did not decrease in the intercaruncular stroma. The intercaruncular stroma is the portion of the endometrium necessary for histotrophic support of the early embryo and throughout gestation. Determining the role of maternal progesterone in embryo survival and development, more importantly the role of genes activated by progesterone, may lead to an improvement in pregnancy rates in cattle.

Another factor that could be responsible for improving early embryo survival in the female reproductive tract is the presence of bovine serum albumin. Bovine serum albumin is a major component of in vitro culture media (Peterson et al., 2003). It has been clearly demonstrated to play a nutritive role to the blastocyst, especially post-compaction, and is the most prevalent extracellular protein in the mammalian reproductive tract (Thompson, 2000).

Early conceptus survival depends highly upon the proper maternal development of uterine glands and secretion of histotroph during this key period in gestation (Gray et al., 2001). Uterine glands found in the intercaruncular area in ruminants are large and branched and secrete a number of different substances such as: enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances commonly classified as histotroph (Gray et al., 2001). In adult animals the uterine wall is histoarchitecturally mature by 2 months after birth however final maturation may not

occur until puberty or even the first pregnancy. During pregnancy, uterine glands exhibit a great deal of hypertrophy and hyperplasia which is presumed to be in response to the increasing demands for histotrophic support by the conceptus (Gray et al., 2001). Previous research has demonstrated that the lack of uterine glands in the ovine uterine gland knockout model results in decreased fertility levels. This is believed to occur due to the absence of uterine secretions that result in the failure of the hatched blastocyst to elongate and further develop (Gray et al., 2001; Gray et al., 2002).

Genes and Pathways Involved in Endometrial Regulation of the Peri-implantation Conceptus Survival and Growth

Several factors have been discovered in the endometrium or uterine lumen and are believed to play a role in peri-implantation conceptus survival, however, for most of these factors vital information regarding certain times of expression and presence of receptors on the blastocyst or endometrium are absent: amino acids, colony stimulating factor 2, chemokine ligand 6, enzymes like acetylglucosaminidase, acetylgalactosaminidase, fucosidase, galactosidase, and mannosidase, fibroblast growth factor 2, gastrin releasing peptide, gonadotropin releasing hormone receptor, growth hormone receptor (GHR), insulin-like growth factor 1 (IGF1), IGF2, IGF binding proteins (IGFBPs), interferon-stimulated gene 15 (ISG15), mucin 16 (MUC16), platelet activation factor, prolacin receptor, PGE2, retinol binding protein, S100 calcium binding protein G, secreted phosophoprotein one, sugars (glucose and fructose), transforming growth factor beta, and uterine serpin (Geisert at al., 1991; Leslie et al., 1991; Harney et al., 1993; Reiswig et al., 1995; Heap et al., 1996; Kirby et al., 1996; MacKenzie et al.,

1997; Schuler et al., 1997; Teixeira et al., 1997; Keller et al., 1998; Wathes et al., 1998; Robinson et al., 2000; Tiemann et al., 2001; Budipitojo et al., 2003; Kojima et al., 2003; McDonnel et al., 2003; Oshima et al., 2003; Arosh et al., 2004; Austin et al., 2004; Emond et al., 2004; Michael et al., 2006 (1); Michael et al., 2006 (2); Singh et al., 2008). Those factors that have been discovered in the post-hatching blastocyst and pre-implantation conceptus are epidermal growth factor receptor, retinol binding protein, and some regulatory genes (Liu et al., 1993; Kleim et al., 1998).

The expression of GHR, IGF, and IGFBP have been observed throughout the female reproductive tract (Giudice, 1992; Hammond et al., 1991; Hammond et al., 1995; Simmen et al., 1993; Spicer et al., 1995). Gene knock-out studies have also been performed in rodents demonstrating the necessary role of the IGF system in conceptus development (Baker et al., 1993). Mice with null mutations of the genes encoding for IGF-1, IGF-2, and type 1 IGF receptor (IGF1R) were observed throughout gestation. It was determined that those embryos with the mutation against IGF-2 production and IGF1R experienced growth deficiencies between d 11-12.5 of gestation (Baker et al., 1993). Insulin-like growth factor 2 was also found to react with an unknown receptor to control placental growth and was deficient within those animals with the null mutation (Baker et al., 1993). Components of the insulin-like growth factor (IGF) axis play an important role in follicular development and more appropriately embryonic development (Kirby et al., 1996). Many reproductive tissues are capable of IGF-1 synthesis allowing the possibility that embryonic growth may be dependent upon autocrine and endocrine derived IGF-1 (Geisert et al., 1991; Giudice, 1992; Green et al., 1995; Hammond et al.,

1991; Hammond et al., 1995; Perks et al., 1995; Simmen et al., 1991; Simmen et al., 1993; Spicer et al., 1995; Stevenson et al., 1994; Watson et al., 1992). Growth hormone may play a role in mediating the localized production of members of the IGF axis due to the fact that GHR is expressed on the bovine CL and an alternate GH receptor is expressed in the CL as well as the endometrium (Lucy et al., 1993; Lucy et al., 1995; Pratt et al., 1995). Insulin-like growth factor 1 and IGF-2 are both expressed in the bovine uterus (Geisert et al., 2001; Kirby et al., 1996) and the conceptus (Wathes et al., 1992). During the embryo elongation phase in porcine, there is an increase in IGF-1 secretion into the uterine lumen (Green et al., 1995; Simmen et al., 1990); in the cow, messenger RNA (mRNA) for IGF-1 does not increase during early pregnancy within the endometrium (Geisert et al., 2001). In vitro studies, IGF-1 and IGF-2 have been observed to have positive effects upon embryonic IFNτ production (Ko et al., 1991) which is a critical protein needed for maternal recognition in ruminants.

Control of reproductive function can be interrelated with actions of IGFBP (Jones et al., 1995). Insulin-like growth factor binding proteins are present on the ovary and within the uterus and have the ability to regulate IGF function (Hammond et al., 1991). Kirby et al. (1996) observed in lactating Holstein dairy cows that IGF-1 mRNA was highest in myometrium and lower amounts were found in the endometrium. Insulin-like growth factor binding protein 2 mRNA was most profuse in the endometrium and IGFBP-3 mRNA was detected in all reproductive tissues but had the least amount in those tissues that had the greatest amount of IGFBP-2 mRNA (Kirby et al., 1996). Cows that were harvested and contained a conceptus also had a higher expression of IGF-1

mRNA than nonpregnant cows (Kirby et al., 1996). It has been observed the GHR expression is decreased in cows treated with GH and administration of GH had no effect on mRNA from IGF-1, IGFBP-1, or IGFBP-3 (Kirby et al., 1996); though this may be a direct effect due to decreased GHR expression. In other research the localization of IGFBP-3 and IGFBP-2 to specific reproductive tissues has been similar (Keller et al., 1998). Concentrations of IGF-1 with in the uterine lumen fluid did not change based upon pregnancy status or stage of cycle (Keller et al., 1998). Variations in expression based upon tissue type and abundance of expression due to the stage of estrous cycle and pregnancy status indicates the potential importance of IGFBP during early pregnancy (Keller et al., 1998).

