THESIS

SELECTION FOR FERTILITY IN LACTATING DAIRY COWS: IMPLICATIONS OF CONCEPTUS-DERIVED SIGNALS

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Bethany Ellen Liebig

Department of Biomedical Sciences

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Master's Committee:

Advisor: Thomas R. Hansen

Milton G. Thomas
Hana Van Campen
Craig S. McConnel

ABSTRACT

SELECTION FOR FERTILITY IN LACTATING DAIRY COWS: IMPLICATIONS OF CONCEPTUS-DERIVED SIGNALS

Infertility is a source of major economic loss in the dairy industry. Selection for fertility in dairy cows is difficult because fertility traits based on a genetic evaluation, such as daughter pregnancy rate (DPR), are lowly heritable ($h^2 \leq 0.04$), influenced by on-farm events, such as services per conception (SPC), and influenced by complex mechanisms that cause embryo mortality (EM). Embryo survival depends on robust interferon tau (IFNT) production and release from the trophectoderm, induction of IFN stimulated genes (ISG) in the endometrium to block the luteolytic, pulsatile release of prostaglandin $F_2\alpha$ (PGF), and continued progesterone production by the corpus luteum throughout maternal recognition of pregnancy. Genes negatively affecting IFNT and ISG expression may increase the occurrence of EM. We hypothesized that selection for high direct genomic value for DPR (DGV-DPR) and low on-farm SPC records would be associated with increased: 1) IFNT production by the conceptus, 2) ISG expression in endometrium and peripheral blood mononuclear cells (PBMC), and 3) embryo survival. Freshening dairy cows (n=86) were sorted by DGV-DPR (determined by Clarifide®, Zoetis) and SPC into high fertile (HF; -1.3 DGV-DPR; 1.4 SPC) nonpregnant (NP) or pregnant (HP), and low fertile (LF; -2.3 DGV-DPR; 3.7 SPC) pregnant (LP) groups (n = 7 each). After the voluntary wait period, cows were estrous synchronized and time-artificially inseminated to a HF bull (+1.8 DPR). NP cows were not inseminated. On day 16 following onset of estrus, embryos were flushed from the uterus and typed as viable or EM based on morphology and length. The

DGV-DPR was negatively correlated ($r = -0.57$; $P < 0.05$) with SPC. Days in milk and number of lactations were not different between groups. Serum progesterone tended ($P < 0.10$) to be lower in the cows carrying EM embryos than NP cows. Two of 7 embryos from HP cows and 3/6 embryos from LP cows were classified as EM. Viable embryos were significantly ($P < 0.05$) longer than EM embryos when fertility group was not considered. Viable HP embryos tended to be longer ($P < 0.10$) than LP embryos. Interferon tau concentrations in uterine flushing (UF) were: 1) greater in HP compared to LP and NP cows ($P < 0.05$), 2) positively correlated with DPR ($r = 0.68$; $P < 0.05$) and 3) negatively correlated with SPC ($r = -0.59$; $P < 0.05$). Interferon stimulated gene 15 mRNA concentrations were significantly: 1) upregulated in endometrium from HP viable compared to LP viable and NP cattle ($P < 0.05$), and 2) upregulated in peripheral blood mononuclear cells from HP compared to LP and NP cows ($P < 0.05$). Furthermore, ISG15 protein concentrations in endometrial tissue were significantly upregulated in HP compared to LP and NP cattle ($P < 0.05$). In conclusion, selection of dairy cows combining DPR and SPC may improve fertility through increased production and action of IFNT.

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CHAPTER 1

LITERATURE REVIEW

INFERTILITY IN DAIRY CATTLE

Infertility is an important problem in livestock impacting the production and economics of all cattle industries, especially the dairy industry (De Vries, 2006). Improved management, adoption of automated milking equipment, better nutrition, superior housing and intense genetic selection (Lucy, 2001; Weigel, 2006) of dairy cows has increased milk production from 5,314 lb per cow (2,415 kg per cow) in 1950 to 18,204 lb per cow (8,275 kg per cow) in 2000 (Blayney, 2002) to 19,932 lb per cow (9,060 kg per cow) in 2014 across all operation types (conventional, grazing, and organic) (USDA-APHIS, 2016). In contrast, during the same time period, first service conception rates declined from 66% in 1951 to 50% in 1975 (Butler and Smith, 1989), and 40% in 1996 (Butler, 1998). Norman et al. (2009) reported the first service conception rate varied between 34% in 1997 and 32% in 2007 in Holstein cows across the United States. In 2012, the reported first service conception rate was 43.4% (SD 16.8%) in the Raleigh, North Carolina Dairy Herd Improvement Association (DHIA) system (Raleigh-DRMS, 2012). Ferguson and Skidmore (2013) conducted a survey of 16 herds in Wisconsin, New York, California, Iowa, Washington, Oregon and South Dakota recognized for having outstanding reproductive performance. First service conception rates in these superior herds were 44.4% (SD 4.8%) and ranged from 37.5% to 51.8% across herds.

The herd average days open is typically an indicator of reproductive performance on the farm and it is inversely related to profitability. As milk production has increased through the years, the herd average days open has also increased. The projected days open was 156.8 days

(SD 42.0) across US herds in 2012 (Raleigh-DRMS, 2012). In a review by Cabrera (2014), the cost of a day open for an average cow between 90 and 120 days in milk (DIM) was $5.20/day. For a cow with 10% more milk production (and therefore a higher value), the cost of a day open during the same DIM was $6.00/day. Depending on the management system, the cost of days open should be avoided to improve the economic efficiency of reproductive programs.

Another reproductive metric that is commonly reported is the 21-day pregnancy rate (21-d PR), which measures the percent of eligible cows that become pregnant in a 21-day period after the voluntary wait period (assumed to be the first 60 days of the lactation) has occurred (VanRaden, 2002). It is nearly the same trait genetically as days open (VanRaden et al., 2004) and considered to be the best single parameter to measure and standardize reproductive performance across herds (Giordano et al., 2011; Giordano et al., 2012; Galvao et al., 2013). In 2012, the mean 21-d PR was 15.9% (SD 5.9%) for 13,885 herds across the US (Raleigh-DRMS, 2012). This parameter is comparable to the percentages reported by Norman et al. (2009) in which 21-d PR ranged from 22.2% to 28.3% depending on the region of the United States. Ferguson and Skidmore (2013) reported higher pregnancy rates for 16 herds with superior reproductive performance. The average 21-d PR was 32.0% (SD 3.9%) and ranged from 26.5% to 39.4%. The curve for 21-d PR versus net return gain ($/cow per year) is a quadratic relationship with diminishing returns at a higher 21-d PR (Cabrera, 2014). However, a 21-d PR greater than 40% still has economic benefits. Cabrera (2012) reported an increased economic net return of $14.40/cow per year when the 21-d PR increased from 10% to 15%, a $10.00/cow per year increase from 15% to 20%, a $7.40/cow per year increase from 20% to 25% and a $5.40/cow per year increase from 25% to 30%.

Reproductive performance on dairy farms directly affects profitability through milk production per cow per day, number of replacement heifers required, and voluntary and involuntary culling (Britt, 1985). Factors that influence the profitability of reproductive programs are labor costs (including training employees and time required for visual heat detection), type of facilities, cost of synchronization hormones (including cost per dose and time required for injections), semen costs, pregnancy diagnosis, and disease prevalence (Caraviello et al., 2006; Olynk and Wolf, 2008; Cabrera, 2014). However, the economic gains from improving reproductive performance positively influence milk production per cow and subsequently increased milk sales, more calf sales and fewer replacement and mortality costs (Giordano et al., 2012; Galvao et al., 2013). Because reproductive programs have complex interactions with farm costs and market prices, it is advised to use decision support tools. The Wisconsin-Cornell Dairy Repro tool (available at http://www.dairymgt.info/tools.php and http://ansci.cals.cornell.edu/extension-outreach/adult-extension/dairy-management/wisconsin-cornell-dairy-repro-giordano) uses the daily Markov chain model (Giordano et al., 2012) to simulate the economics of all the cows in the herd and computes the net return associated with reproductive performance parameters.

EMBRYO MORTALITY

Decreased reproductive efficiency is multifactorial and associated with high milk production, stress, increased herd size, labor shortages and higher inbreeding percentages (Lucy, 2001). Fertilization rates are highly variable (55-88%) among high yielding dairy cows in comparison to rates in moderately productive or non heat-stressed cows (80-90%) (Wiebold, 1988; Ryan et al., 1993; Sartori et al., 2002). In comparison to fertilization rates, first service calving rates average about 40-55%, suggesting embryo mortality (EM) rates of 35-50% (Royal

et al., 2000; Diskin et al., 2006). Early embryonic death prior to day 24 of gestation has been estimated to be approximately 43% in Holstein-Friesian cattle (Diskin et al., 2006), a percentage much higher than estimates of late (day 28-84 of gestation) embryonic death rate of 7% (Silke et al., 2002). Thus, EM is one of the largest contributors to decreased reproductive efficiency.

Early EM is most common between days 7 to 16 of gestation (Diskin and Sreenan, 1980; Roche et al., 1981; Berg et al., 2010). During this period, the spherical blastocyst hatches from the zona pellucida (day 9-10) and grows into an ovoid shape (day 12-14) (Betteridge, 1988). The conceptus begins elongation and doubles in length every day between days 9 and 16 (Berg et al., 2010) at which time point, the conceptus begins the initial process of attachment to the uterine epithelium.

There are numerous reasons that have been postulated to explain EM. Possible causes include physiological, endocrine, environmental, management, and genetic factors that will be discussed further in this text. One explanation for EM is an insufficient or poorly-timed interferon tau (IFNT) secretion by the conceptus and resulting failure of maternal recognition of pregnancy.

INTERFERON TAU

INTRODUCTION

During elongation in ruminants, it is critical that the conceptus produce robust amounts of IFNT for maternal recognition of pregnancy to occur. Interferon tau is produced by the trophectoderm epithelial cells of the trophoblast, and secreted into the lumen of the uterus to initiate maternal recognition of pregnancy. Interferon tau acts in a paracrine manner on endometrial epithelial cells resulting in the attenuation of the release of prostaglandin $F_2\alpha$ (PGF). By inhibiting the release of PGF from the endometrium, the luteolytic effect of PGF on the

corpus luteum (CL) of the ovary is prevented, progesterone production by the CL is sustained and pregnancy can persist (Thatcher et al., 1984; Knickerbocker et al., 1986a; Bazer et al., 1996). Collectively, robust IFNT production and release is essential for survival of the embryo.

HISTORY

Bartol et al. (1985) reported a protein (bovine conceptus secretory protein; bCSP) transiently secreted by bovine conceptuses from day 16 to day 24 of gestation and suggested it might be similar to ovine trophoblast protein-1 (oTP-1) (Godkin et al., 1984a; Godkin et al., 1984b), which was determined to be required for maternal recognition of pregnancy. When bCSP was injected into the uterus of cyclic cows, it significantly extended the CL lifespan and interestrous interval by attenuating endometrial PGF production (Knickerbocker et al., 1986a; Knickerbocker et al., 1986b). Bovine trophoblast protein-1 (bTP-1) was isolated from bCSP and first described by Helmer et al. (1987). Bovine TP-1 is an acidic, low molecular weight polypeptide with two predominant molecular weight classes (22 and 24 kDa) due to differences in glycosylation. Bovine TP-1 exhibits several isoelectric points ranging from 6.3 pI to 6.8 pI, and shares epitopes with oTP-1 (Helmer et al., 1987; Anthony et al., 1988). Using oTP-1 antiserum in an immunoblot analysis to detect bTP-1 from culture medium, the glycoprotein was determined to be secreted by bovine conceptuses from at least day 19 to day 38 of gestation (Godkin et al., 1988). Secretion is maximal between days 16 and 19 of pregnancy; however, mRNA can be detected as early as day 12 (Bazer et al., 1996).

Purified oTP-1 was determined to bind to type 1 IFN receptors and had antiviral and antiproliferative properties similar to IFN-α (Pontzer et al., 1988; Hansen et al., 1989; Roberts et al., 1989). Bovine TP-1 was later classified as an interferon composed of 172-amino acids because its sequence was 85% identical to the cDNA of oTP-1 and 79% identical to bovine IFN-

α_{II} (Imakawa et al., 1989; Klemann et al., 1990). The bTP-1 gene was first sequenced by Hansen et al. (1991). Hansen and coauthors proposed that the trophoblast protein genes represented a distinct IFN-α subclass for three reasons: 1) the organization and sequences of the bTP-1 genes upstream of the TATA box were more similar to oTP-1 than to other bovine IFN-α_{II} genes, 2) the trophoblast IFN had high expression in trophectoderm but poor viral inducibility in leukocytes which made it distinct from other IFN-α genes, and 3) the promoter regions of the trophoblast IFNT genes were highly conserved across species, yet showed only limited sequence identity with the IFN-α_{II} promoter due to a deleted tandem IRF-1 binding site and IFN response element GAAATG motif.

After cloning the gene and inferring the amino acid sequence, bTP-1 was renamed IFNT by the International Interferon Society in 1992 (Roberts et al., 1992a). Interferon tau is only produced in ruminant species in the Artiodactyla order and most likely evolved from the IFN-ω class around 36 million years ago (Roberts et al., 1992b; Roberts et al., 1998).

ACTIONS

Interferon tau has a paracrine effect on the endometrium and binds to the type I IFN receptor subunits, IFNAR1 and IFNAR2, which are expressed primarily in endometrial luminal epithelium (LE), and superficial ductal glandular epithelium (GE) cell types (Rosenfeld et al., 2002). The binding of IFNT to the receptor stimulates the JAK-STAT pathway by activating a dose and time-dependent tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1), STAT2 and interferon regulatory factor 1 (IRF-1) (Hansen et al., 1999; Binelli et al., 2001). STAT homodimer and heterodimer complexes form and migrate to the nucleus where they bind interferon response elements (IRSE) in the regulatory region of the interferon-stimulated genes (ISG) resulting in their expression.

There are more than 100 known ISG (Garcia-Sastre and Biron, 2006) and many are increased in the pregnant uterus in ruminants. Some of these include ISG15 (Austin et al., 1996; Austin et al., 2004), MX1 (Charleston and Stewart, 1993; Ott et al., 1998; Hicks et al., 2003), β2-microglobulin (β2MG) (Vallet et al., 1991), and 2'5' oligoadenylate synthetase 1 (OAS1) (Johnson et al., 2001). Interferon stimulated gene 15, formerly known as ubiquitin cross-reactive protein, is a tandem ubiquitin repeat that conjugates to proteins in the cytosol of endometrial epithelial cells to aid in maternal recognition of pregnancy (Hansen et al., 1999). Oliveira et al. (2008) performed an elegant study where ISG were examined in uterine and extrauterine tissues. They reported that ISG15 and OAS1 mRNA were significantly increased in the: 1) endometrium of d15 pregnant ewes, 2) jugular vein blood, uterine vein blood, and uterine artery blood of d15 pregnant sheep, and 3) CL on d15 of pregnancy. In a similar study, Bott et al. (2010) infused IFNT into the uterine vein in sheep and found that the infusion extended the life of the CL as well as significantly increased mRNA (luteal, endometrial and liver tissue) and protein concentrations (uterine flushing) of ISG15. These studies indicate that in addition to local effects on the uterus, IFNT may escape from the uterus and travel through the blood stream to the CL exerting effects that secure the pregnancy.