With fertility being a quantitative trait many of the aforementioned pathways may or may not be involved in maintaining a successful pregnancy. It could be argued that if one or more important pathways are absent or mutated in certain animals, those animals may present with an altered phenotype, in this study, sub-fertile versus highly fertile dairy heifers. Performing whole genome analysis may help to reveal some of the differences between the two phenotypes via SNP associations in or near the aforementioned genes.

Whole Genome Analysis and Heritability of Fertility Traits

The heritability of fertility remains low ranging from 1 to 4%, and fertility traits may have sex-limited expression, or be expressed later in life (Höglund et al., 2009; Lui et al., 2008). Through selection of animals with superior production traits, fertility traits have been compromised. The genetic correlation between milk yield traits and fertility

traits ranges from -0.2 to -0.5 (Höglund et al., 2009). Villumsen et al. (2008) discovered that as heritability decreased so did reliabilities. However, high reliabilities could be obtained for low heritable traits when utilizing the correct model; this indicated that genomic selection of cattle could be useful for selection of low heritability traits (Villumsen et al., 2008). In turn, it may become more important to identify QTL, SNP, and CNV that may allow for genomic selection of dairy cattle for superior fertility prior to progeny testing. Whereas genes controlling fertility in bulls are present earlier in life, traits in relation to daughter fertility are expressed later in life and would benefit the most from genomic selection and the identification of genetic markers (Berglund, 2008). It must be understood that while detection of QTL, SNP, and CNV will be useful; it may only be applicable for a finite number of generations since haplotypes will change over time (Berglund, 2008). Quantitative trait loci relating to fertility have been identified on BTA 3 in relation to nonreturn rate estimated 90 days after A.I. (Druet et al., 2008). Druet et al. (2008) concluded that the fertility QTL found on BTA 3 ($P < 0.001$) had large effects on insemination results.

With the decline in dairy cow fertility, the areas of embryo survival and fertilization rates appear to be of the utmost importance (Khatib et al., 2009). Candidate genes have been identified that affect quantitative traits such as fertility. Single gene association analysis has revealed that signal transducer and activator of transcription 5, uterine milk protein, and osteopontin and, to a lesser extent POU class 1 homeobox 1 are associated with fertilization rate while GHR, prolactin receptor, and signal transducer and activator of transcription 5 are associated with embryo survival rate (Khatib et al.,

2009). Höglund et al. (2009) recently genotyped a number of Holstein grandsires for 416 microsatellite markers. Twenty-six QTL were discovered on 17 different chromosomes with the most significant for reproduction being found on BTA 1, BTA 7, BTA 10, and BTA 26. One of the fertility traits analyzed was artificial inseminations per conception (AIS) for both heifers and cows and it was determined that 2 QTL had effects on AIS. A QTL for AIS in heifers was found on BTA07 ($P = 0.04$). Regarding AIS for cows, a QTL ($P = 0.01$) was located on BTA 24. However, in this study no convincing evidence was found for the presence of an overlap between QTL affecting heifer traits versus cow traits (Höglund et al., 2009), in turn, suggesting that specific genes affecting fertility and specifically inseminations per conception are expressed at different stages of the animal's life. Once a QTL is identified, further determination of the causative mutation, quantitative trait nucleotide (Braunschweig, 2010) and the relationship between the genotype and phenotype of the causative agent is important but remains difficult due to the unclear relationship (Andersson et al., 2004). The availability of SNP chips that can detect tens to hundreds of thousands of SNP has recently allowed for further elucidation of the possible causative mutations affecting fertility and the confirmation of those responsible for milk production (Pimentel et al., 2010; Pryce et al., 2010; Clempson et al., 2011; Schulman et al., 2011). Pryce et al. (2010) tested 39,048 SNP and determined mutations affecting milk production, but also identified several novel regions including one located on BTA 18 associated with fertility. Similar research has recently been published identifying 10 SNP that are involved with the antagonistic relationship between milk production and fertility, 4 SNP were associated with fertility and yield

traits, 2 SNP associated with positive effects on fertility and percentage traits, and 2 SNP associated with antagonistic effects on fertility (Pimentel et al., 2010). Furthermore, Schulman et al. (2011) recently reported 16 SNP on 9 different chromosomes that had significant associations with one or more fertility traits. Further elaboration upon the previous study conducted by Schulman (2008) confirmed that there were SNP associated with various fertility traits on BTA 1, 2, 3, 8, 12, 13, 20, 24, and 27 (Schulman et al., 2011). The SNP identified were linked to traits such as non-return rate for heifers and cows, first to last insemination in days for heifers and cows, number of inseminations for cows and heifers, and time from calving to first insemination in days for cows (Schulman et al., 2011). Clempson et al. (2011) identified SNP responsible for leptin production and leptin receptor expression in dairy cows and heifers. It has been suggested that by the association of leptin SNP with fertility traits in both heifers and lactating cows, the effects on fertility may be direct instead of being mediated by altered tissue mobilization (Clempson et al., 2011). Other research has demonstrated the positive effects of leptin on oocyte quality and subsequent early embryo development (Boelhauve et al., 2005).

Inclusion of previously mentioned CNV with SNP in association analyses may be useful because chromosomal rearrangements may play a role in the expression of complex traits such as fertility (Braunschweig, 2010) and CNV allow for detection of some variations not detected by SNP microarrays (Stranger et al., 2007). Research regarding the role of CNV in dairy cattle has been limited; however some characterization has been made regarding the role of CNV in understanding and

accelerating improvement of complex or quantitative traits (Hou et al., 2011). Hou et al. (2011) determined that certain CNV regions overlap with cattle genes and are significant for immunity, lactation, reproduction, and rumination. Liu et al. (2010) performed the first systematic and genome-wide analysis of CNV within the modern domesticated cattle genome involving beef, dairy, and dual purpose breeds. Determination that 67% of CNV regions were spanning or partially spanning cattle genes was confirmed, with similar implication as Hou et al. (2011) towards immunity, lactation, reproduction, and rumination, CNV possibly becoming useful in genomic selection for quantitative traits.