The major effect of IFNT on the endometrial LE and GE of the sheep is to suppress increases in the estrogen receptor 1 (ESR1) and oxytocin receptor (OTR) expression. As a result, the pulsatile release of PGF is inhibited while the basal production of PGF is unaffected (Spencer and Bazer, 1996). Subsequent to the action of IFNT on ESR1/OTR receptors, luteolysis does not occur and the ewe does not return to estrus. The resulting preservation of the CL and production of progesterone is necessary for the maintenance of pregnancy.

GENES

In the bovine genome, three functional IFNT genes (IFNT, IFNT2 and IFNT3) have been identified within the locus on bovine chromosome 8 (BTA8) by Walker and Roberts (2009). However, none of these genes exactly matched the cDNA sequences that were previously reported by Ealy et al. (2001). The latter reported 12 novel bovine IFNT cDNA sequences that encoded for six distinct proteins (τ1c, τ2a, τ2b, τ3ca, τ3b, and τ3e) and an additional 21 cDNA sequences that may represent additional polymorphic sequences or are by-products of RT-PCR-induced base mutations. Walker and Roberts (2009) suggested that due to incomplete sequence coverage across the bovine genome, there is potential for more than three functional IFNT genes. They reported the possibility of six IFNT genes with four or more alleles. The IFNT type I interferon may have evolved to permit the unique, synepitheliochorial placentation characterized by the Ruminantia sub-order that allows powerful conceptus signaling prior to trophoblast attachment to the uterine lining (Roberts et al., 2008).

ENDOCRINE

PROGESTERONE

Of the endocrine factors thought to play a role in EM, progesterone and its actions are the most studied. The corpus luteum (CL) is a transient endocrine organ that develops following ovulation of the dominant follicle. The small and large luteal cells of the CL secrete progesterone, a steroid that plays a central role in the establishment and maintenance of pregnancy. Progesterone begins to rise following ovulation and is maximal by day 5 when the luteal cells are fully developed. Progesterone persists through metestrus and wanes during diestrus, around day 18 of the 21-day estrous cycle, if there is no maternal recognition of pregnancy signal.

Progesterone is derived from cholesterol. Cholesterol used for steroidogenesis is derived from multiple sources. In ruminants however, high-density lipoproteins are the major source of cholesterol for luteal cells (Grummer and Carroll, 1988). Steroidogenic acute regulatory protein (StAR) transports the hydrophilic cholesterol through the cell membrane (Stocco and Clark, 1996). The P450 side chain cleavage (P450scc) enzyme catalyzes the conversion of cholesterol to pregnenolone and is the rate-limiting step in steroidogenesis. Then, 3-β hydroxysteroid dehydrogenase (3βHSD) converts pregnenolone to progesterone. Progesterone can then diffuse from the luteal cells into the bloodstream and be delivered to target tissues. In ruminants, large luteal cells produce and secrete high levels of progesterone while the small luteal cells contribute minimally to progesterone production (Hoyer and Niswender, 1985).

Progesterone levels are controlled by metabolism of the steroid by the liver. Cytochrome P450 (2C and 3A) and aldo-keto reductase are involved in progesterone inactivation before it is conjugated and excreted as a metabolite (Lemley et al., 2010). The rate of progesterone metabolism is controlled by the changes in blood flow to the liver. Numerous studies have demonstrated the relationship between dry matter intake, liver blood flow, and circulating concentrations of progesterone in the lactating dairy cow (Sangsritavong et al., 2002; Wiltbank et al., 2006). Increased circulating levels of progesterone are dependent on increased luteal tissue volume and progesterone production, and reduced blood flow to the liver and decreased rate of progesterone metabolism (Wiltbank et al., 2011; Wiltbank et al., 2014).

Progesterone concentrations directly affect fertility. For instance, high serum progesterone concentrations during preovulatory follicular growth have been associated with ~10% increase in fertility to the subsequent artificial insemination (AI) (Silva et al., 2007; Bisinotto et al., 2010a; Bisinotto et al., 2010b; Denicol et al., 2012; Stevenson and Lamb, 2016).

In contrast, incomplete luteolysis can lead to elevated levels of serum progesterone at ovulation which have been shown to be detrimental to fertility when breeding cows using synchronization and timed AI (TAI) protocols (Souza et al., 2007). High serum progesterone levels also seem to be necessary after AI for increased fertility, although results are conflicting (Bulman and Lamming, 1978; Stronge et al., 2005; Lonergan et al., 2007; Morris and Diskin, 2008). A recent study reported 34% higher serum concentrations of progesterone in high fertile cows (based on genetic merit) compared to low fertile cows from day 5 through day13 of the estrous cycle (Cummins et al., 2012). Interestingly, they also found that luteolysis occurred 2.9 days earlier in the fertile cows compared to the subfertile cows indicating genetic merit was associated with the length of the estrous cycle and alter the ability to undergo spontaneous luteolysis. A logistic regression analysis by Parr et al. (2012) showed that the optimum progesterone concentration on day 4 to day 7 to ensure the greatest probability of pregnancy per AI was 2.5-5.2 ng/ml.

There are a number of studies in beef and dairy cattle that indicated a delayed rise in circulating progesterone lead to retarded embryonic elongation, early embryonic death and low pregnancy rates (Mann and Lamming, 2001; Spencer et al., 2008; Forde et al., 2011; Forde et al., 2012; Dorniak et al., 2013). However, Minten et al. (2013) did not find differences in circulating serum progesterone between high fertile, subfertile and infertile *Bos taurus* x *Bos indicus* beef heifers between day 3 and day 16 of the estrous cycle. McMillan and Donnison (1999) did not correlate ovarian follicular turnover and circulating progesterone profiles with pregnancy rate in fertility-classified heifers from serial ET. The lack of correlation between serum progesterone levels and fertility suggested that embryo survival is dependent on more than postovulatory circulating progesterone concentration.

ENVIRONMENT

STRESS

There are numerous environmental stressors, such as heat (ambient temperature and humidity), transportation and handling stress that a dairy cow may experience. Minimizing these stressors during early lactation ensure the greatest reproductive success.

Exposure to high ambient temperature and relative humidity (heat stress) leads to rising body temperatures, increased maintenance costs, decreased dry matter intake (DMI) and depressed milk production in the cow (Huber et al., 1994). Heat stress has negative effects on steroidogenesis and oocyte quality (Zeron et al., 2001), fertilization rate (Sartori et al., 2002) and early embryo development leading to increased EM. When the average daily maximum temperature increased from 14.3°C to 34.0°C 50 to 20 days prior to AI, the CR decreased from 31.3% to 23.0%. There was no temperature effect on CR 20 days prior to AI after AI (Chebel et al., 2004). Cartmill et al. (2001) reported that increasing heat stress resulted in depressed conception rates and increased rates of EM. They found that Ovsynch initially improved pregnancy rates at d27 to d30 during elevated temperatures, but these cows experienced greater EM by d40 to d50 of gestation. They also reported that for every 10-unit increase in the temperature humidity index on the day of GnRH injection during synchronization, embryo survival decreased by 4.9±2.5%. Furthermore, transferring embryos from non-heat stressed cows may attenuate EM in cows experiencing heat stress. Drost et al. (1999) reported that transferring in vivo produced embryos from thermoneutral donors to heat stressed recipients increased pregnancy rates compared to heat stressed cows subjected to AI.

Few studies have examined the conceptus under heat stressed conditions. The conceptus wet weight was reduced in heat stressed beef cows compared to controls; however, the in vitro

synthesis and release of IFNT from cultured conceptuses (37°C) was not affected (Geisert et al., 1988). It is unknown if the in vivo synthesis and release of IFNT was altered in the heat stressed cow with a uterine temperature of 39.8°C. More research is needed to examine how heat stress alters the embryo's ability to produce and release IFNT as well as the dam's ability to respond.

ADVERSE REACTIONS TO INJECTIONS

According to the 2014 National Animal Health Monitoring System (NAHMS) Dairy Cattle Management Practices in the United States, 9.9% of dairy operations in the United States reported at least one cow had an adverse reaction to an injection. The majority of these reactions (76.2%) were reported as local swelling at the injection site. However, 14.6% of the adverse reactions contributed to abortion and 1.9% contributed to infertility (USDA-APHIS, 2016). Appropriate vaccinations and herd health programs to prevent infectious disease likely outweigh the risk associated with adverse reactions to the injection but consulting with a veterinarian is advised.

MANAGEMENT

NUTRITION

Dry matter intake, energy balance, protein and fat levels during the dry period and post-calving transitional period play a significant role on herd health, productivity and reproductive efficiency. Nutrient deficiencies must be minimized in the diets of breeding animals to decrease subfertility. It has been shown that EM rates increase with the age of the cow as well as with body condition score (BCS) (Starbuck et al., 2004). After parturition, the dairy cow's nutrient demands increase dramatically. As she approaches peak lactation yield, the cow will typically experience a state of negative energy balance (NEB) due to lactation demands exceeding dietary intake. At this time, body reserves are mobilized to meet the maintenance and lactation demands,

BCS may decrease, and ovarian function and reproductive performance can diminish (Beam and Butler, 1999). Increased DMI during the first 28 days in milk (DIM) and greater plasma concentration of insulin-like growth factor I (IGF-I) are positively associated with first service conception rate and cows having a poor BCS (≤ 2.25) at the time of first service had lower first service conception rates (Patton et al., 2007). Nebel and McGilliard (1993) reported that the timing and magnitude of NEB negatively alters the hypothalamic secretion of gonadotropin releasing hormone (GnRH), which alters the gonadotropins (luteinizing hormone; LH) release (Canfield and Butler, 1990) and therefore affects estrus expression and uterine support during early pregnancy.

Ingesting large quantities of protein, especially rumen degradable protein (RDP), have been shown to reduce fertility. Increased RDP has been shown to decrease ovarian activity. The average number of follicles (2.1 versus 1.7), size of the dominant follicle (24.8 versus 18.9 mm), total follicular area (27.9 versus 21.3 mm), number of CL (1.1 versus 0.7), size of the largest CL (17.6 versus 11.8 mm) and total luteal area (18.4 versus 12.2 mm) were depressed in multiparous lactating Holstein cattle fed high RDP diets (Garcia-Bojalil et al., 1998). Elrod and Butler (1993) and Elrod et al. (1993) reported high crude protein diets blocked the luteal phase rise in uterine pH that naturally occurs between estrus and day 7 of the cycle. This effect of uterine pH could have detrimental effects on a developing embryo. High protein diets elevate plasma urea nitrogen (PUN) levels and decrease first service conception rates (Canfield et al., 1990; Elrod and Butler, 1993). PUN was elevated at all time points (0-24 hours after feeding) in cows fed a high RDP diet versus a balanced diet (Elrod et al., 1993). Likewise, Butler et al. (1996) reported an 18% and 21% decrease in pregnancy rates when concentrations on milk urea nitrogen and PUN were greater than 19 mg/dL. Supplementing calcium salts of long-chain fatty acids into the diet may

reverse some of these effects as well as increase pregnancy rates from 52.2% to 86.3% (Garcia-Bojalil et al., 1998).

Feeding supplemental fats has also been shown to alter fertility. It is thought that high concentrations of cholesterol act to increase the size of the pre-ovulatory follicle as well as its production of estradiol (Lucy et al., 1991; Beam and Butler, 1997). Increased follicular size may increase oocyte quality and CL function (Vasconcelos et al., 2001) due to enhanced progesterone secretion, which aids to support a developing embryo (Ryan et al., 1992). Furthermore, supplementation with polyunsaturated fatty acids has been shown to prolong the lifespan of the CL by suppressing PGF secretion (Staples et al., 1998; Cheng et al., 2001; Thatcher et al., 2006). Thatcher et al. (2006) examined the effects on PGF secretion and reproductive performance when post-partum dairy cows were fed calcium salts of fatty acids containing 28% linoleic acid (Megalac-R®). Plasma PGF profiles revealed that cows fed Megalac-R® pre-partum increased substrate concentrations for arachidonic acid, the precursor for PGF. Furthermore, these cows had fewer health problems (retained fetal membranes, metritis, or mastitis) in the first 10 DIM than control cows. Cows fed Megalac-R® pre- and post-partum tended to experience higher first service conception rates (40%, 70%, and 63.6% depending on time of supplementation) than controls (27.8%) when TAI following the Ovsynch protocol (~72 DIM) (Thatcher et al., 2006). This group also reported a tendency for increased first service conception rates when supplementing pre- and post-partum cattle with C18:2 and *trans* C18:1 fatty acids (EnerG-1 Transition Formula®) (28.9%) compared to controls (25.9%). This research indicates that supplementing by-pass fats enriched in selected unsaturated fatty acids during the dry period and post-partum transitional period may improve post-partum health and pregnancy rates. However,

a conclusive result of the effects of increasing certain types of fats in the lactating cow ration on reproductive performance needs further investigation.

Many plants produce toxins that can harm cattle. Reproductive problems associated with plant toxins, such as mycotoxins (Coppock et al., 1990; Shappell et al., 2012), nitrates (Page et al., 1990; Laven et al., 2002), endophyte infected fescue (Porter and Thompson, 1992; Browning et al., 1998; Schuenemann et al., 2005), locoweed (Panter et al., 1999; Wang et al., 1999) and ponderosa pine (Ford et al., 1992; Short et al., 1992; Short et al., 1994; Wang et al., 2004) have been widely studied. Depending on location in the country, grazing cattle probably encounter these plant toxins more frequently than cattle fed a TMR but it is important to understand the physiological effects. It is worthwhile to note that mycotoxins are abundant in moldy feed so eliminating it from the diet of pregnant cows is the simplest way to avoid mycotoxin-related reproductive issues.

ESTRUS SYNCHRONIZATION

Ovsynch is one of the most commonly used protocols for estrus synchronization. It consists of three injections prior to timed ovulation and artificial insemination. The timing and hypothetical action of Ovsynch is as follows: 1) an intramuscular (IM) injection of GnRH (100μg) is administered to stimulate the ovulation of current large follicles (> 10mm) and to induce a new follicular wave, 2) PGF (25mg) is injected IM 7 days later to regress the corpora lutea, 3) a second GnRH (100μg) is injected IM 30 to 48 hours later to cause the preovulatory, dominant follicle to ovulate, and 4) TAI 16 to 24 hours later (Pursley et al., 1995; Pursley et al., 1997).

Timed ovulation allows cows to be bred by appointment, even if visual heat is not detected. Furthermore, the protocol results in pregnancy rates similar to those obtained through

AI after visual heat detection (AM-PM breeding) when cows were 60 to 75 DIM (39.4% and 26.0%, respectively). In addition, once the cows were 76 DIM or greater, the pregnancy rate for a single insemination increased to 43.4% using the Ovsynch protocol (Pursley et al., 1995; Pursley et al., 1997).