Lactation and Fertility Interactions

The decline in dairy cattle fertility may partially be attributed to the heavy selection pressure for superior milk production. There are roughly 50 QTL for milk production and, through intense selection, alleles affecting fertilization and embryo survival that were in repulsion phase with the desirable alleles for milk production may have been lost by the hitchhiking effect (Khatib et al., 2009). The hitchhiking effect refers to the footprint that is placed on a population following natural or artificial selection for a specific mutation; with the selection away from animals with low milk production the loss of loci associated with high fertility also occurred (Hilton et al., 1994). In the dairy industry, through the selection for animals with high production; a footprint of animals with above average milk production and subsequently a population of animals with poor fertility has developed. Milk production in the United States has increased by 20% in the last 10 years, but the first-service insemination rate has decreased approximately 15% from 60% to 45% (Lucy, 2001). The decline in fertility

within the dairy industry has only been observed in lactating dairy cows and heifer fertility rates have changed very little over time, also indicating that bull fertility has too experienced little change (Butler et al., 1989); subsequently displaying the negative effects of lactation on fertility. High production cannot be confused or correlated to NEB due to the fact that every animal experiences some form of NEB with nutrient partitioning and adipose tissue mobilization independent of production level (Lucy, 2001). An extended period of NEB may result in a delay in the onset of estrus in lactating animals but this does not explain the increase in services per conception and increase in embryo mortality; it has also been found that cows that experience a slight decrease in body condition score exhibited earlier first ovulation and improved reproductive performance thereafter (Dochi et al., 2010). Higher producing animals generally consume more feed, which leads to an increase in blood flow through the liver that results in an increase in steroid catabolism and more specifically the catabolism of progesterone and estradiol resulting in lower circulating levels (LeBlanc, 2010). This increase in progesterone catabolism may help to explain the increase in embryo mortality by leading to a decrease in progesterone below the threshold for maintenance of pregnancy.

Prolactin, a hormone produced by the placenta during lactation is multifunctional, affecting uterine function (Young, 1989), lactation, and ovarian function (McNeilly et al., 1982). Receptors for prolactin are found on the endometrium of various placental species including sheep (Young, 1989). McNeilly et al. (1982) states that suckling in humans results in a release of large quantities of prolactin to maintain milk

production, it could be argued a similar affect is experienced in lactating dairy cows. Furthermore, the level of prolactin found in blood is much higher than that required to maintain lactation and places the animal in a constant hyperprolactinaemic state (Young, 1989). In spite of the elevated levels of prolactin during lactation it has been found in dairy cows that normal CL function and ovulation occurs regardless (Carruthers et al., 1980). Unlike primates, most livestock species including cattle have been found to have little role for prolactin when it comes to maintenance of the CL during pregnancy and the diestrus stage of the estrous cycle (McNeilly et al., 1982). Young (1989) induced hypoprolactinaemia in gilts by decreasing circulating prolactin by 40% and resulted in decreased leucine aminopeptidase activity and total recoverable calcium, sodium, potassium, and chloride. This suggests that hypoprolactinaemia decreases endometrial secretory activity and modulated ion changes in pigs (Young, 1989) In addition, it was determined that the interaction between estradiol and prolactin enhances uterine secretion and not the relationship between progesterone and prolactin (Young, 1989). Taylor et al. (2000) utilized neonatal ewes to evaluate the uterine gland genesis and reverse transcriptase-polymerase chain reaction analysis was used to detect prolactin receptor (PRL-R) expression. Prolactin receptor was detected on the glandular epithelium on day 7 and increased between days 7 and 56, ultimately indicating that PRL-R on the endometrium stimulates and maintains endometrial gland genesis and branching (Taylor et al., 2000). The application of the previous findings to the dairy industry and the effects of lactation on fertility and early embryonic mortality is in the determination of whether dairy cows selected for greater milk production have a

decreased amount of PRL-R expression on the endometrium compared to on the secretory cells of the mammary gland but maintain a consistent PRL secretion level to create a competitive hormone binding situation. Further research is needed in this particular area to determine the validity of PRL-R expression as a possible negative effect of the selection pressure found within the dairy industry.

During early lactation dairy cows mobilize body fat due to the demands of high milk production; subsequently a small amount of body protein is mobilized as well which results in elevated plasma urea concentrations (Jorritsma et al., 2003). There is also an accumulation of triacylglycerides in the liver which may result in high ammonia concentrations due to the fact that ureagenesis is inhibited (Zhu et al., 2000). The negative effects of urea and ammonia occur at different stages of development, with ammonia having a negative effect on the oocyte prior to ovulation and urea having a negative effect during cleavage and blastocyst formation (Jorritsma et al., 2003); this detriment having the greatest effect during the time which repeat breeder cows have been observed to experience the greatest amount of embryo mortality (Ayalon, 1978; Thatcher, et al., 1994).

With increases in milk production it has been well documented that a decline in fertility has also been observed (Berglund et al., 2008; Janson et al., 1981). The antagonistic relationship between these two traits has been supported by genetic correlations ranging from 0.2 to 0.4 (Roxström et al., 2001). The correlations were observed to increase with lactation number possibly as a result of increased production and increased energy demand (Berglund et al., 2008). Due to the fact that selection has

been mainly focused around those animals with high milk production, a loss of favorable reproductive traits occurs if they are not also included in the breeding goal (Berglund et al., 2008). Liefers et al. (2002) investigated polymorphisms involving the leptin gene and their effects upon milk production, energy balance, feed intake, and fertility in Holstein heifers. All animals were genotyped for fragment length polymorphisms and the microsatellite BM1500, all of which were located at the leptin gene locus (Liefers et al., 2002). It was observed that heifers with the RFLP1 B-allele experienced increased milk production and dry matter intake while having no negative effects upon reproduction and more specifically luteal function (Liefers et al., 2002). Findings such as the previous may lead to the finding of further solutions to help repair the reversible damage done through single trait selection in the dairy industry. Genomic selection serves to eliminate single trait or single gene selection by taking into account all of the information within a genome due to causality by one gene being rarely proven in cattle.

CHAPTER III

MATERIALS AND METHODS

Identifying Highly Fertile and Sub-fertile Herd Populations

Dairy Comp 305® (Tulare, CA) records were analyzed from 2 large Texas commercial dairy farms (Dairy 1 and Dairy 2) to determine Holstein-Friesian heifers that conceived either on the first AI or those that conceived after ≥4 AI. Heifers that conceived on the first AI were classified as highly fertilee and those with a SPC ≥4 were classified as sub-fertile. All heifers were currently pregnant and greater than 60 days post conception and those heifers with a history of uterine infection, disease, or excessive body condition were not used.

Sample Collections and Processing

Whole blood (approximately 7 to 10 mL) was collected in standard blood tubes with ethylenediaminetetraacetic acid (EDTA) from 40 heifers from 2 dairy farms that were analyzed to determine groups of highly fertile and sub-fertile animals (n = 20 highly fertile, n = 20 sub-fertile). The blood was centrifuged at 3,000 x g for 10 min and the buffy coat of white blood cells was removed and placed into a corresponding 1 mL microtube. The protocol for lymphocyte extraction was adapted from CRI Laboratory Manual: RFLPs Project (1989). Approximately 1200 µL of Sucrose Triton Buffer was added to the microtubes, which were then inverted to mix the solution and incubated for 10 min on ice. Following incubation, the tubes were centrifuged at 8,000 x g for 5 min, with subsequent removal of the supernatant. Approximately 600 µL of Sucrose Triton

was added to microtubes and pellet was resuspended using vortex. The microtubes were then centrifuged at 8,000 x g for 2 min and supernatant was collected. Approximately 600 µL of saline was added to each microtube and pellets were resuspended using vortex. The tubes were centrifuged again at 8,000 x g for 2 min. Following the final centrifugation, supernatant was decanted and microtubes with pellets were stored in a -80 °C freezer.

The DNA was extracted from white blood cells following a protocol modified from Short Protocols in Molecular Biology (2002). Half of the white blood cell pellet was placed into a microtube and 250µl of sodium tris EDTA buffer was added. The pellet was dispersed via pestle and 300µl of a solution comprised of 250µl sodium tris EDTA, 25µl 10 mg/mL Proteinase K, and 25 µl 20% w/v sodium dodecyl sulfate was added to each sample. The samples were then vortexed and incubated at 55 ºC for one hour. Following incubation, samples were vortexed again and incubated at 55 ºC overnight. Following the second incubation, samples were extracted twice with 25:24:1 phenol-chloroform-isoamyl alcohol (PCI) and twice with chloroform. Approximately, 550 µl PCI were added to each sample and centrifuged for 5 min at 8,000 x g. The top layer was removed into a new microtube, and the above process was repeated. Subsequently, 550µl chloroform was added to each sample and centrifuged for 5 min at 8,000 x g. The upper aqueous layer was removed and placed into a new microtube and the above process was repeated a second time. To each sample, 1/10 volume of each sample of 2N sodium chloride and 2.5 volumes of cold absolute ethanol were added and mixed by inversion. Samples were then incubated at -80 ºC for 30 min, centrifuged at

13,000 x g for 15 min, and supernatant was decanted. To each sample, 250 µl of 70 % ethanol was added and centrifuged at 13,000 x g for 5 min. After supernatant was decanted, pellets were placed into a speedvac to dry and resuspended in 400µl of tris EDTA (10mM Tris, 1mM EDTA, pH 8.0). The DNA quality was assessed by running 1µl of each sample on an agarose gel. Concentration was then determined utilizing a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Samples were shipped to GeneSeek, Inc. (Lincoln, NE) where genotyping with an Illumina BovineHD Infinium assay (http://www.illumina.com/products/bovinehd_whole-genome_genotyping_kits.ilmn) was performed utilizing 250 ng DNA per sample. Data were obtained from GeneSeek, Inc. in PLINK format for whole-genome association (WGA) analysis using PLINK (Purcell et al., 2007). A gender code was added for each sample and then data filtered by: missingness by individual (0.1), minor allele frequency (0.05), missingness by SNP (0.1), Hardy Weinberg equilibrium at $\alpha = 0.0001$, and Hardy Weinberg equilibrium 2 (exact) at $\alpha = 0.0001$. A gender verification was performed to verify that all samples were taken from females and a genome file was created. Identity-by-descent proportion matrix was constructed to determine the genetic distance between all pairs of individuals in the populations. A phenotype file was created assigning animals to case (sub-fertile) or control (highly fertile). Data were clustered with a pairwise population concordance of 0.01 and 4 cluster files were created. Following confirmation of no stratification, basic association, Fisher's exact, and haplotype based associations were performed. Basic association and Fisher's exact association were ordered based upon a P-value that

would result in a reasonable amount of SNP to be analyzed (< 50); those SNP with a P-value ≤ 1 x 10-5 were selected. The sequence containing each SNP was obtained using the NCBI website because data were in the University of Maryland (UMD) 3.1 format and at the time this research was completed the NCBI and UCSC browsers were only displaying the Baylor (Btau 4.0) assembly. The sequence was input into University of California Santa Cruz's Genome BLAT (Baylor 4.0 format) to reveal the presence of any candidate genes for that particular SNP. Those SNP that appeared on the same chromosome and did not result in any specific location on a gene were entered into BLAT with their range to determine any significant Reference Sequences (RefSeqs). Those SNP that resulted in a candidate gene had their sequence entered into BLAT to determine the specific location of the SNP on the particular gene.

CHAPTER IV

RESULTS AND DISCUSSION

Highly fertile animals had an SPC of 1 and an average age of 416 ± 1 day. Those animals that were sub-fertile had an average SPC of 6.05 ± 1.23 with an average age of 535 ± 32 days). Distribution of animals within the 2 herds was not normal and was positively skewed towards those animals that were highly fertile (Figure 1). This is due to many sub-fertile or infertile animals not remaining on the farm and being culled, and due to the high fertility of heifers, very few animals receive a 4th service because they became pregnant to a previous insemination. The average conception rates during the time period of sample collection were 59% for both Dairy 1 and Dairy 2 and one technician performed 90% of the breeding on each farm.

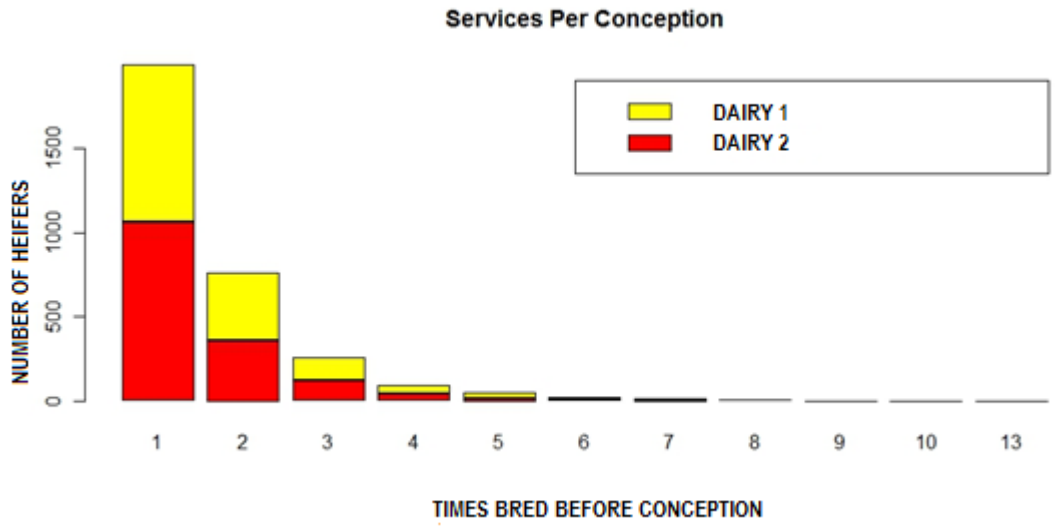

Figure 1: Population distribution of services per conception in both experimental farms.

Agarose gel electrophoresis was used to demonstrate the quality and integrity of DNA, and revealed that there was no indication of RNA contamination (Figure 2). The missingness test resulted in one sub-fertile animal being excluded from further analysis due to failure of 130,541 SNP to be detected. Following all filtering, it was determined that 570,620 SNP remained for analysis (Figure 3).