In a survey of 102 dairy farms across the US, Ovsynch was the most popular synchronization program used (38% in cows and 33% in heifers, respectively). Other popular programs were controlled intravaginal drug-releasing (CIDR) insert containing progesterone with a single injection of PGF (19% and 20%), Presynch (13% and 7%), and a single injection of PGF followed by visual estrus detection and AI (13% and 20%). However, the program that yielded the highest net present value (NPV) for each farm was dependent on farm costs, labor efficiencies, reproductive performance, and lactation number (Olynk and Wolf, 2008). Caraviello et al. (2006) reported that 78% of large, high yielding dairy farms used estrus detection and 87% used hormonal synchronization programs. It is common practice to use a combination of hormonal synchronization and visual estrus detection.

Olynk and Wolf (2008) also found the hormone cost per dose varied significantly. They reported that PGF costs per dose ranged from $1.25 to $6.00/dose. This was similar to what Nebel and Jobst (1998) reported where PGF hormone ranged from $2.50 to $5.50/dose and averaged $3.30/dose. With such a large variation in hormone costs per dose, it may alter reproductive management program decisions on individual farms.

It is important to note that some programs are more sensitive to increased labor costs than others. For example, visual heat detection requires more labor hours than Ovsynch. Depending on the labor cost and reproductive efficiency, a visual heat detection program may not be suitable for all farms. It has been reported that Ovsynch with a 30% CR and 2.1 minutes per injection had

a greater NPV than AI submission rate of 65% with visual heat detection (2.15h/day) when labor costs were ≥$19.00/h in first lactation cows, ≥$11.50/h in second lactation cows, and ≥$10.00/h in third lactation cows. Furthermore, Ovsynch with a 30% CR and 2.1 minutes per injection had a greater NPV than AI submission rate of 65% with visual heat detection (2.6h/day) when labor costs were ≥$15.00/h in first lactation cows, ≥$9.00/h in second lactation cows, and ≥$8.50/h in third lactation cows (Olynk and Wolf, 2008). Due to the vast differences between individual farms, determining which reproductive management program will be most effective can be difficult. The main goal is to increase reproductive success while minimizing direct (hormones) and indirect (labor) costs.

RECOMBINANT BOVINE SOMATOTROPIN

Bovine somatotropin (bST) has a positive effect on glucose metabolism and increases readily available glucose as a result (Peel and Bauman, 1987; Bauman et al., 1988). Administration of recombinant bST (rbST) during the lactation can increase milk production by 15% but due to consumer demands, it has restrictive use in supply chains. According to the NAHMS survey reflecting dairy cattle in 2014 (USDA-APHIS, 2016), 14.7% of all cows in the US (9.7% of operations) received rbST during the most recent lactation. Large herds (> 500 cows) used more rbST (28.6%) than small herds (< 30 cows; 1.5%).

Treatment with rbST has been shown to decrease estrus expression and thus decrease reproductive performance in lactating dairy cows (Cole et al., 1992; Zhao et al., 1992). However, when reproductive management eliminates the need for visible estrus detection, rbST increases pregnancy rates (Moreira et al., 2000; Moreira et al., 2001; Moreira et al., 2002). Pregnancy rates at day 32 of gestation were significantly increased in cows treated with rbST at 63 DIM (39.1%) and at 73 DIM (50.6%) compared to control cows with similar DIM (34.4%) (Moreira et al.,

2001). Recombinant bST affected early embryonic development in superovulated cows by decreasing the number of unfertilized ova per flush. Furthermore, rbST treated recipient cows had increased pregnancy rates over controls (25.6% for control-recipient/control-embryo, 43.2% for rbST-recipient/control-embryo, 56.1% for control-recipient/rbST-embryo, and 43.3% for rbST-recipient/rbST-embryo) but there was no additive effect when rbST-recipients received rbST-embryos (Moreira et al., 2002). Thatcher et al. (2006) reported cows that were fed fish oil-enriched diets and received rbST had increased pregnancy rates (40% versus 83%), conceptus length (34 cm versus 45 cm), and IFNT concentrations in uterine flushings (5.3 μg versus 9.4 μg).

GENETICS

CONTRIBUTIONS TO EMBRYO MORTALITY

Genetic causes of EM include chromosomal defects, individual gene defects, and deleterious genetic interactions (VanRaden and Miller, 2006). Maternal inbreeding has been shown to decrease the 70 day non-return rates by 2% for every 10% of inbreeding of the dam (Cassell et al., 2003). Furthermore, inbreeding of the embryo results in a reduction of the 70-day non-return rate by 1% per 10% increase in the level of inbreeding (Cassell et al., 2003; VanRaden and Miller, 2006). Another genetic disorder, aneuploidy, is an abnormal number of chromosomes in some or all of the embryonic cells. A common cause for this genetic defect is polyspermy (fertilization of an egg by multiple sperm) and the condition is lethal. Saacke et al. (2000) reported a higher incidence of polyspermy when AI occurred closer to the time of ovulation instead of the optimal time, 12 hours after the onset of estrus. Further research is required to completely understand the genetic contribution to EM and infertility in dairy cattle.

GENOMICS

Traditional genetic evaluations were based on milk production records and programs for type traits from breed associations. Predicted transmitting abilities (PTA) were developed to predict the performance of the future offspring relative to the population mean and were mainly used to predict the difference in an animal's offspring due to the genes transmitted by the parent. The widespread use of superior bulls through AI allowed immense genetic progress in dairy cattle, however, identifying these bulls was costly and time consuming because of the reliance on data from milking daughters (Wiggans et al., 2011). Fortunately, genomic evaluation has allowed the genetic merit of the animal to be predicted earlier in life with higher reliability than parent averages.

Genomic evaluations are based on single nucleotide polymorphisms (SNP) and haplotypes within the DNA of the animal. These markers are used to calculate a direct genomic value (DGV) for all traits currently used by the industry (milk production, type, fertility, etc.). The DGV is then added to the traditional PTA model creating the genomic PTA (gPTA). The gPTA reflects the animal's true genetic merit and represents the genetic predictions from the SNP, pedigree, progeny, and performance records. Overall, genomics increases the reliability of a trait. Reliability is the measure of how much information contributes to the evaluation. For example, in genomic evaluations, reliability combines genomic daughter equivalents, parent average, and traditional pedigree-based evaluation information (Wiggans et al., 2011). Comparing August 2006 traditional parent averages for young sires to their August 2006 genomic evaluations plus traditional parent averages demonstrated an increase in evaluation reliability. Reliability gains for Holsteins above parent average ranged from 2.7% (daughter stillbirth) to 47.6% (fat %). Furthermore, the reliability for milk (kg) increased by 29.4

percentage points (from 38.1% to 67.5%) while DPR increased by 17.0 percentage points (from 29.8% to 46.8%) (Wiggans et al., 2011). This improvement in reliability is evidenced by the increasing use of semen from young sires (0.8 to 3.9 years of age) compared to first-crop (4.0 to 7.9 years of age) and old, proven bulls (≥8.0 years of age). In 2007, nongenotyped, Holstein young sires accounted for 28% of breedings in the dairy industry. By 2012, 51% of Holstein breedings were from genotyped young bulls. During these years, first-crop sire breedings decreased from 56% to 40% and old sire breedings decreased from 16% to 9%. Furthermore, the average use of young sires in Holstein herds across the U.S increased from 28.5% to 50.9% between 2006 and 2012 (Hutchison et al., 2014).

Studying linkage disequilibrium (LD), non-random association between two loci within a population, and creating HapMaps (haplotype block maps) is an active area of research in genetics. Haplotype blocks are regions of the chromosome with high LD and low haplotype diversity flanked by recombination hotspots. In the past, microsatellite markers were used to estimate LD in dairy cattle populations. More recently, however, LD has been estimated using high-density SNP genotyping (Gautier et al., 2007; Khatkar et al., 2007; McKay et al., 2007). Khatkar et al. (2007) was one of the first to describe haplotype blocks in the bovine genome utilizing this method. Using Holstein-Friesian bulls, they found 727 haplotype blocks comprised of three or more SNP that covered 50,638 kb of the bovine sequence map. These sequences corresponded to 2.18% of the combined length of all the autosomes in the cow. Also identified were 1068 haplotype blocks comprised of two SNP. Another study performed by Kim and Kirkpatrick (2009) identified haplotype blocks in Holstein cattle using SNP genotyping and familial relationships. They detected a total of 119 haplotype blocks comprised of at least four SNP that were equally distributed in both intergenic and intragenic regions across the bovine

genome. Constructing haplotypes and using them directly in genome-wide association studies involving a particular region of the genome (Druet and Georges, 2010) could prove to be a useful tool in trait selection.

Weigel et al. (2010) performed an experiment to evaluate the predictive ability of DGV for commonly used and economically important traits (milk yield, protein percentage, and daughter pregnancy rate (DPR)) when a large number of SNP genotypes had been imputed based on haplotypes in the populations rather than measured directly. In this study, 1,446 Jersey bulls were used in the training set and 316 in the low-density genotyping testing set. When 96.6% of SNP were masked in the testing set, the correlation between DGV and PTA was 0.62. They found low-density genotyping of about 3,000 equally spaced SNP, followed by imputation of missing high-density genotypes from a reference panel could provide approximately 95% of the predictive ability achieved through high-density genotyping. This approach has practical implications for commercial dairy farms because accurate imputation from a low-density chip to a high SNP level can increase the reliability of the PTA at a reduced cost. For example, the cost-effective, low-density SNPchip could be used to screen young sires, identify genetically superior cows in the lactating herd, or select heifers to breed with high quality or sexed semen.

CONTRIBUTIONS TO INFERTILITY

Daughter pregnancy rate is one of the most commonly used fertility traits. It is defined as the percentage of nonpregnant cows that become pregnant during each 21-day period. However, conventional approaches to improving fertility by genetic selection are difficult because most reproductive traits are polygenic (complex) and lowly heritable (h^2 = ~0.04) (Oseni et al., 2004; VanRaden et al., 2004; Weigel, 2006; Berry et al., 2012). Mechanisms such as pleiotropic gene effects, linkage, and physiological associations likely contribute to the complexity of fertility

(Veerkamp and Beerda, 2007). Furthermore, it may take years to acquire phenotypes for some of the fertility traits, such as calving interval (Berry et al., 2012). However, with the use of genomics, gains in reliability are apparent. Wiggans et al. (2011) reported the observed reliability from parent averages for DPR was 29.8% in August 2006 but increased to 46.8% (17.0% gain) when the genomic evaluation (including SNP and polygenic effects) were added to the traditional parent averages.

Utilizing genomic tools to analyze the parental contributions to the offspring genome could advance genetic selection. Kropp et al. (2014) proposed that as fertility declines, SNP biomarkers could be generated from SNP analysis and used as predictors for embryo selection, improved fertility, and healthier offspring. Genome-wide association study (GWAS) is one genomic tool to identify parental genotypes as predictors for the progeny phenotypes (Feugang et al., 2009; Cole et al., 2011; Berry et al., 2012).

Daughter pregnancy rate has been reported to be positively correlated with heifer conception rate, cow conception rate, productive life, and net merit, and negatively correlated with milk yield, fat yield, and protein yield (Cochran et al., 2013). Cole et al. (2011) conducted a GWAS analysis for 31 production, health, reproduction and body conformation traits in high-producing Holstein dairy cows. This study found 40 genes significantly associated with DPR on *Bos taurus* chromosome (BTA) 3, BTA7, and BTAX. Interestingly, not all SNP associated with DPR showed association with milk production traits. Cochran et al. (2013) also found 40 SNP linearly related to DPR using a candidate gene approach, but only 11 were negatively associated with milk yield, fat yield or protein yield. These findings are encouraging as it may be possible to select for DPR without compromising current milk production. An Italian study selected 58 SNP in 25 candidate genes located on 14 chromosomes to evaluate their association with six

single traits for milk production, such as milk yield and milk fat percentage, as well as three complex indexes: longevity, fertility, and productivity-functionality (Fontanesi et al., 2014). Of the 25 genes analyzed, only two genes showed significance for the fertility index: Acetyl-CoA carboxylase alpha (ACACA) on BTA19 and prolactin receptor (PRLR) on BTA20. It is worthwhile to note that Fontenasi and collaborators reported 22 SNP that were associated with more than one trait. This finding indicates pleiotropic gene effects and confirms there are positive and negative genetic correlations between traits. An example of negative association is that some favorable milk traits are unfavorable for fertility and longevity traits.

To define mechanisms contributing to suboptimal reproductive performance in dairy cattle, Moore et al. (2016) combined transciptomic data from endometrium and CL on day 13 of the estrous cycle with GWAS data. Layering technologies allows for SNP discovery that causes a mutation or change in protein expression. Differentially expressed genes in the endometrium were involved in uterine inflammation, energy status, and PGF synthesis and secretion. Differentially expressed genes in the CL were involved in PGF response, steroidogenesis, and mRNA processing. This study also found 93 quantitative trait loci (QTL) primarily located on BTA5, BTA7, BTA8, BTA18, BTA29 associated with various fertility traits. Other studies provide evidence that fertility associated SNP (Hoglund et al., 2014) and QTL (Hoglund et al., 2015) are concentrated on BTA1, BTA4, BTA6, BTA7, BTA9, BTA11, BTA13, BTA15 and BTA24.

Minten et al. (2013) also combined microarray and GWAS data to understand fertility. Endometrial samples on day 14 of the estrous cycle did not cluster based on fertility classification (high fertile, subfertile, or infertile) in a bovine microarray using a false discovery rate (FDR) and showed homogenous gene expression. However, when analyzed without

controlling for FDR, nominal differences were found between the high fertile versus infertile (15 upregulated, 40 downregulated), high fertile versus subfertile (31 upregulated, 107 downregulated), and subfertile versus infertile groups (40 upregulated, 19 downregulated). This study also conducted a GWAS on DNA from 38 fertility-classified heifers analyzing 707,971 SNP. This study reported moderate evidence for fertility QTL on BTA1, BTA8, BTA9, and BTA19 (Minten et al., 2013). BTA8 is of particular importance to our study as the IFNT genes reside on chromosome 8. Unfortunately, this chromosome has poor sequence and annotated areas so IFNT-related SNP have yet to be identified.

The most efficient way to increase reliability of the PTA will be to add predictor animals that have traditional evaluations but have yet to be genotyped. (Wiggans et al., 2011) Furthermore, if enough high-density genotypes are evaluated, it is hypothesized that most haplotypes will be represented within the breed. This information could allow the discovery of SNP that are quantitative trait nucleotides (causal mutations) or accurately track QTL alleles. Collaboration is the least expensive way to accomplish these goals because thousands of genotypes will be required. Current collaboration projects include Interbull (http://www.interbull.org/ib/interbull) EuroGenomics (https://global.crv4all.com/information/genomicselection/eurogenomics/), the 1000 bull genomes project (http://www.1000bullgenomes.com) and individual countries trading genotypes for specific breeds.