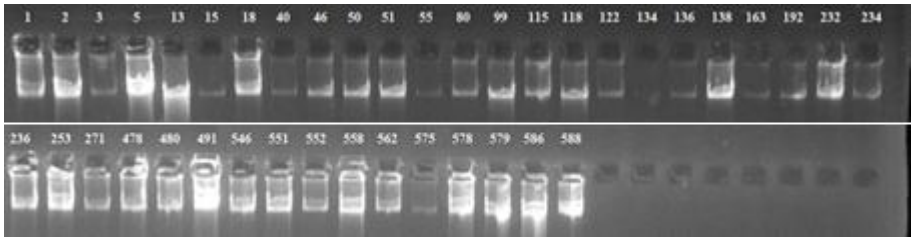

Figure 2: Agarose gel demonstrating quality and integrity of DNA samples.

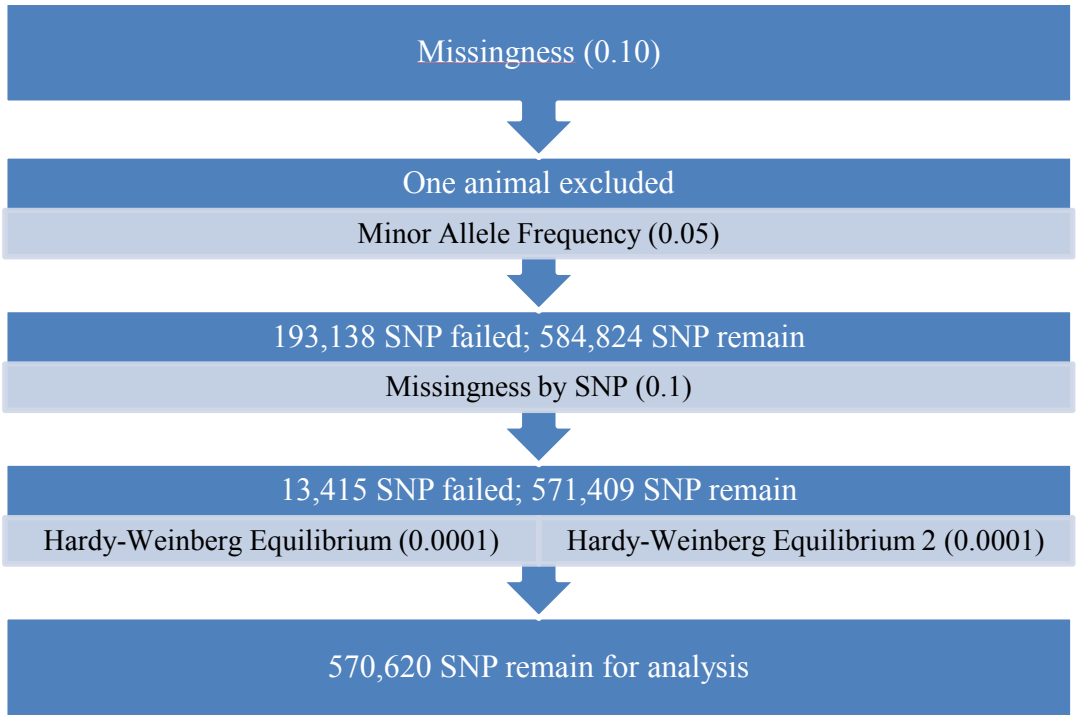

Figure 3: Filtering performed utilizing PLINK resulted in the final set of SNP to be analyzed for genomic association as it pertains to fertility.

acceptable as females with the greatest *F* value being 0.2514, which was lower than the 0.8 value assigned to males. Identity-by-descent revealed that no numbers greatly stood out beyond those that would be expected by chance and current inbreeding within the dairy industry. Identity-by-state was determined to show no significant stratification beyond that which would be expected by chance (Table 1).

Table 1: Identity-by-state to eliminate the possibility of stratification and determine any identical, one-shared, or no-shared alleles. Values obtained conclude no significant stratification beyond that expected by chance.

IBS	Mean	Standard Deviation
Between-group	0.7023	0.0186
Sub-fertile	0.7001	0.0164
Highly Fertile	0.7035	0.0206

Basic and Fisher's exact were used to determine WGA between cases and controls. The Q-Q (quantile) plots were constructed for both basic association and Fisher's exact tests to determine any significant differences between case and control groups. For both methods, there were no significant differences between sub-fertile and highly fertile (Figure 4), assuming that each SNP association test was independent. However, it is improbable that each test is independent because of the extent of linkage disequilibrium across the bovine genome (The Bovine HapMap Consortium, 2009).

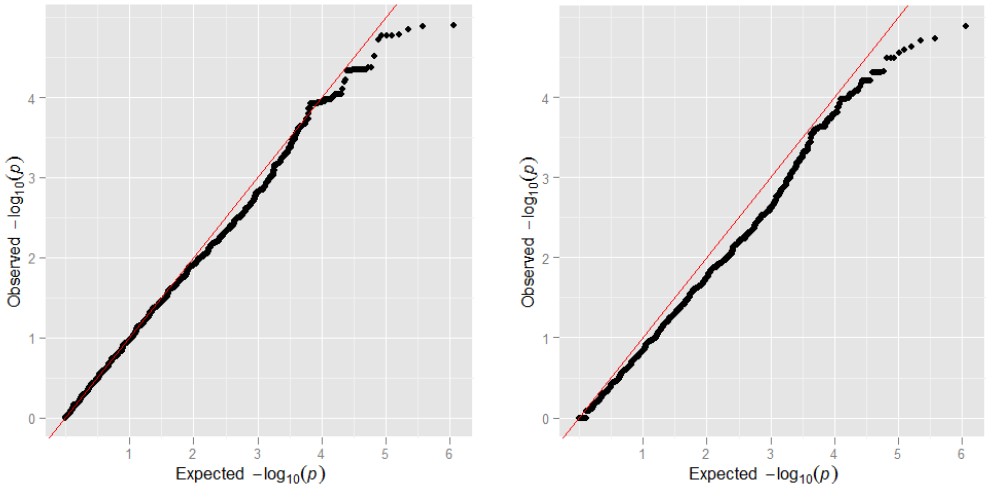

Figure 4: Q-Q plots from basic association (left) and Fisher's exact association (right). Plots indicate no definitive difference between case and control SNP.