New SNP could be identified through both traditional and functional genomics and added to the evaluations to increase reliability of the PTA. Although the Illumina BovineSNP50 BeadChip features 54,001 informative SNP evenly spaced across the bovine genome, 63% of these are intergenic and there are over 14,000 genes that have no SNP on the chip (Matukumalli

et al., 2009; Michelizzi et al., 2010). Incorporating SNP for specific genes involved in fertility, milk production, and herd health is one way to improve PTA reliability. This addition could result in a noticeable increase in the rate of genetic gain because selection could be applied at birth (or before) to increase selection intensity. The future use of genomics in dairy cattle is bright. Genomics may be used commercially to identify heifers and cows that are genetically superior that should stay in the herd, select cows to be inseminated with semen from young sires or sexed semen, determine which cows should be used for embryo flush and transfer, and control inbreeding with more accurate parentage information (Wiggans et al., 2011).

RATIONALE, EXPERIMENT AND HYPOTHESIS

It is evident that genetic selection has significantly increased milk production in dairy cattle over the last 60 years. Unfortunately, fertility has declined during this same time period. Infertility, including EM, greatly limits reproductive efficiency and is a major cost factor to the cattle industry. This experiment was designed to combine DPR, a fertility trait with low heritability and reliability, and on-farm production records, such as services per conception (SPC), to increase genetic selection for fertility in lactating Holstein cows. The hypothesis of this study was that selection based on greater DGV-DPR and decreased SPC can effectively predict improved fertility in lactating dairy cattle through enhanced IFNT release and action. This experiment was designed to 1) determine if the IFNT protein expression is depressed in low compared to high fertility dairy cows, 2) determine if the paracrine action of IFNT on the endometrium is impaired in low compared to high fertility dairy cows, and 3) determine if the endocrine action of IFNT on PBMC is decreased in low compared to high fertility dairy cows.

CHAPTER 2

SELECTION FOR HIGH FERTILITY MAY BE DEPENDENT ON CONCEPTUS-DERIVED SIGNALS IN LACTATING HOLSTEIN COWS

INTRODUCTION

Infertility is problematic across all mammalian species and EM is considered to be the greatest limitation to reproductive efficiency (Bazer and First, 1983). These issues negatively impact the production and economics of all cattle industries, especially dairy (De Vries, 2006). Improved management, adoption of technologies, better nutrition, and intense genetic selection of dairy cows has led to increased milk production. From1950-2000, milk production per cow rose from 2,415 kg to 8,275 kg per cow (Blayney, 2002). In contrast, during the same period, first service conception rates declined from 66% in 1951 to 50% in 1975 (Butler and Smith, 1989), and 40% in 1996 (Butler, 1998).

Fertilization rates are highly variable (55-88%) among high yielding dairy cows in comparison to rates in moderately productive or not heat-stressed cows (80-90%) (Wiebold, 1988; Ryan et al., 1993; Sartori et al., 2002). In comparison to fertilization rates, first service calving rates average about 40-55%, suggesting EM rates of 35-50% (Royal et al., 2000; Diskin et al., 2006). Early embryonic death prior to day 24 of gestation has been estimated to be 43% in Holstein-Friesian cattle (Diskin et al., 2006), a figure much higher than estimates of late (day 28-84 of gestation) embryonic death rate of 7% (Silke et al., 2002). Early EM is most common between days 7-16 of gestation (Diskin and Sreenan, 1980; Roche et al., 1981; Berg et al., 2010). During this period, the spherical blastocyst hatches from the zona pellucida (day 9-10) and grows into an ovoid shape (day 12-14) (Betteridge, 1988). The conceptus begins elongation and

doubles in length every day between days 9 and 16 (Berg et al., 2010) at which time point, the conceptus begins attachment and placentation. During elongation, it is critical that the conceptus produce robust amounts of interferon tau (IFNT). In ruminants, IFNT attenuates the release of endometrial prostaglandin $F_2\alpha$ (PGF) in a paracrine manner to initiate maternal recognition of pregnancy. By inhibiting the luteolytic release of PGF from the endometrium, the production of progesterone from the CL of the ovary is sustained and pregnancy persists (Thatcher et al., 1984; Knickerbocker et al., 1986a; Bazer et al., 1996). IFNT from the conceptus has endocrine action on immune cells and the CL that contributes to sustaining pregnancy. Collectively, robust IFNT production and release is essential for survival of the embryo.

Decreased reproductive efficiency is multifactorial and associated with high milk production, stress, increased herd size, labor shortages and higher inbreeding percentages (Lucy, 2001). There are numerous postulated causes for EM, including genetic, physiological, endocrine and environmental factors. Experiments to examine each causative factor of EM in dairy cow infertility are limited. Also, genetic selection for improved fertility is particularly difficult because fertility traits are polygenic and tend to have low heritability (h^2 = ~0.04) (Oseni et al., 2004; VanRaden et al., 2004). For example, the fertility trait DPR is lowly heritable ($h^2 \leq 0.10$). Clarifide® (Zoetis), a genetic screening tool based on gene haplotype and SNP analysis, provides the direct genomic value for DPR (DGV-DPR) from the individual animal's DNA. This information is then interrogated along with analysis of progeny and production records to calculate the gPTA-DPR. These new genomic selection tools, in addition to standard production data such as SPC, may lead to selecting cattle with greater fertility and more robust signaling between the embryo and dam. This technique could curtail early embryonic death and increase genetic improvement. To investigate the genetic influences on infertility and EM in dairy cattle,

DGV-DPR analysis of DNA was coupled with the previous lactation SPC records for each cow to effectively select for greater fertility.

It was hypothesized that selection based on greater DGV-DPR and decreased SPC can effectively predict improved fertility in dairy cattle through more advanced development of the conceptus and enhanced production and action of IFNT. Greater IFNT release, paracrine action on the endometrium and endocrine action on maternal immune cells and the CL would promote maternal recognition of pregnancy through sustained survival of the CL and ensure placentation and implantation of the embryo.

MATERIALS AND METHODS

ANIMAL CARE AND SELECTION OF LACTATING DAIRY COWS

Cattle handling, housing and tissue sampling procedures were reviewed and approved by the Colorado State University Animal Care and Use Committee (protocol #14-5190). White blood cell DNA was isolated from blood collected in healthy, freshening U.S. Holstein dairy cattle (n = 86) entering the 2^{nd}-3^{rd} lactation. DNA for each cow was analyzed (Clarifide®; Zoetis, Kalamazoo, MI) to estimate the DGV-DPR, which was a summation of the effects of ~19,000 SNP and haplotypes. The SNP-effects were estimated (September 1, 2014) from the reproductive performance records and breeding values of approximately 10,000 U.S. Holstein cows. After identifying the top 20% and bottom 10% of cows based on DGV-DPR, they were further ranked for fertility based on the number of SPC from the previous lactation. Fourteen cows with the highest DGV-DPR and lowest number of SPC were selected for the HF group and randomly assigned to non-pregnant and pregnant groups (n = 7 non-pregnant: NP; and 7 pregnant: HP) (Table 1). Cows with the lowest DGV-DPR and greatest number of SPC were selected for the LF group (n = 7 pregnant: LP). Cows with conflicting DGV-DPR and SPC values, as well as

TABLE 1.
Trait and lactation information (mean ± standard error) in 21 Holstein cows classified as low or high fertility.

Trait	Fertility Classification		
	NP[1]	LP[2]	HP[1]
Number of cows	7	7	7
DGV-DPR[3]	-1.50 ± 000.06[a]	-2.31 ± 000.17[b]	-1.10 ± 000.18[a]
gPTA-DPR[4]	0.22 ± 000.70[a]	-0.61 ± 000.35[a]	-0.09 ± 000.64[a]
gPTA-DPR Reliability[5]	36.57 ± 000.81[a]	34.43 ± 002.59[a]	34.83 ± 001.87[a]
SPC[6]	1.29 ± 000.18[a]	3.71 ± 000.42[b]	1.43 ± 000.20[a]
gPTA-Milk[7]	1627.90 ± 371.11[a]	1185.14 ± 376.89[a]	1782.20 ± 328.88[a]
gPTA-Milk Reliability[8]	62.14 ± 000.99[a]	61.86 ± 001.40[a]	63.00 ± 000.63[a]
Days in Milk	18.00 ± 003.00[a]	16.00 ± 002.77[a]	15.00 ± 002.16[a]
Number of lactations	2.43 ± 000.20[a]	2.71 ± 000.18[a]	2.43 ± 000.20[a]
Julian Calving Date	223.00 ± 003.00[a]	225.00 ± 002.77[a]	226.00 ± 002.16[a]

[ab] Within rows are declared significant at $P < 0.05$.
[1]High fertility group (high fertile nonpregnant, NP; high fertile pregnant, HP).
[2]Low fertility group (low fertile pregnant, LP).
[3]Direct genomic value for daughter pregnancy rate (9/30/14).
[4]Genomic predicted transmitting ability for daughter pregnancy rate (3/3/15).
[5]Reliability of the genomic predicted transmitting ability for daughter pregnancy rate (3/3/15).
[6]Services per conception from previous lactation (10/1/14).
[7]Genomic predicted transmitting ability for milk production (3/3/15).
[8]Reliability of the genomic predicted transmitting ability for milk production (3/3/15).

unhealthy cows were omitted from the study. Cows were managed together in a single pen of 200 cows prior to selection. After selection, cows were managed as a group of 21 cows in a single pen. The study was initiated in November 2014 to eliminate the impact of heat stress. However, for cows that required multiple cycles to produce an embryo, minimal heat stress may have been experienced in early Spring (March 2015). The 21 cows were daughters of 6 sires. One LP cow was removed from the study and all subsequent analysis due to a uterine abscess and inability to produce an embryo.

EMBRYO FLUSH AND COLLECTION OF ENDOMETRIUM, PBMC AND MILK SAMPLES

Cows were bred by TAI following the 60-day voluntary wait period (60-days post calving). Estrous cycles were synchronized using a variation of the Ovsynch program (GnRH, 7 days, Prostaglandin, 52 hours, GnRH, 16-18 hours, TAI). Cows that did not have a CL two weeks in a row were enrolled in a 7-day CIDR-synch program with the same intervals as the standard Ovsynch program (Pursley et al., 1997). The cows that were bred to standing estrus were inseminated on an "AM-PM" schedule. All cows assigned to the pregnant groups were inseminated using semen from the same HF bull (Ronelee Gold Digger; Accelerated Genetics, Baraboo, WI). The relevant PTA for this bull were DPR (+1.8), heifer conception rate (HCR; +2.9) and cow conception rate (CCR; +3.5). Furthermore, this bull is not a carrier for any of the known detrimental fertility haplotypes. All NP cows were not inseminated and considered day 0 at the onset of estrus. On day 16 of pregnancy, embryos were collected from LF (n = 7) and HF (n = 7) dairy cows using a non-surgical embryo flush technique (Elsden et al., 1976; Rowe et al., 1976) with Dulbecco's Phosphate Buffered Saline (PBS) (Sigma; St. Louis, MO) containing 0.1% polyvinyl acetate (Sigma; St. Louis, MO) and Bioniche Vigro Complete Flush media

(MWI Veterinary Supply; Boise, ID). The uteri of 7 HF NP cows were flushed to serve as negative controls.

Embryos were examined for the following morphologic criteria: color, size and tubular structure using a stereo-microscope (Stereo Star Zoom, American Optical) at 7X magnification. Embryos were photographed beside a ruler to determine length at a later time. Then, embryos were typed as viable (clear or white, translucent, tubular and > 75 mm in length) or undergoing EM (pink or red, opaque, collapsed and < 85 mm in length). The conceptus was cut into halves with a sterile scalpel blade. One half of each embryo was placed into a microcentrifuge tube, snap-frozen in liquid N_2 and stored at -80° C until RNA, DNA and protein isolation and analysis. The other half of each embryo was cultured at 38.6° C, 5% CO_2, 5.0% O_2, 90% N_2 for 24 h in DMEM without serum + 1% amphotericin B, penicillin, streptomycin antibiotic mix (VWR International; Radnor, PA). Following culture, the supernatant and embryonic tissue was frozen and stored at –80° C. Uterine luminal flushings (UF) were snap-frozen in liquid N_2 and stored at -80° C for protein analysis. After collection of the embryo, endometrial biopsies were obtained using transcervical Jackson uterine biopsy forceps with a cutting area of 4 mm x 28 mm (Jorvet, Jorgensen Laboratories, Inc.; Loveland, CO). Each biopsy was divided; one half was immediately frozen in liquid N_2 and stored at -80°C until processed for RNA, DNA and protein analysis and the other half was cultured for 24 h, frozen and stored at – 80° C. Blood from the jugular vein was collected into serum collection tubes, centrifuged at 2,000 rpm for 10 min at 4°C and the serum was aliquoted and frozen at -80° C.

For isolation of peripheral blood mononuclear cells (PBMC), blood was collected in 10 ml EDTA blood collection tubes (Becton, Dickinson and Company; Franklin Lakes, NJ), centrifuged at 2,000 rpm for 10 min at 4° C. The buffy coat was transferred to a 15 ml centrifuge

tube containing 20 ml of ammonium chloride lysis buffer and incubated at room temperature for 15 min. PBMC were pelleted by centrifugation at 2,000 rpm for 10 minutes at 4° C, washed in 5 ml of sterile BioWhittaker Hank's Balanced Salt Solution without calcium, magnesium or Phenol Red (Lonza; Walkersville, MD). The PBMC were then frozen in liquid N_2 and stored at -80° C until processed for RNA, DNA and protein analysis.

Whole milk (100 ml) was centrifuged at 5,000 rpm for 20 min at 4° C. The milk fat was removed, the liquid portion decanted and the pelleted milk cells washed in 10 ml of PBS by centrifugation at 5,000 rpm for 5 minutes at 4° C. The milk cells were resuspended in 1 ml of PBS, transferred to a 1.5 ml microcentrifuge tube (Safe-Lock Tubes, Eppendorf; Hauppauge, NY) and the cells were pelleted by centrifugation at 5,000 rpm for 5 minutes at 4° C. The supernatant was decanted and milk cells were frozen in liquid N_2 and stored at -80° C until RNA, DNA and protein isolation and analysis.

CELL CULTURE

The conceptus and endometrial tissue was washed in culture media three times and minced with a scalpel blade. Minced explants were distributed into three culture wells of a 12-well Falcon tissue culture plate (Corning Inc; Durham, NC). Each well had a 6 ml well volume. Conceptus was cultured in 3 ml of DMEM/F12 (Gibco, Life Technologies; Carlsbad, CA) + 1% amphotericin B, penicillin, streptomycin antibiotic mix (VWR International; Radnor, PA) per well for 24 hours in a tri-gas incubator (38.6° C, 5.0% CO_2, 5.0% O_2, 90% N_2). Endometrial tissue was incubated in 3 ml of DMEM (Gibco, Life Technologies; Carlsbad, CA) + 1% amphotericin B, penicillin, streptomycin antibiotic mix (VWR International; Radnor, PA) per well for 24 hours at 37.0°C, 5.0% CO_2. Following culture, supernatant (conceptus secretory

protein; CSP and endometrial secretory protein; ESP) was removed, frozen in liquid N_2 and stored at -80° C for subsequent protein analysis.

PROGESTERONE RADIOIMMUNOASSAY

Whole blood was centrifuged at 3,000 rpm for 20 minutes in a refrigerated centrifuge (4° C) to separate clotted blood from serum. Concentrations of progesterone in serum were determined by radioimmunoassay as previously described (Niswender, 1973). All samples were analyzed in two assays. The average sensitivity of the assays was 14.7 pg/ml; the intra-assay coefficient of variation was 4.64%.

WESTERN BLOT

Uterine flush samples were centrifuged for 20 minutes at 4° C and 3,200 rpm to reduce cellular debris. Samples were concentrated and desalted using two Amicon Ultra-4 Centrifugal filters (EMD Millipore; Billerica, MA). The protein concentration was quantified utilizing a Pierce BCA Protein Assay Kit (Thermo Scientific; Waltham, MA). Forty-five μg of protein from each UF sample were loaded into a lane of 12% SDS-PAGE gels and electrophoresed for 1.5 h at 200 volts. Proteins were transferred to a nitrocellulose blotting membrane (Amersham Protran 0.2 μm NC; GE Healthcare; Pittsburgh, PA) for 1 h at 100 volts. Membranes were incubated for 1 h in 5% non-fat dry milk in Tris-buffered saline + Tween 20 with a pH of 7.5 (TBST) at room temperature, incubated with rabbit anti-bovine IFNT primary antibody (1:5000; from Dr. Michael R. Roberts, University of Missouri, Columbia, Missouri) or mouse anti-bovine ISG15 primary antibody (1:1000; (Austin et al., 2004) diluted in TBST with 5% non-fat dry milk overnight at 4° C, washed three times in TBST for 5 min and incubated with donkey anti-rabbit or donkey anti-mouse secondary antibody diluted in TBST with 1% non-fat dry milk (1:2000; Santa Cruz Biotechnology Inc.; Santa Cruz, CA) for 1 h at room temperature. Following three

more washes in TBST for 5 minutes each, proteins were detected using the Amersham ECL Prime Western Blotting Detection Reagent Kit (GE Healthcare; Pittsburgh, PA). Quantification was performed using the optical densitometry program, Image Lab 4.1, on a ChemiDoc XRS+ System with Image Lab Software (Bio-Rad Life Science; Hercules, CA).

RNA, DNA AND PROTEIN EXTRACTION

TRIzol Reagent (Life Technologies; Carlsbad, CA) was used to extract RNA and DNA from conceptus, endometrium, milk cells, and PBMC samples following the manufacturer's instructions. Fifty to 100 mg of conceptus, endometrium, PBMC or milk cells were homogenized in 1 ml of TriZol Reagent and RNA pellets were dissolved in 87.5 µl nuclease-free water. Remaining DNA was removed from the RNA fractions by treatment with RNase-Free DNase (Qiagen; Valencia, CA) and RNeasy MinElute Cleanup Kit (Qiagen; Valencia, CA). The RNA was quantified and the A260/280 and A260/230 determined using NanoDrop (NanoDrop Technologies; Wilmington, DE); A260/280 ratios greater than 1.7 and A260/230 ratios greater than 1.8 were considered clean. The DNA pellets were resuspended in 1 ml of 8 mM NaOH 86 µl and the pH adjusted to pH 8.4 with 0.1 M HEPES. The DNA concentration was quantified utilizing the NanoDrop; A260/280 ratios greater than 1.7 and A260/230 ratios greater than 1.8 were considered "pure".

Protein pellets were dissolved in 200-400 µl of 1% SDS, the insoluble material was sedimented via centrifugation at 10,000 x g for 10 minutes at 4° C and the supernatant diluted 1:10 in PBS for quantification. Protein was quantified utilizing a Pierce BCA Protein Assay Kit (Thermo Scientific; Waltham, MA) as per the manufacturer's instructions.

SEMI-QUANTITATIVE RT-PCR AND PRIMERS

Single stranded cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad Life Science; Hercules, CA). Synthesized cDNA was diluted five-fold with RNase-free water for the qRT-PCR reaction. Primer3 software was used to design primers that were optimized for a 61° C melting point and lacked self-annealing or folding at high temperatures. Primers used generated amplicons ranging from 80-120 bp. Amplicons were sequenced to confirm identity to the target gene. qRT-PCR reactions were performed in duplicate using 2 µl of cDNA, 5 µL of iQ SYBR green supermix (Bio-Rad Life Science; Hercules, CA), 1.5 µl of RNase-free water and 1.5 µl of 7.5 nM solution of each primer set per well of a 384-well plate and amplified on a LightCycler480 (Roche; Basel, Switzerland). Amplification of PCR products was performed at 95° C for 3 minutes for denaturation, followed by 40 cycles of 95° C for 30 seconds, 61° C for 30 seconds, and 72° C for 15 seconds. The reaction products were assessed for quality by melting curve analysis on the LightCycler 480 SW 1.5.1.62 SW program (Roche; Basel, Switzerland). The qRT-PCR results were analyzed using the comparative Ct (ΔCt) method described by Livak and Schmittgen (2001). The data are presented relative to the house keeping genes, GAPDH and 18s, concentrations and the values are represented as $2^{-\Delta Ct}$. Housekeeping genes, target genes, and primers are listed in Table 2**.**

STATISTICS

The statistical analyses were performed using R statistics (version R.3.2.3; R Foundation for Statistical Computing, Vienna, Austria) (Team, 2015). Correlations between variables, such as DGV-DPR, SPC and IFNT, were completed using the lm and anova procedures to fit the regression and test for analysis of variance (ANOVA). Nonpregnant HF, pregnant HF and pregnant LF were entered as equal main effects and analyzed for analysis of variance using the

TABLE 2.
Oligonucleotide primer sequences used for semi-quantitative RT-PCR

Target	**Accession**	**Primer Sequences**
ISG15	NM_174366	F: ggtatccgagctgaagcagtt
		R: acctccctgctgtcaaggt
GAPDH	NM_001034034	F: tgacccttcattgaccttc
		R: cgttctctgccttgactgtg
18s Ribosomal RNA	NR_036642	F: gaacgagactctgggcatgc
		R: ctgaacgccacttgtccctc

aov, lsmeans, and cld procedures in the lsmeans (Lenth, 2016) and multcompView (Graves et al., 2015) packages. Differences between groups were considered a tendency when $P < 0.10$ and significant when $P < 0.05$. Main effects of pregnancy (NP versus P), fertility (LF versus HF) and embryo quality (viable versus EM) were also examined using ANOVA where appropriate. Other than correlation data, values represent the arithmetic mean ± standard error.

RESULTS

ANIMAL MODEL

There were no differences in gPTA-DPR, gPTA-MILK, days in milk, number of lactations, and Julian calving date between NP, HF and LF groups (Table 1). The DGV-DPR was greater ($P < 0.05$) and SPC from the previous lactation was lower ($P < 0.05$) in the high fertile NP and HP groups compared to the low fertile LP group (Table 1). As predicted, the DGV-DPR was negatively correlated with SPC ($r = -0.57$; $R^2 = 0.33$; $P < 0.05$) (Fig. 1).

EMBRYO CLASSIFICATION

Embryos were classified based on morphology and length (Fig. 2A). Healthy embryos were translucent, long, and did not collapse. EM embryos were darker and red in appearance, narrower and shorter. EM embryos were sticky and often collapsed during manipulation with a pipette. High pregnant cows had more viable embryos (n = 5) than the LP cows (n = 3) (Table 3; panel B in Fig. 2). Viable embryos (114.5 ± 8.5 mm) were more than double the length of the EM embryos (50.4 ± 11.5 mm) ($P < 0.05$, Fig. 3A). There was no difference in length of LP compared to HP embryos when all embryos were examined (Fig. 3B). However when only viable embryos were examined, HP (124.5 ± 5.6mm) tended ($P < 0.10$) to be longer than LP (94.5 ± 17.5mm) embryos (Fig. 3C). There was no difference in length of EM embryos (Fig. 3D). Due to damaged DNA and limitation of methods, definitive parental information for the EM

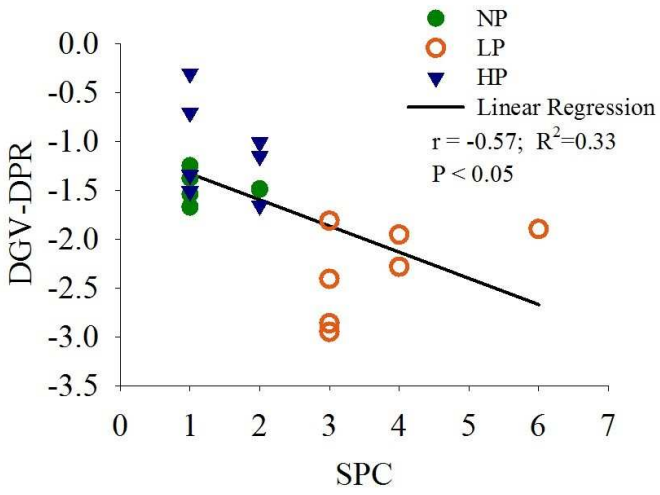
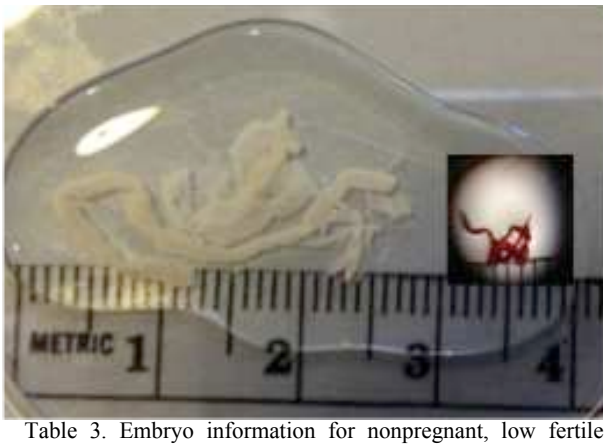


FIGURE 1.
Relationship between DGV-DPR and SPC in lactating dairy cows. The coefficient of determination (R^2) is the percent of variance explained by the model.

A



B

Table 3. Embryo information for nonpregnant, low fertile pregnant and high fertile pregnant Holstein cows.

Trait	Fertility Classification		
	NP[1]	LP[2]	HP[1]
Number of cows	7	7	7
Total embryos	0	6	7
Viable embryos	0	3	5
Embryo mortality	0	3	2

[1]High fertility group (high fertile nonpregnant, NP; high fertile pregnant, HP).
[2]Low fertility group (low fertile pregnant, LP).

FIGURE 2.
Embryo classification (A) and numbers (B) of embryos from low and high fertile pregnant dairy cows. Panel A shows a viable, long and translucent conceptus compared to a collapsed, narrow, short and dark red EM conceptus (insert). The EM conceptus was photographed through the ocular of a stereo-microscope (Stereo Star Zoom, American Optical) at 7X magnification. Note the guides on the metric ruler are aligned in both photos to provide perspective regarding the size of these conceptuses.

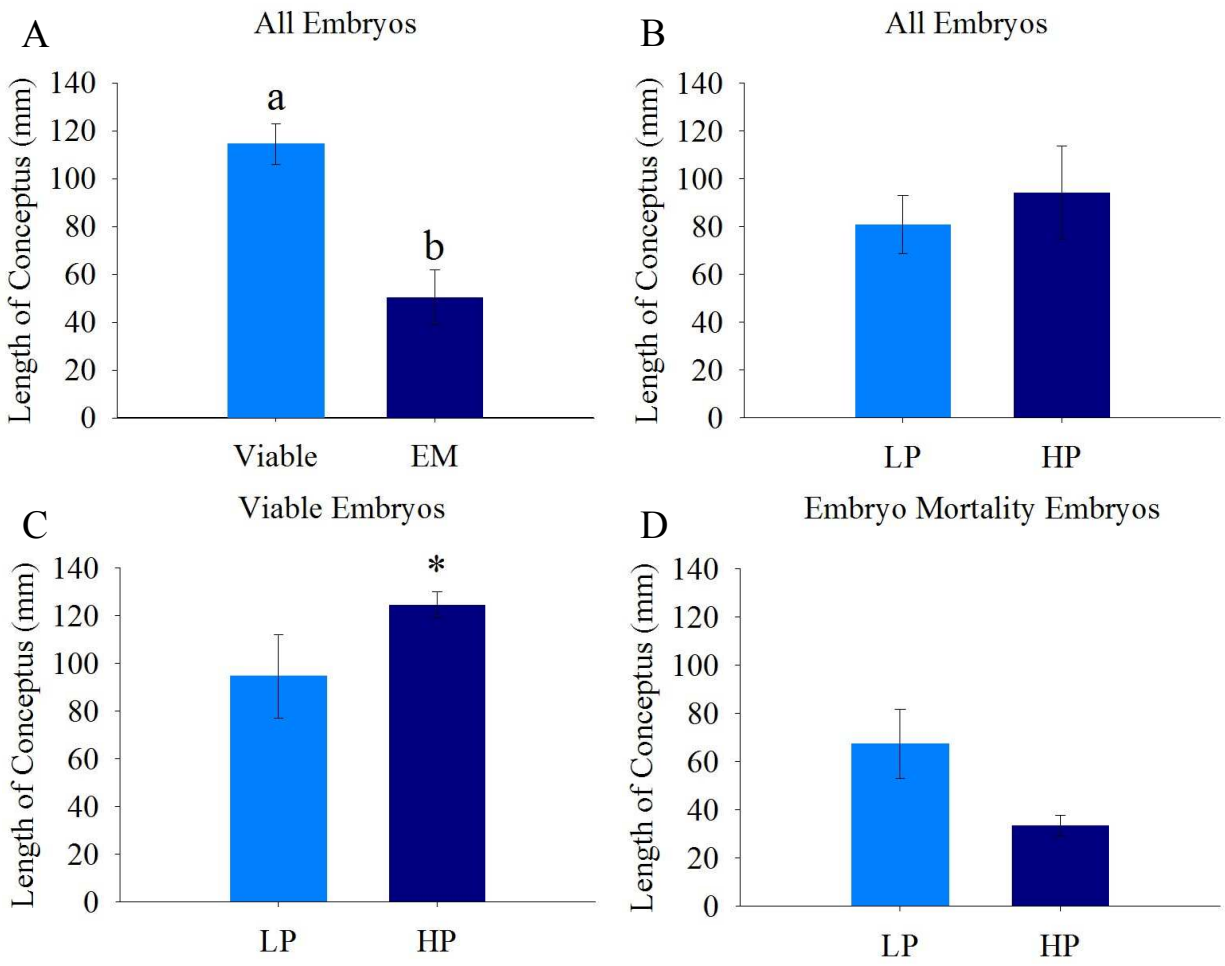


FIGURE 3.
Embryo length (A-D) from low and high fertile pregnant dairy cows. Values represent mean ± standard error. Means marked with different superscript letters differ ($P < 0.05$). * - Represents a tendency ($P < 0.10$).

conceptuses was not obtained. The amount of conceptus tissue was limiting and used as a priority for RNAseq analysis. Briefly, analysis of conceptus RNA revealed that all conceptuses, whether LP or HP, viable or EM, contained detectable IFNT mRNA concentrations (Liebig and co-workers; unpublished data). However, IFNT levels in EM conceptuses were significantly diminished compared to those found in LP or HP conceptuses. This result is interpreted to mean that tissues collected in uterine flushings and analyzed were indeed conceptuses, rather than other cellular debris collected from the uterine lumen.