A Manhattan plot was also constructed for each association, and it was determined that there was more clustering of SNP present on BTA02, 04, 09, 19, and 26 than would be expected by random chance (Figure 5). Further analysis was conducted of significant individual SNP and SNP ranges for correlations to genes involved with reproduction in dairy heifers

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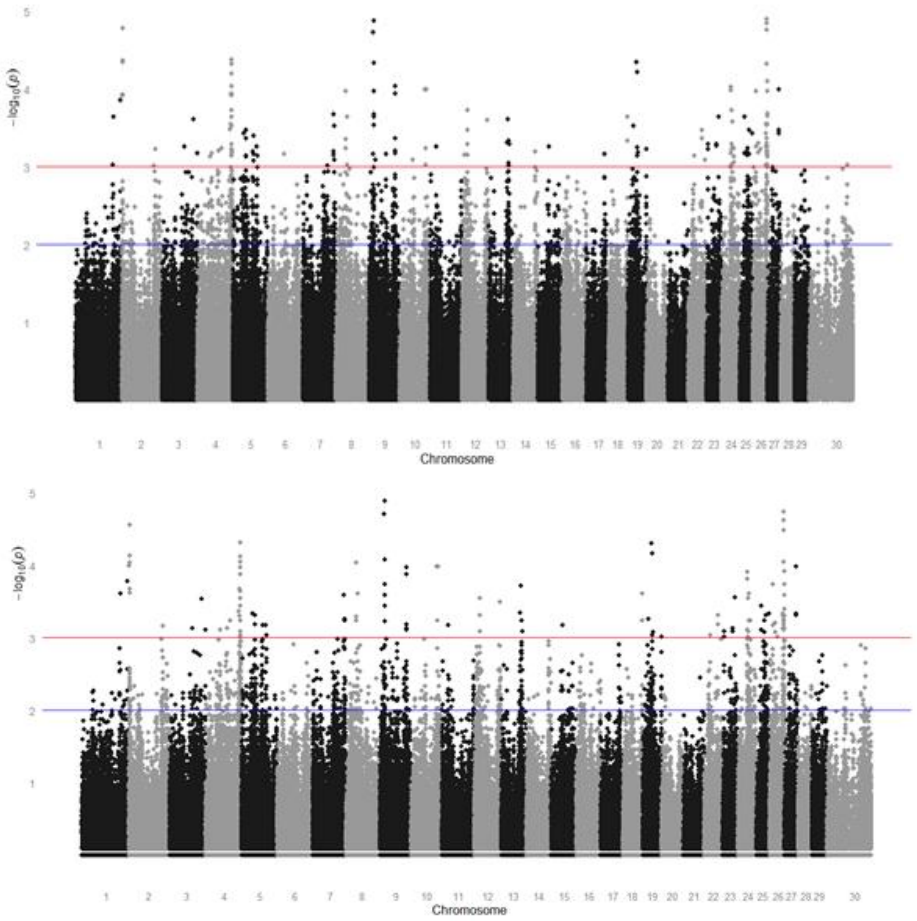

Figure 5: Manhattan plots from basic association (top) and Fisher's exact association (bottom) to demonstrate SNP clustering in designated chromosomes. Significance of SNPs is displayed by negative logarithm of *P*-value (y-axis).

As determined by PLINK analysis, 44 SNP had significance of $P \leq 1.0 \times 10^{-5}$ between highly fertile and sub-fertile (Table 2). On BTA02, 5 SNP were found to be located within two genes. The SNP, BovineHD0200000256 was located within the non-imprinted Prader-Willi/Angelman syndrome 2 (NIPA2) gene, which encodes for a magnesium transporter (Pruitt et al., 2009). Four additional SNP on BTA 2 were located within the gene, CYFIP1. The CYFIP1 gene encodes for cytoplasmic FMR1 interacting protein 1; FMR1 being associated with altered reproductive function in humans. Three SNP on BTA 9 were observed within the gene MTHFD1L, encoding for a protein involved in the synthesis of tetrahydrofolate in mitochondrion. Tetrahydrofolate is important for de novo synthesis of purines, thymidylate, and in regeneration of methionine from homocysteine. Several transcript variants encoding different isoforms have been found for this gene (Pruitt et al., 2009). Further research has also linked MTHFD1L mutations to neural tube defects during gestation (Parle-McDermott et al., 2009). Six SNP on BTA 4 were located within vacuolar protein sorting 41 homolog, which plays an important role in segregation of intracellular molecules and in formation and fusions of transport vesicles from the Golgi complex (Pruitt et al., 2009). However, to date, no significant correlation between vacuolar protein sorting 41 homolog and fertility has been elucidated. As previously mentioned, those SNP that were not found within a specific gene on a chromosome were evaluated by BLAT analysis. On BTA 19, 4 RefSeqs were found in the vicinity of the 6 SNP; of these 4 RefSeqs, 3 were genes (ZNF18, MAP2K4, and elaC homolog 2) and one was microRNA (MIR744). The ZNF18 gene is involved with heart development during early embryo development (Guo

et al., 2005). The MAP2K4 gene interacts with toll-like receptors (Pruitt et al., 2009) including those involved with GnRH signaling and cytokines involved in pregnancy. The range between the 7 SNP on BTA26 resulted in one RefSeq gene, GLRX3. The GLRX3 gene encodes for a protein (glutaredoxin) that binds to and modulates the function of protein kinase C θ (PKCθ); which may play a role in inhibition of apoptosis and cellular growth (Pruitt et al., 2009). All significant SNP associated with a candidate gene or alteration in allele frequency were found to lie within the non-coding regions associated with introns with the exception of BovineHD0200000275 which lands on exon 19 of CYFIP1 and is a synonymous SNP. The elaC homolog 2, microRNA 744, and GLRX3 located on BTA19 and 26 respectively were not found to demonstrate any significance or involvement in reproductive pathways.

Table 2: 44 SNP with significance at $P \leq 1.0 \times 10^{-5}$. Highlighted are those SNP that were located on or near genes.

CHROMOSOME	SNP	GENE TYPE	GENE/REFSEQ
2	BovineHD0200000256	protein coding	NIPA 2
2	BovineHD0200000260	protein coding	CYFIP1
2	BovineHD0200000264	protein coding	CYFIP1
2	BovineHD0200000275	protein coding	CYFIP1
2	BovineHD0200000298	protein coding	CYFIP1
4	BovineHD0400033990		
4	BovineHD0400033994		
4	BovineHD0400034028		
4	BovineHD0400034032		
4	BovineHD0400034033		

Table 2 continued.

4	BovineHD0400034034		
4	BovineHD0400034035		
4	BovineHD0400034036		
4	BovineHD0400034037		
4	BovineHD0400022880	protein coding	VPS41
4	BovineHD0400022883	protein coding	VPS41
4	BovineHD0400022884	protein coding	VPS41
4	BovineHD4100003061	protein coding	VPS41
4	BovineHD0400022886	protein coding	VPS41
4	BovineHD0400022888	protein coding	VPS41
8	BovineHD0800009877		
8	BovineHD0800009876		
9	BovineHD0900003363		
9	BovineHD0900004164		
9	BovineHD0900004165		
9	BovineHD0900004170		
9	BovineHD0900004171		
9	BovineHD0900025137	protein coding	MTHFD1L
9	BovineHD0900025141	protein coding	MTHFD1L
9	BovineHD0900025143	protein coding	MTHFD1L
19	BovineHD1900008920		4 RefSeqs
19	BovineHD1900008925		4 RefSeqs
19	ARS-BFGL-NGS-64718		4 RefSeqs
19	BovineHD1900008928		4 RefSeqs
19	BovineHD1900008930		4 RefSeqs
19	BovineHD1900009433		4 RefSeqs
24	BovineHD2400008869		
26	BovineHD2600014006		1 RefSeq

Table 2 continued.