SERUM PROGESTERONE CONCENTRATION

Serum progesterone concentrations were lower ($P < 0.05$) in LP compared to NP cows and tended ($P < 0.10$) to be lower in HP compared to NP cows (Fig. 4A). In cows with viable embryos only, serum progesterone concentrations did not differ (Fig 4B). In only cows with EM embryos, serum progesterone tended to be lower in LP compared to NP cows (Fig. 4C). Serum progesterone concentrations may be limiting in LP cows with EM because of luteal insufficiency, premature luteolysis or both. The serum progesterone concentrations are within normal ranges for dairy cows at this stage of gestation or estrous cycle (Plante et al., 1991).

IFNT CONCENTRATION

IFNT appeared as a doublet migrating at about 22-24 kDA on western blots (Fig. 5A). Higher ($P < 0.05$) concentrations of IFNT were discovered in UF from all HP compared to LP cows (Fig. 5B). In only cows with viable embryos, IFNT concentrations were higher ($P < 0.05$) in UF from HP compared to LP cows (Fig. 5C). A small amount of IFNT was detected in the UF from a single HP cow with an EM embryo (IFNT Optical Density = 2.4×10^6). All other cows with EM embryos had non-detectable IFNT concentrations (Fig. 5D). Also, IFNT was not detected in UF from any NP cows.

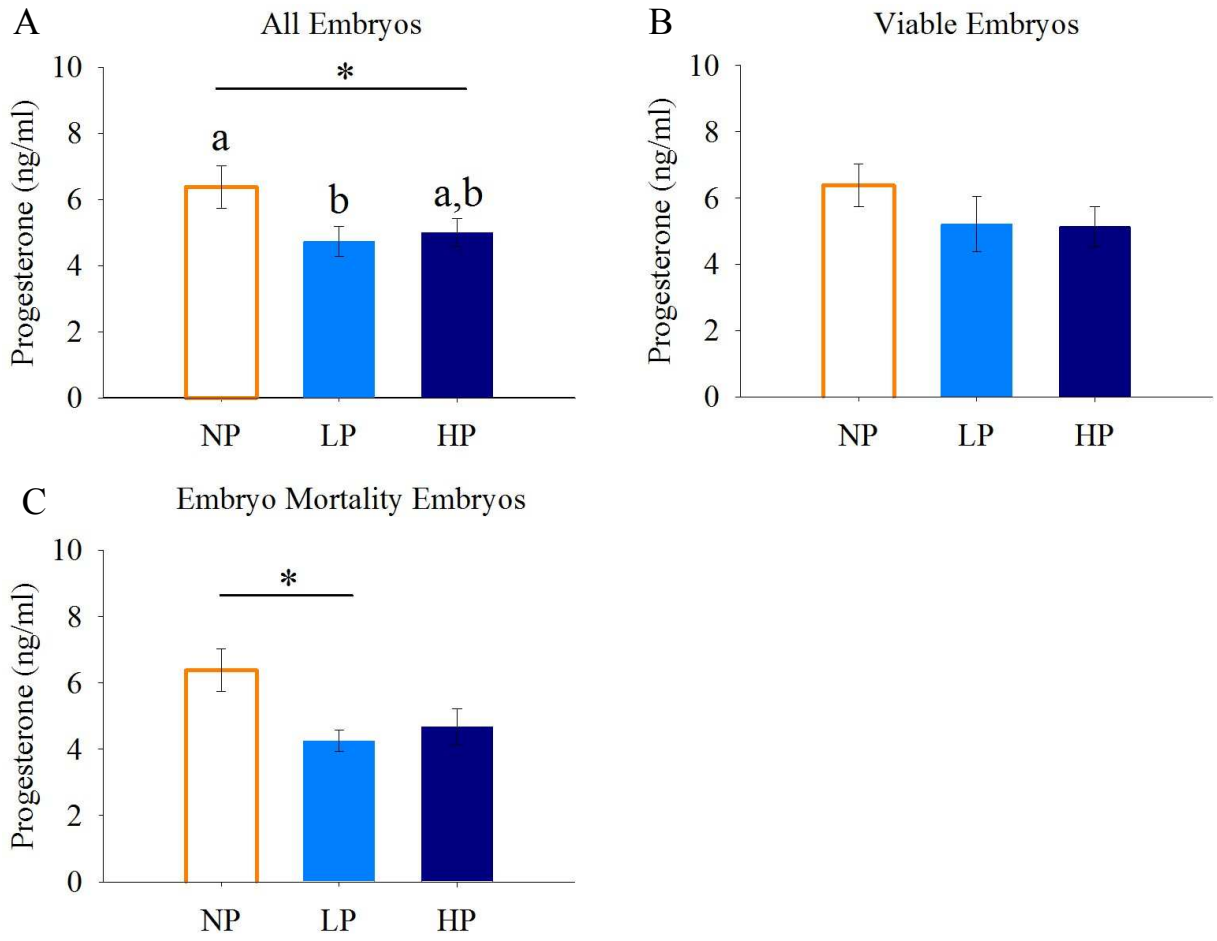


FIGURE 4.
Serum progesterone concentrations in lactating dairy cows. Quantitation of serum progesterone is presented in all cows (A), cows with viable embryos (B) and cows EM embryos (C). Values represent mean ± standard error. Means marked with different superscript letters differ ($P < 0.05$). * - Represents a tendency ($P < 0.10$).

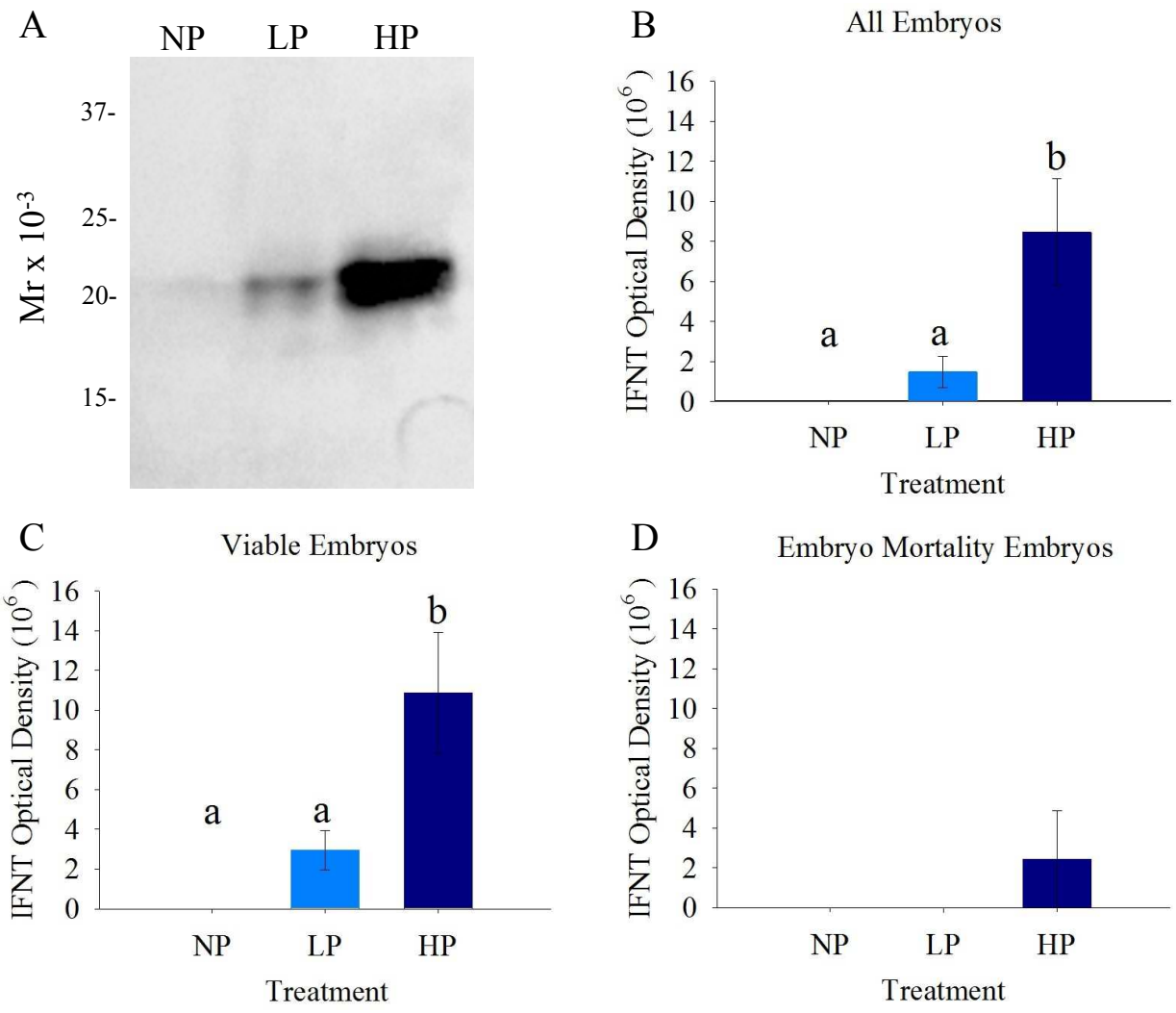


FIGURE 5.
IFNT detected from uterine flushing in lactating dairy cows. Image of IFNT western blot (A). Quantitation of IFNT release is presented in all cows (B), cows with viable embryos (C) and cows EM embryos (D). Values represent mean ± standard error. Means marked with different superscript letters differ ($P < 0.05$).

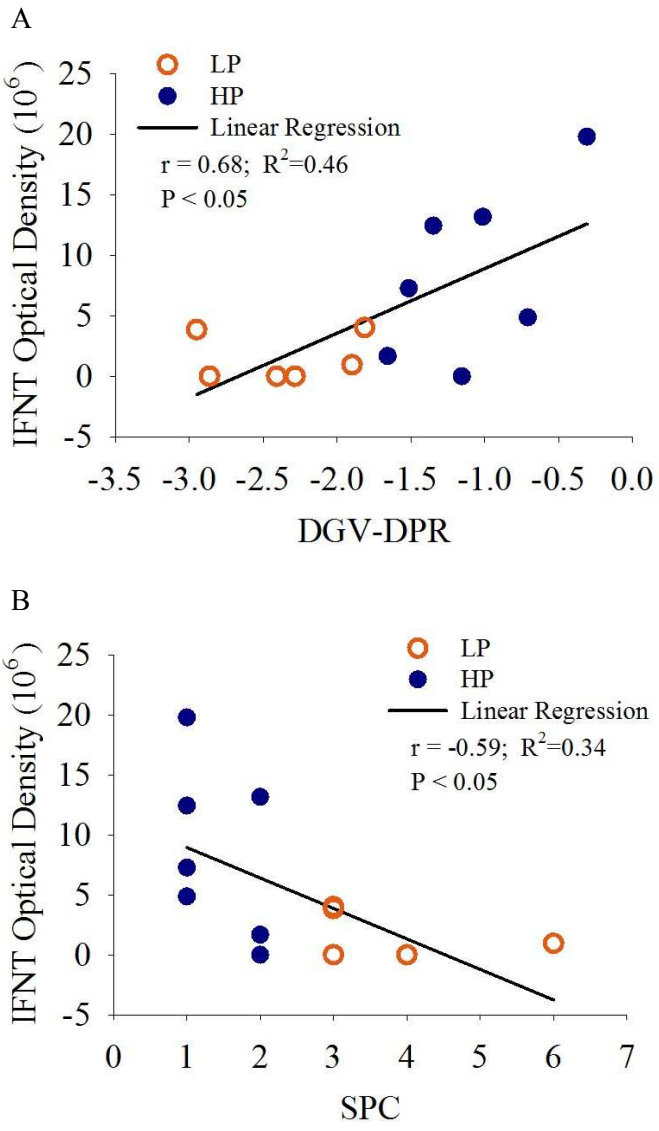


FIGURE 6.
Relationship between IFNT detected in uterine flushing and DGV-DPR (A) or SPC (B). The coefficient of determination (R^2) is the percent of variance explained by the model.

Increased IFNT release from the conceptus was associated with increased DGV-DPR (Fig. 6A) and decreased SPC (Fig. 6B) in HP compared to LP cattle. IFNT concentrations in UF were positively correlated with DGV-DPR ($r = 0.68$; $P < 0.05$) and explained approximately 46% ($R^2 = 0.46$) of the variation in DGV-DPR values. Furthermore, IFNT concentrations in UF were negatively correlated with SPC ($r = -0.59$; $R^2 = 0.34$; $P < 0.05$).

PARACRINE AND ENDOCRINE ACTION OF IFNT

Interferon stimulated gene 15 (ISG15) mRNA concentrations tended ($P < 0.10$) to be greater in endometrium from HP compared to NP cows (Fig. 7A). When analyzing only cows with viable embryos, ISG15 mRNA concentrations in the endometrium were higher ($P < 0.05$) in HP compared to NP and LP cows (Fig. 7C). The ISG15 mRNA concentrations in LP cows with viable embryos were intermediate and did not differ from NP or HP cows. Interferon stimulated gene 15 mRNA concentrations were very low (baseline) in cows with EM embryos and did not differ due to pregnancy or fertility groupings (Fig. 7E).

When isolating RNA from endometrial tissues, the protein fraction was saved and analyzed using western blot to examine ISG15 protein concentrations. In early responses to IFNT, almost all of the free ISG15 becomes conjugated to targeted proteins in the correct conditions (i.e., presence of conjugating enzymes) in a mechanism that is similar, but not identical, to ubiquitin. In tissues, ISG15 exists in free form (non-conjugated) at about 15 kDa on 1D-PAGE gels, whereas conjugated ISG15 can range in size from 40 kDa to > 250 kDa (Fig. 8) (Johnson et al., 1998). The recombinant ISG15 prep that we used as a standard for this analysis also had some higher molecular weight bands. These bands represent dimers formed after long-term storage of the protein at 4° C (Sorensen et al., 2007). Free ISG15 was not detected in endometrial proteins. It is possible that all free ISG15 was incorporated into conjugate forms in

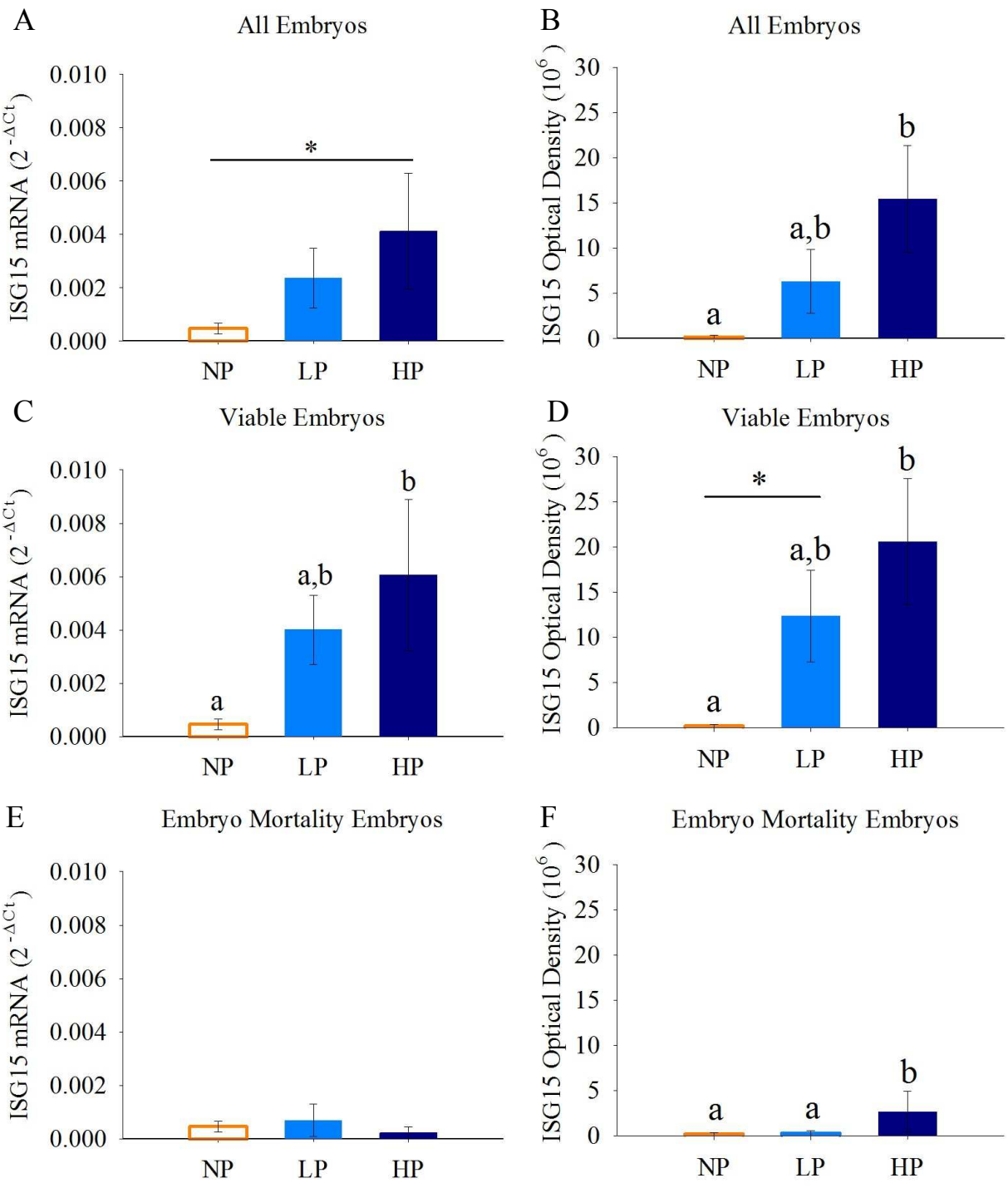


FIGURE 7.
Relative concentration of ISG15 mRNA (A, C, E) and protein (B, D, F) in endometrium from lactating dairy cows. Quantitation of ISG15 is presented in all cows (A, B), cows with viable embryos (C, D) and cows EM embryos (E, F). Values represent mean ± standard error. Means marked with different superscript letters differ ($P < 0.05$). * - Represents a tendency ($P < 0.10$).

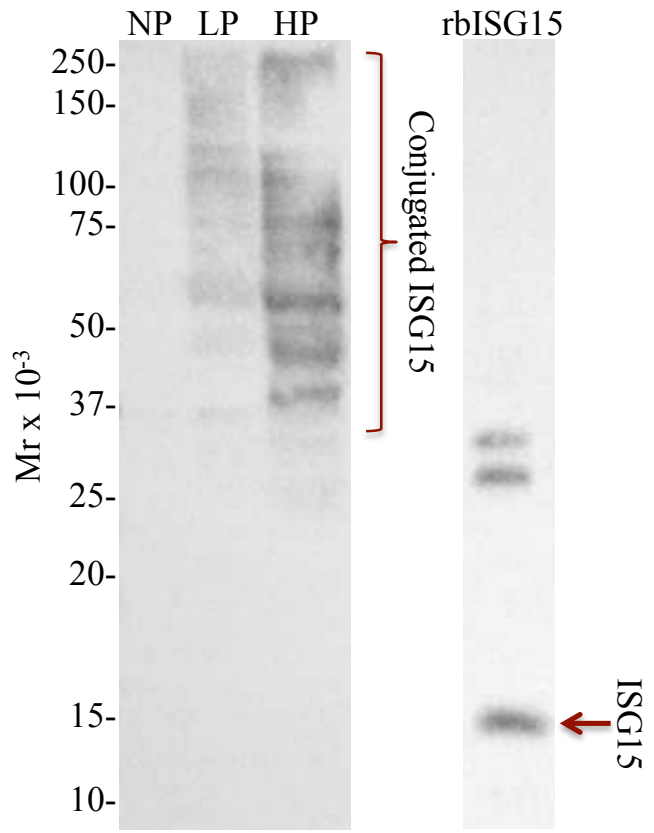


FIGURE 8.
Western blot depicting conjugated (40 to > 250 kDa) and free (17 kDa) ISG15 in endometrium from lactating dairy cows. rboISG15 = recombinant bovine ISG15.

response to conceptus-derived IFNT during this early stage of pregnancy. However, conjugated ISG15 was not detected in endometrium from NP cows, was intermediate in LP cows and increased significantly ($P < 0.05$) in HP cows. This difference is reflected in the representative western blot in Fig. 8, as well as the densitometric readings provided in the right panels (B, D, F) of Fig. 7. The expression pattern of ISG15 protein was very similar to the detection of ISG15 mRNA concentrations in endometrium from NP, LP and HP cows.

Further study of endocrine action of IFNT in PBMC revealed upregulation ($P < 0.05$) of ISG15 mRNA concentrations in all HP cows compared to LP or NP cows (Fig. 9A). In cows with viable embryos, ISG15 mRNA concentrations in PBMC from HP was diminished to a tendency ($P < 0.10$) of upregulation when compared to LP or NP cows (Fig. 9B). Interferon stimulated gene 15 mRNA concentrations in cows with EM embryos were greater ($P < 0.05$) in HP compared to LP and NP cows (Fig. 9C).

DISCUSSION

Conventional approaches to improving fertility are difficult because most reproductive traits are polygenic and lowly heritable (Weigel, 2006; Berry et al., 2012). Mechanisms such as pleiotropic gene effects, linkage, and physiological associations likely contribute to the complexity (Veerkamp and Beerda, 2007). Furthermore, it may take years to acquire phenotypes for some of the fertility traits, such as calving interval (Berry et al., 2012). Therefore, utilizing genomic tools to analyze the parental contributions to the offspring genome could advance genetic improvement. Kropp et al. (2014) proposed that as fertility declines, biomarkers could be generated and used as predictors for embryo selection, improved fertility, and healthier offspring.

Clarifide®, a relatively new genetic screening tool from Zoetis Inc., is based on SNP and haplotype analysis to provide the DGV, which is combined with traditional PTA data to calculate

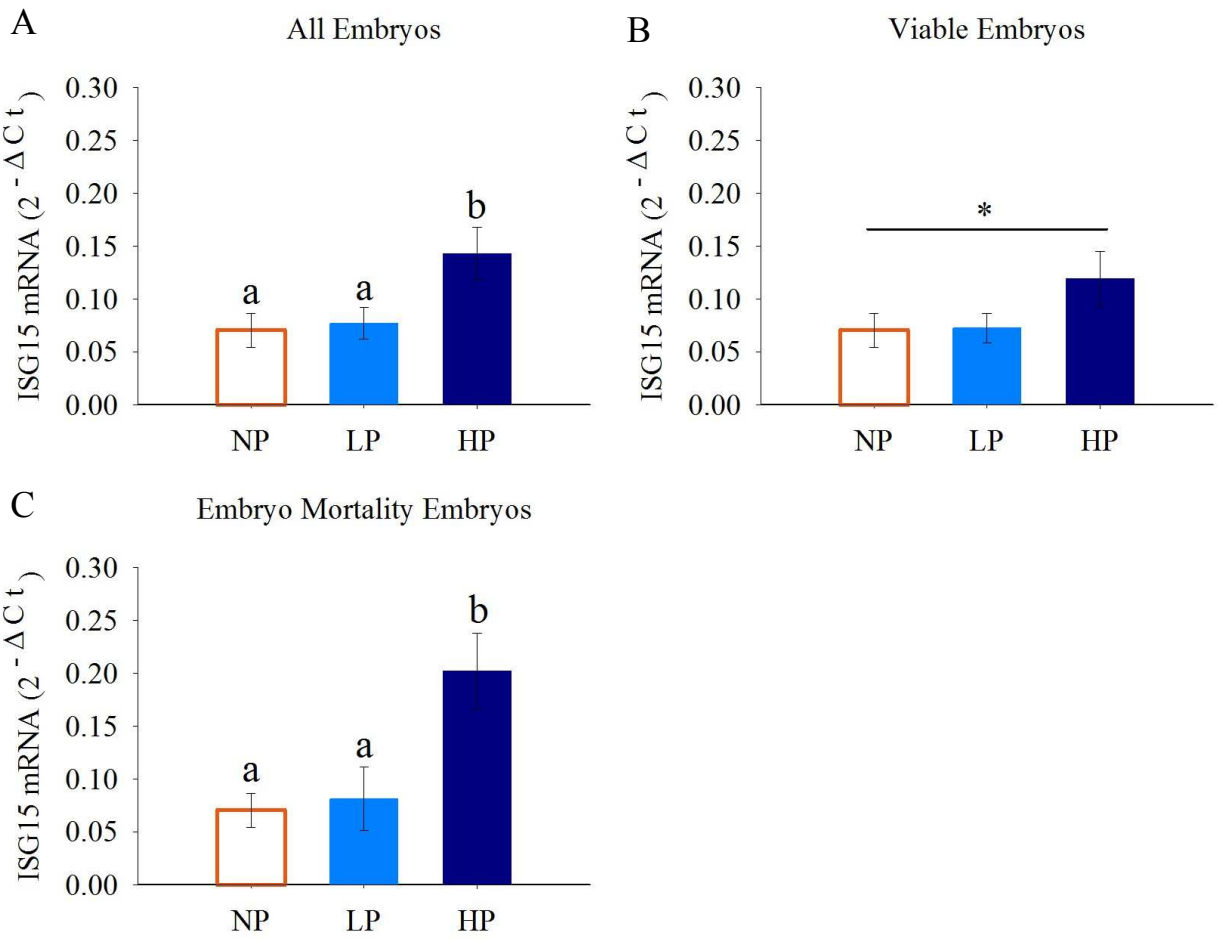


FIGURE 9.
Relative concentration of ISG15 mRNA in PBMC from lactating dairy cows. Quantitation of ISG15 is presented in all cows (A), cows with viable embryos (B) and cows EM embryos (C). Values represent mean ± standard error. Means marked with different superscript letters differ ($P < 0.05$). * - Represents a tendency ($P < 0.10$).

the gPTA for various production, health and fertility traits, including DPR. The gPTA values are derived from the Council on Dairy Cattle Breeding (USDA-CDCB; https://www.cdcb.us/Main/site_main.htm) dairy genetic evaluation system using Clarifide® data. It was reasoned in the present study that the DGV-DPR along with the on-farm SPC record could be used to select cattle with greater fertility and that these cows would have 1) more robust conceptus development, 2) increased production and action of IFNT and 3) no negative impacts on other important traits, such as milk production.

In the present study, 21 out of 86 cows were pre-selected as HF or LF based on the DGV-DPR analysis using the Clarifide® DNA screening tool. Cows were then sorted by the on-farm SPC from the previous lactation. Fourteen of the 21 cows were assigned randomly to the NP or HP groups while the 7 LF cows were assigned to the LP group. All cows assigned to the pregnant groups were inseminated using semen from a single HF bull. These sorting criteria did not impact economic traits or reliabilities, such as the gPTA-milk. Furthermore, DIM, number of lactations and Julian calving dates were not different among groups indicating that selection for fertility was not confounded by production traits or environmental effects. The negative correlation between the DGV-DPR and the SPC indicates that these selection criteria are inversely related when selecting for extremely high and low fertility in lactating dairy cows. It was unexpected that the gPTA-DPR was not different between fertility groups. A PTA is a genetic prediction for sires with a multitude of daughter records; however, for cows with only a few records, the variance from combining DGV and PTA was most likely too large to observe a difference in the gPTA-DPR. Regardless, the selection criteria using the DGV-DPR and SPC appeared to be a valid approach for selection of fertility in the present study. To our knowledge,

this is a unique approach to predict and select for the top (HF) and bottom (LF) extremes of fertility in dairy cows.

After sorting the 21 cows into LF and HF groups, the 65 remaining cattle were examined. The inverse DGV-DPR relationship with SPC was not clear because the extreme HF and LF cows had been removed (data not shown). For example, some cows with a high DGV-DPR had high SPC, perhaps due to negative health events in the previous lactation that prolonged SPC. Also, some low DGV-DPR cows had a low SPC and became pregnant on the first or second insemination. Inclusion of these cows in the base 65-cow data set further explains why these cows showed no difference in DGV-DPR and SPC and could not be sorted effectively into HF and LF groups.

Cows were bled for DNA, genotyped, and sorted into LF or HF groups at about 15-18 DIM. After the voluntary wait period, estrous cycles were synchronized and cows were TAI so that embryos could be flushed on day 16 of pregnancy. As conceptuses were non-surgically flushed from pre-determined LP and HP cows, they were further classified into viable or EM embryos based on morphologic criteria. In this experiment, 6 embryos were collected from the LP group (3 viable, 3 EM) and 7 embryos were collected from the HP group (5 viable, 2 EM). HP cows had longer and more viable embryos compared to LP cows. Exactly why embryo size and quality differed between HP and LP cows and which responses during early pregnancy were involved with this difference in fertility is the focus of future experiments. The increased conceptus growth seen in HP cows (Fig. 3) could be the result of a higher quality oocyte, a more efficient blastocyst, advantageous gene expression for elongation (such as LIN28; (Seabrook et al., 2013), an optimal uterine environment, maternal growth factors, histotroph quality, the amount of IFNT and subsequent response of maternal ISG, or a combination of these factors

(Lonergan and Forde, 2014; Angulo et al., 2015; Spencer et al., 2015; Spencer and Hansen, 2015). Also, there is the possibility of SNP or epigenetic gene suppression that disrupt regulatory promoter or enhancer regions in LF cows. Selection for these deleterious gene modifications might explain critical gene (i.e., IFNT and, consequently, ISG) suppression in LP compared to HP cows.