26	BovineHD2600014025		1 RefSeq
26	BovineHD2600014026		1 RefSeq
26	BovineHD2600014028		1 RefSeq
26	BovineHD2600014032		1 RefSeq
26	BovineHD2600014033		1 RefSeq
26	BovineHD2600014476		1 RefSeq

NIPA2 and Magnesium Transport

The NIPA2 gene has been associated and named for non-imprinting Prader-Willi/Angelman syndrome 2, however it has also been suggested that it codes for a magnesium transporter (Pruitt et al., 2009). The under-expression of magnesium transporters on the endometrium and myometrium could result in early embryo death in mammals. Under-expression of magnesium transporters may result in an animal that is termed a repeat breeder and requires multiple AI services to achieve successful pregnancy until there is adequate magnesium transporter expression in the reproductive tract. The increased availability of magnesium within the uterine environment prevents myometrial activity (Lemancewicz et al., 2000). In animals with inadequate expression of magnesium transporters, a reduced availability of magnesium could occur, in turn, causing early embryo death in dairy animals. Lemancewicz et al. (2000) measured the diffusion of calcium and magnesium across the chorioamniotic membranes in humans who experience term and preterm labor. The transport coefficient for calcium was 0.203 h^{-1} and .0223 h^{-1} for term and preterm labor respectively. Similarly, magnesium transport coefficients were -0.017 h^{-1} and 0.051 h^{-1}. The effects on calcium transport and

availability may subsequently result in a decrease in nitric oxide synthase, and a reduction in nitric oxide production which has been linked to control of uterine contractility, and more specifically myometrial quiescence via production of PGE (Chaud et al., 1997; Pearson et al., 1998; Lemancewicz et al., 2000). Chaud et al. (1997) evaluated the effects of inhibition of nitric oxide synthase on oxytocin stimulated contractions and PGF2α synthesis in rats and determined that inhibition of nitric oxide synthase resulted in stronger sustained contractions stimulated by oxytocin by day 13 of pregnancy. The production of nitric oxide has a relaxing effect upon myometrial activity; in incidences of decreased nitric oxide production caused by inadequate magnesium transporter expression, myometrial activity may be increased (Lemancewicz et al., 2000). Myometrial quiescence is necessary for adequate attachment and adhesion of bovine embryos to the endometrium.

The gene, NIPA2, codes for a magnesium transporter in humans, and possibly in the bovine as well. Magnesium transport can positively affect calcium transport. In the incidence of poor magnesium transport, calcium transport may be compromised leading to decreased nitric oxide production and the subsequent increase in PGF2α synthesis leading to uterine contractions resulting in pre-term abortion in humans and possible early spontaneous abortion in cattle.

CYFIP1, FMR1, and Premature Ovarian Failure

The CYFIP1 genes codes for a cytoplasmic fragile X mental retardation 1 (FMR1) interacting protein. The FMR1 gene, in humans, codes for proteins that may be involved in mRNA trafficking. A trinucleotide repeat (CGG) is normal in humans, with

6-53 repeats present naturally on the 5' untranslated region with no adverse effects (Pruitt et al., 2009). However, certain individuals possess 55 to 200 repeats and this occurrence may manifest as fragile X syndrome. Fragile X syndrome is an error in the functionality of the FMR1 gene on the fragile portion of the X chromosome and presents as a mental disability with various symptoms; more importantly, the excess repeats may also be associated with premature ovarian failure (POF) (Pruitt et al., 2009). When the full mutation of 55 to 200 trinucleotide repeats exists, fragile X syndrome occurs, however; there is an incidence of individuals possessing hyper-repeats that are unaffected and deemed "unaffected carriers" possessing a "premutation" (Wittenberger et al., 2007). While the research regarding the association of the FMR1 premutation with premature ovarian failure and ovarian activity is still vague; of those individuals exhibiting POF, 13 to 26% were found to have the FMR1 premutation (Wittenberger et al., 2007), and those individuals with the premutation prior to POF had significantly higher follicle stimulating hormone levels across the entire follicular phase when compared to controls (21.9 ±3.5 vs. 11.2 ±0.5 IU/L) and demonstrated hormone (inhibin B, inhibin A, and progesterone) levels that were associated with impaired follicular and luteal function (Welt et al., 2004).

The application to dairy cow fertility problems may lie in the alteration of the CYFIP1 gene since it interacts with FMR1. Possible errors in the pathway leading to these interacting proteins may manifest in ways similar to premutations of FMR1 which is associated with altered ovarian function and POF. Those animals that are categorized as repeat breeders may have a mutations leading to improper CYFIP1 coding and

production hence leading to altered FMR1 functions and possibly altered follicular and luteal function. Altered luteal function may result in an inadequate embryo environment during the crucial period of maternal recognition. Further research is needed regarding FMR1's exact role in dairy cattle reproduction, as well as, research investigating the presence and actions of CYFIP1 and FMR1 in bovine reproduction.

MTHFD1L Causation of Neural Tube Defects and ZNF18's Connection to Early Heart Development

Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like (MTHFD1L) is the monofunctional gene that codes for C-1 Tetrahydrofolate Synthase enzyme which is localized in mitochondria (Parle-McDermott et al., 2009). The lack of folate supplementation (Laurence et al., 1981) and errors in folate metabolism (Beaudin et al., 2009) can result in a higher prevalence of neural tube deformities developing during early embryo development in humans. A similar problem may occur in dairy cattle due to the presence of the polymorphism on the MTHFD1L gene. If folate metabolism is interrupted, embryos may experience neural tube deformities resulting in possible early embryo death.

Limited research is available regarding ZNF18 in the human and mouse, and nearly non-existent for the bovine. However, the ZNF18 gene located within the human genome on chromosome 17 is very similar to the mouse Zfp535 (77%), and in the bovine it is located on BTA19 (Guo et al., 2005). Expression of ZNF18 in adult mouse tissues is widespread, with the exception of no expression in the heart (Guo et al., 2005). Expression within the mouse embryo was highly variable. Within an embryonic d 7.5

mouse embryo, expression was mainly isolated to the extraembryonic membranes (Guo et al., 2005). By d 8.5, expression began to spread and was present in the embryonic heart by d 9.0 and heavily expressed in the heart by d 10.5 (Guo et al., 2005). Therefore, it has been suggested that ZNF18 may play a crucial role in embryonic heart development (Guo et al., 2005). Application to the bovine may be in the role of ZNF18 in heart development within the bovine embryo. If there is a failure in expression of this particular protein, embryonic heart development may be altered resulting in early embryonic death or malformation leading to complications post parturition. Further research needs to be conducted regarding ZNF18's exact relationship to embryonic heart development in the mouse followed by elaboration into the role in bovine embryo heart development.

Involvement of MAP2K4 in Toll-like Receptor (TLR) Activation and TLR Mediated Abortion

Several studies have shown MAP2K4's involvement in immune response, as well as, involvement in reproductive diseases such as ovarian and endometrial cancers as it pertains to humans (Davis et al., 2011;, Ishikawa et al., 2010; Jiang et al., 2011; Yeasmin et al., 2011). Davis et al. (2011) screened for mutations of MAP2K4 in ovarian tumors in mice. It was observed that MAP2K4 homozygous inactivation was present in 5.6% of tumors overall, and the hemizygous deletion of MAP2K4 was present in 38% of samples (Davis et al., 2011). Similar decreased expression of MAP2K has been observed in 63.2% of endometrial tumors found in mice, indicating that MAP2K4 acts as a tumor suppressor (Ishikawa et al., 2010). TheMAP2K4 gene has also been illustrated to have a

direct effect on toll-like receptor function as it pertains to immunology and more applicably the GnRH signaling pathway (Naor et al., 2000).

Toll-like receptors are a family of innate immune receptors that recognize pathogen associated molecular patterns expressed by microorganisms and mediate an immune response (Abrahams et al., 2006). It has been suggested that the placenta may play a role in the immunological response based upon its response to pathogens through TLR and serving as an active barrier during pregnancy (Abrahams et al., 2006). First trimester trophoblast cells have been found to produce IFN-beta which serves as a leukocyte protease inhibitor (Abrahams et al., 2006). Kannaki et al. (2011) reviewed the role of TLR in animal reproduction; it has been characterized that the endometrium of cattle expresses TLR 1-10 and tissues associated with gestation also express TLR; however, the role in pregnancy of domestic animals is still vague. Activation of TLR can lead to the activation of cytokines and chemokines (IL-6 and IL-1β) that are related to pregnancy. In addition, it has been illustrated that upregulation of TLR-2 and TLR-4, most often in the cases of infection or obesity, led to placentitis and abortion (Kannaki et al., 2011). Cytokines are present during normal gestation; however the incidence of excessive pro-inflammatory cytokine expression at the maternal-fetal interface may inflict harmful effects on pregnancy causing pre-term parturition. Also, TLR expressed at the interface play a role in the pathogenesis of reproductive complications (Kannaki et al., 2011).

The G-protein-coupled receptors transmit signals from a vast selection of external stimuli (Naor et al., 2000), and the receptor for GnRH is a specialized G-

protein-coupled receptor that uses the Gq protein for downstream signaling (Naor et al., 2000). The GnRH hormone activates all four MAPK signaling pathways leading to the hypothesis that MAPK may be involved in the expression of genes responsible for gonadotropin synthesis (Kegg Pathway, 2009; Naor et al., 2000).

The observed results and previously published research illustrate that MAP2K4 expression may play a fundamental role in dairy cattle fertility and maintenance of pregnancy past the crucial maternal recognition stage. The involvement of MAP2K4 in GnRH signaling pathways and over expression or lack of expression in dairy cattle may lead to incomplete synthesis of gonadotropins. Errors in synthesis of luteinizing hormone, possibly resulting in inadequate CL development and a subsequent sacrifice in progesterone production may lead to failure to maintain pregnancy, specifically during the early embryo stage when progesterone production is crucial to promote histotrophic nutrition from the endometrium prior to embryo attachment. Decreased levels of follicle stimulating hormone could have negative implications as it pertains to follicular development, oocyte maturation, and ovulation, all of which could help to explain the decline in dairy cow fertility observed over the past decade.

The MAP2K4 gene's involvement in reproductive immunology, especially during the time of pregnancy is not extensively characterized in domestic animals. However, if MAP2K4 is overexpressed, an increase in expression of inflammatory cytokines could result in a proinflammatory immune response, in turn, causing placentitis and early parturition resulting in fetal abortion. With an elicited immune response, there may be negative effects on the embryo if it is not recognized as self and

rather identified as a foreign body, leading to abortion as a result of an immune attack on the embryo. If there is low or no expression of MAP2K4 there may be a failure of TLRs to cause the necessary immune response at the site of the placenta which would lead to a failure in protection from harmful pathogens during pregnancy, inevitably leading to embryonic death and abortion.

CHAPTER V

CONCLUSIONS AND IMPLICATIONS

As previously mentioned, one of the major factors plaguing efficiency and profitability in the dairy industry is reproductive failure. Fertilization rates following natural service or artificial insemination remain high with positive results between 80 to 90%; however conception rates following a single insemination remain at 35 to 45% when pregnancy determination is performed between days 27 and 31 post breeding (Diskin et al., 1980; Lucy, 2001; Santos et al., 2004). The aforementioned results indicate that the cause in decline of fertility between fertilization and conception may involve other causative agents beyond oocyte quality and sperm quality, such as genetic factors that lead to early embryonic death which would result in the heifer returning to estrus and necessitating additional inseminations to achieve a successful pregnancy. Utilizing services per conception as a determination of fertility, dairy cattle can be classified into categories of highly fertile, sub-fertile, and infertile.

Whole genome analysis on highly fertile and sub-fertile dairy cattle was utilized to determine the presence of genetic variations between the two groups. The results from whole genome analysis did not indicate any specific SNP that were directly linked to fertility in dairy heifers as it pertains to services per conception and the status of "repeat breeder". However, it was determined that more clustering was present on particular chromosomes that would have been expected by random chance. Genes involved in various aspects of reproduction were identified on BTA 2, 4, 9, 19, and 26 (NIPA2,

CYFIP1, MTHFD1L, ZNF18, and MAP2K4) all of which, upon further investigation, were obvious candidates for early embryonic death in dairy cattle and the overall decline in dairy cattle fertility. The implication of genomic research for the dairy industry is the allowance of genetic testing once the establishment of markers pertaining to heifer and subsequent cow fertility has been established. Producers may also have the capability of making reproductive advances by only retaining animals that test positive for specific fertility markers versus those that test positive for sub-fertility. Eventually, genetic testing may be performed on-farm and the fertility status of an animal could be determined within the first few weeks of life when the animal is still prepubertal. In turn, this would improve profitability since the dairy farm could cull heifers at an early age before enduring the cost of rearing. Understanding the quantitative agents that are involved in fertility may further allow for the understanding of the inverse relationship between lactation and fertility, and the means by which to preserve fertility in high lactation animals.

Previous research has indicated the presence of QTL found on BTA 3 that had large effects upon insemination results (Druet et al., 2008). Höglund et al. (2009) also determined the presence of 26 QTL that were significant for reproductive performance; these QTs were found on BTA 1, BTA 7, BTA 10, and BTA 26. Current literature has also revealed the presence of SNP that are involved in cattle reproduction and also involved in lactation (Pimental et al., 2010, Pryce et al., 2010, Schulman et al., 2011). Even so, further research is needed in the area of genomic variation and its control on reproductive traits. More concise results may be observed if a larger sample size is

studied allowing for further analysis towards the direction of CNV to allow for greater coverage and detection of differences over the entire bovine genome. Continued research could also involve a similar whole genome analysis of heifers, and then a subsequent whole genome analysis of those same animals following onset of lactation to allow for the determination of possible conditionally expressed genes that are a result of lactation. Additional research could be conducted regarding the expression of the genes characterized in this study during different stages of pregnancy and the prevalence of expression in the conceptus and reproductive tract tissues.

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