The DGV and gPTA are derived from the training and predicting process with the current genotypes on the SNP chip at the relative time that the DNA analysis is completed. These values change over time as the process is retrained. In this study, we used the DGV-DPR and other genomic values from the 09/30/2014 iteration of these data to sort cows into HF and LF groups. The 19,000 SNP, a subset of the original 50,000 SNP, are equally spaced across the chromosomes and not necessarily associated with functional genes (Matukumalli et al., 2009). Consequently, there is great interest in associating SNP with specific genes that function in conferring differences in fertility (i.e., functional genomics). Three possible functional and critical responses to pregnancy include serum progesterone, conceptus-derived IFNT, and ISG in endometrium and peripheral tissues, such as PBMC.

High levels of progesterone produced by the CL are necessary for creating a uterine environment that is conducive to embryo survival and the maintenance of pregnancy. The uterine epithelia secrete amino acids, glucose, cytokines, enzymes, growth factors, lymphokines, transport proteins for vitamins and minerals and extracellular matrix molecules that constitute histotroph which supports the early expanding blastocyst. If progesterone becomes limiting, this luteal insufficiency is a cause for low pregnancy rates (Wiltbank et al., 2014). In this study, serum progesterone concentrations on day 16 were lower in LP compared to NP cows. When only cows with viable embryos were examined, this difference disappeared. However, in EM

cows, serum progesterone concentrations tended to be lower in LP compared to the NP cows. This result is interpreted to mean that the lower serum progesterone concentrations in the LP cows was caused by the EM cows that were either luteal deficient or entering early luteolysis compared to the NP cows. In the cows with viable embryos, serum progesterone concentrations were consistent with those reported from other groups (Gifford et al., 2007) describing no difference in serum progesterone concentrations in bred, nonpregnant and pregnant dairy cows on days 16 or 18. The sustainability of progesterone production beyond day 16 of gestation was not analyzed and would require additional HF and LF cows to be examined at later time points. The lower serum progesterone concentrations in LP cows may reflect decreased CL function as well as other factors impacting conceptus survival such as genetic influences on the production and timing of IFNT release. For example, critical signals from the conceptus, such as IFNT, cooperate with progesterone to fully activate a nurturing uterine environment during elongation of the conceptus, formation of the placenta and attachment/implantation to the luminal epithelium of the endometrium (Spencer and Hansen, 2015).

The conceptus-derived IFNT gene family is one example of a cluster of genes that are critical to recognition of pregnancy. Currently, there are no known SNP on the chip that span the IFNT genes. Based on the original bovine genome sequence from the inbred Hereford cow, Dominette, there are limited sequence reads on chromosome 8 surrounding the IFNT loci. Therefore, there is conflicting information that can be obtained from the current assemblies (Btau_4.0 and UMD3.1) of the bovine reference genome. There are at least three known genes for IFNT (Hansen et al., 1991; Walker and Roberts, 2009). However, there is about 1 million bp of DNA between two of the three described IFNT genes based on NCBI sequences. Therefore, there may be more genes that have not been identified due to the significant gaps in the reference

genome. Also, we have preliminary data that the number of IFNT genes might vary from individual to individual (unpublished results). The increase in IFNT concentrations in the UF from HP versus LP cows suggests that IFNT genes control successful maternal recognition of pregnancy and therefore fertility. Perhaps low fertile cows produce conceptuses with less IFNT gene expression. However, the minimal threshold of IFNT required to induce a successful maternal response to the conceptus is unknown. There may be a dosage effect of IFNT gene expression from the conceptus and the maternal response (i.e., ISG) to the conceptus. Future studies will examine the cause of LF pregnancies. Perhaps there are fewer IFNT genes in these cows, suppression of IFNT gene expression (i.e., SNP or epigenetic silencing), or a shorter conceptus with a reduced number of trophoblast cells secreting IFNT.

IFNT was not expressed in four out of five EM embryos, indicating that these embryos ceased production of IFNT and were undergoing apoptosis. One EM embryo did express some IFNT into the UF media and we suspect that this embryo was in the process of dying. Perhaps if the UF were collected on day 17 or 18, IFNT would not have been detected in the UF from this EM cow. We hypothesize that healthier embryos have more robust synthesis of IFNT, which may be programmed through epigenetic mechanisms, increased numbers of genes or increased gene expression. Also, one IFNT gene may be preferentially expressed over another and may encode an IFNT protein that has greater activity compared to other IFNT gene products. For example, when testing 8 different recombinant IFNT, it was reported that only one of these proteins retained cross-species antiviral activities in bovine, murine and human cells (Alexenko et al., 1999). The antibody against IFNT that was used in these western blots recognizes the glycosylated IFNT doublet (22 kDa and 24 kDa) found in native proteins (Anthony et al., 1988) but it does not delineate between the IFNT genes. Determining which genes contribute to the

robust production of IFNT, or if specific genes are attenuated in LF versus HF cows is still to be examined.

The positive correlation between IFNT and DGV-DPR suggests DGV-DPR is a good selection tool for increasing fertility in dairy cattle; at least in context of selecting for a more advanced conceptus that produces greater concentrations of IFNT. The difference in IFNT expressed in UF explained 46% of the variation of DGV-DPR values. Likewise, the negative association of IFNT and SPC suggests that farm breeding records could be used more frequently to select for fertility. Therefore, IFNT levels may be a major driving factor for fertility in lactating dairy cattle. Perhaps low fertile cows have a similar conception rate as high fertile cows but the low fertile embryos experience retardation in elongation that decreases the IFNT produced and released by the conceptus. If the expressed IFNT do not fully stimulate maternal responses (such as ISG) within the recognition of pregnancy window, then the embryo begins to die and undergoes apoptosis. If this occurs, it would help explain the higher SPC in the low fertile group.

Due to the differences in IFNT expression by the conceptus, the paracrine action of IFNT on the endometrium and the endocrine action on the PBMC were examined. There are hundreds of endometrial and blood cell genes expressed in the presence of a conceptus (Hansen et al., 2010). One of these genes is ISG15. Formerly known as ubiquitin cross-reactive protein, ISG15 is a tandem ubiquitin repeat that conjugates to proteins in the cytosol of endometrial epithelial cells to aid in maternal recognition of pregnancy (Hansen et al., 1999; Hansen and Pru, 2014). In this experiment, ISG15 was used as a marker for IFNT action. It could be reasoned that if there is an increased dosage (number of active genes or increased concentration released from the conceptus) of IFNT, then there should be a more active signal transduction downstream of the

IFNT receptor and greater increase of ISG. This enhanced maternal response to the conceptus may also contribute to the increased fertility in the HF group. Indeed, HP and LP cows had increased ISG15 mRNA concentrations in endometrial tissues when compared to NP cows. Austin et al. (1996) showed that ISG15 was detectable by day 15 of pregnancy in cows using western blot. ISG15 levels increased to day 18 and remained high until day 26 of pregnancy. Data from the present experiments describe upregulation of ISG15 in endometrium by day 16 in HP compared to NP cows. However, the LP cows had ISG15 protein concentrations that were not different from NP or HP cows. This intermediate, but low-level maternal paracrine response to the conceptus in LP cows may help explain why a greater number of embryos die when compared to HF embryos.

When analyzing maternal endocrine responses to IFNT, ISG15 mRNA concentrations were upregulated in the PBMC of HP cows compared to the NP and LP cows. This difference is interpreted to mean that IFNT not only has more robust action on the endometrium, but may also enhance ISG in PBMC and other tissues peripheral to the uterus, such as the CL. An endocrine role for IFNT has been proposed in ruminants where it may be involved with modulating maternal immune responses (Hansen et al., 2010) and conferring resistance of the CL to lytic effects of PGF (Oliveira et al., 2008; Bott et al., 2010; Antoniazzi et al., 2013). Our findings are supported by other publications that have described the endocrine action of IFNT and its upregulation of ISGs in circulating blood cells. Han et al. (2006) found that ISG15 mRNA concentrations increased by day 16 of pregnancy and peaked by day 20 in blood cells. Furthermore, the abundance of ISG (i.e., ISG15, MX1, MX2, OAS1) in PBMC increased from day 15 to day 20 post-AI in beef cattle. These ISG dramatically decreased from day 20 to day 22 of gestation (Pugliesi et al., 2014). However, Gifford et al. (2007) reported that ISG15 mRNA

concentrations from peripheral blood leukocytes were not sufficiently upregulated until day 18 of pregnancy. ISG15 was found to be significantly higher in pregnant cattle on day 18 and 20 post AI. Although ISG increased statistically in pregnant cows, a parity status interaction was detected. For some multiparous cows, the increase in ISG was nearly undetectable, while the ISG response was fairly great in primiparous cows (Green et al., 2010). It is unknown why the parity difference exists.

There are still many questions that need to be answered. Are the IFNT genes or alleles modified in high versus low fertile cattle? What other paracrine roles exist for IFNT? Are there other endometrial secretory proteins that play a role in these mechanisms? What pathways are involved to induce IFNT responses in the CL? Future studies of female fertility need to be conducted. Our lab plans to use this animal model to analyze methylation across IFNT genes between high and low fertile groups, which may give insight to persistent infertility. This animal model could be used to increase fertility among lactating dairy herds. Determining new SNP along known fertility genes could greatly enhance genetic improvement for fertility traits. It may also aid in diagnosing animals that are infertile and culling them from the herd.

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CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

Several key discoveries related to the preselection of cattle based on fertility as well as determination of a viable pregnancy are described herein. Preselecting cattle based on DGV-DPR and on-farm SPC could be used in the future to sort cows into high and low fertile groups. The producer may use this selection tool to identify high fertile animals to be bred to high quality semen or create a list of low fertile animals to be culled from the herd.

Serum progesterone was not different on day 16 of the estrous cycle/gestation between NP, LP and HP groups when cows were gestating a viable conceptus. However, the LP cows with EM embryos tended to have decreased serum P4 compared to the NP group, indicating that the dying embryos had initiated luteolysis early. These findings strengthen the idea that embryo survival is a function of the conceptus, uterine environment, and the postovulatory circulating progesterone concentration in normal, healthy cattle.

Not surprisingly, viable embryos were significantly longer than EM embryos when treatment group was ignored. Viable embryos from HP cows tended to be longer than viable embryos from LP cows suggesting faster growth. Further research is needed to identify why these embryos grew faster: genetics, uterine environment, protein synthesis and secretion, or a combination of factors. Nonetheless, IFNT synthesis and release was greater in viable embryos from HP cattle compared to LP cattle while the majority (4/5) of EM embryos did not contain detectable IFNT in the UF. As expected, IFNT was not detected in any NP UF samples as IFNT is produced solely by the conceptus. Interferon stimulated gene 15 mRNA concentrations were upregulated in the endometrium and PBMC on day 16 of pregnancy due to the paracrine and

endocrine actions of IFNT as described previously. Interferon tau production was positively correlated with DGV-DPR and negatively correlated with SPC. This correlation supports the observation that high fertile cows, selected on the basis of high DGV-DPR and low SPC, produced more viable embryos with faster growth and greater IFNT production and secretion than low fertile cows.

Perhaps the most important finding in this study is that high fertile cows, sorted based on high DGV-DPR and low SPC several months prior to AI, actually had 1) longer embryos with greater release of IFNT, 2) increased paracrine response to IFNT in context of upregulated endometrial ISG15 mRNA and protein and 3) increased peripheral endocrine response to IFNT in context of increased relative concentrations of ISG15 mRNA in PBMC. Whether these IFNT mediated responses are due to IFNT and ISG gene dosage, increased numbers of trophoblast cells and consequently greater IFNT release because of more advanced conceptus development, or increased maternal gene expression in response to pregnancy and IFNT remains to be determined.

This high-low fertility cow model can be used extensively in the future to identify SNP for functional genomics as well as provide a way to examine differences in the IFNT/maternal recognition of pregnancy pathway between groups. Our lab is currently performing a proteome analysis of conceptus secretory protein, endometrial secretory protein, and UF media from NP, LP and HP cows to understand the uterine milieu. This approach will identify proteins that are expressed from the conceptus and endometrium that are commonly found in UF and identify protein families or pathways that may have different abundance between fertility groups.

Another current study in our lab is the analysis of RNA-seq data obtained from EM, LP and HP groups in conceptus tissue and NP, EM, LP, and HP groups in endometrial, PBMC and

milk tissues. These analyses will allow identification of differentially expressed genes (DEG) between pregnancy and fertility groups. Furthermore, we may be able to determine why HP cows produced more viable embryos with greater IFNT production compared to LP cows. For instance, perhaps the IFNT transcripts for the three known IFNT genes are expressed differentially in the embryos from HP cows versus LP cows. Alternatively, if the IFNT genes are not differentially expressed, perhaps one or more of the IFNT genes are methylated or influenced by a miRNA, which alters the synthesis and release of IFNT from the trophectoderm of embryos from HP compared to LP cows.

On the current SNPchip platform, there are no SNP within or in close proximity to the IFNT genes. This deficit in the SNP data is partially due to the poor coverage along chromosome 8 in the reference genome to the Hereford cow, Dominette. In the near future, SNP discovery analysis could identify unique SNP along the IFNT genes and the IFNT pathway, such as IFNAR1/2, STAT1, IRF, ISG15, OAS1/2, MX1/2, which could be used as functional genomic markers for fertility. A custom fertility-specific SNPchip or genotyping assay could be constructed based on this IFNT SNP data to increase genetic gain for fertility and improve reproduction in dairy cows. This study focused on the female, however there is untapped potential examining these same IFNT and IFNT pathway genes in bulls. Sorting bulls into high and low fertile groups and performing the same IFNT SNP analyses could determine the paternal effects on fertility. Recently it has been discovered that there may be more than three IFNT genes based on full genome sequence in Holstein bulls (unpublished data). There are an ever-increasing number of DNA sequences available from bulls with known genotypes. Utilizing this additional genomic resource could substantially increase the reliability of the fertility genetic predictions as well as provide insight to how specific SNP impact functional protein expression.

Future studies could reveal a novel detection method (ELISA or RIA) for IFNT in the blood. Ultimately, IFNT would make the best biomarker for a pregnancy as it is only produced and secreted in large enough concentrations by the viable embryo. Unlike pregnancy diagnosis by transrectal ultrasonography (day 32+) or ELISA for pregnancy associated glycoproteins (PAG) in the blood or milk (DHIA, 2015) (day 35+), identifying IFNT around day 16 would allow for pregnancy diagnosis prior to the next ovulation. This early pregnancy diagnosis would save producers money by allowing them to identify open (nonpregnant) cows on day 16 post-insemination and monitor follicular status prior to ovulation and subsequent insemination. This early pregnancy test could greatly increase breeding efficiency because producers would not have to wait until day 32 to segregate open cows. We believe the experiments included in this thesis can lead to a functional genomic SNPchip and new diagnostic tests, as well as expand the general knowledge of pregnancy in lactating dairy cattle.

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