Characterization of novel reproductive phenotypes in dairy cows

by

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Abstract

A reproductive phenotype that has high variability, repeatability, heritability, and association with fertility could become an excellent candidate for genetic selection to augment fertility in dairy cows. Therefore, the general objective of this research was to evaluate the aforementioned characteristics for five novel reproductive phenotypes in dairy cows, namely gonadotropin releasing hormone (GnRH)-induced luteinizing hormone (LH) response, antral follicle count (AFC), anti-Müllerian hormone (AMH), insulin-like growth factor-1 (IGF-1), and ano-genital distance (AGD). The secondary objective was to identify genetic markers (single nucleotide polymorphisms; SNP) associated with three of the above reproductive phenotypes (AMH, IGF-1, and AGD).

The first study was designed to determine the variability and repeatability of GnRH-induced LH responses and the associations among plasma LH, follicle stimulating hormone (FSH), estradiol (E2), and progesterone (P4) concentrations. The association between LH response categories and reproductive outcomes was also tested. I hypothesized that cows have variable responses to GnRH treatment, which are repeatable, and cows with a high LH response to GnRH would have increased likelihood of ovulation and consequently improved reproductive outcomes compared to cows with a low LH response. The GnRH-induced LH responses were normally distributed and highly variable but unrepeatable. Plasma concentrations of LH were positively associated with FSH and E2, and negatively associated with P4. Cows with high LH responses had numerically greater ovulation rate and pregnancy to first artificial insemination (P/AI); however, the association with reproductive outcomes needs to be evaluated in a larger population.

The second study was designed to test the repeatability of AFC and AMH concentrations at an unknown stage of follicular growth and at an expected day of follicular wave emergence and the association between AFC and AMH at the above two stages in dairy cows. I hypothesized that the repeatability for AMH would be higher than the repeatability for AFC and the association between AFC and AMH would be strong at both stages. The repeatability was high for AMH and moderate for AFC, and a moderate correlation was found between AFC and AMH at both stages.

The third study was designed to evaluate the distribution and variation of AMH, identify factors associated with variation in circulating AMH, determine heritability, establish an optimum AMH threshold predictive of P/AI, examine the relationship between AMH and fertility, and identify SNP associated with the phenotypic variation of AMH in dairy cows. Circulating AMH was positively skewed and highly variable, had a quadratic relationship with parity, moderately heritable, and was associated with numerous genetic markers. The relationships between AMH and fertility outcomes, however, were not significant despite numerical indications of better fertility outcomes in cows with high AMH concentration.

The fourth study had similar objectives as the third study, with IGF-1 as the hormone of interest. Circulating concentration of IGF-1 was positively skewed and highly variable, influenced by herd, age, parity, pre-calving body condition score (BCS), and season of sampling. It was also positively associated with P/AI, and linked to a few genetic markers related to previously identified candidate genes of fertility in dairy cows.

The fifth study was designed to characterize the distribution and variability of AGD, determine the relationships among AGD, age and height, and evaluate the associations between AGD and P/AI and cumulative pregnancy by 250 d in milk (DIM) within parity groups (first, second, and third + parities) in Canadian Holstein cows. I hypothesized that cows with short AGD,

presumably exposed to low in-utero testosterone during fetal life, would have greater fertility than cows with long AGD. The AGD was normally distributed and highly variable, weakly-related to cow age and height, and inversely related to P/AI and pregnancy by 250 DIM in first and second parity cows, but not in third + parity cows.

The sixth study, with similar objectives as the fifth study, was conducted in Irish Holstein cows. Despite its normal distribution, high variability, moderate heritability, and weak associations with cow age, height, weight and BCS, the inverse relationship between AGD and fertility found in Canadian Holsteins, was not evident in the Irish Holsteins.

Overall, this doctoral thesis provided a deeper understanding of the characteristics of five novel reproductive phenotypes and their potential for selection in dairy cows, and opened new avenues for further research.

Preface

This thesis is an original work by Mohanathas Gobikrushanth. The research project, which this thesis is a part of, received research ethics approval from the University of Alberta Animal Care and Use Committee (ACUC) for Livestock, Project Name "Reproductive phenotypes in dairy cattle", No. AUP00001272, dated October 22, 2014.

This thesis is the result of an international research collaboration, led by Professor Divakar Ambrose at the University of Alberta, Edmonton, Canada along with Dr. Stephen Butler as the lead collaborator from Teagasc, Moorepark, Ireland.

Chapter 3 of this thesis has been published as **M. Gobikrushanth**, P.A. Dutra, T.C. Bruinjé, M.G. Colazo, S.T. Butler, and D.J. Ambrose (2017), "Characterization of the variability and repeatability of gonadotropin releasing hormone-induced luteinizing hormone responses in dairy cows within a synchronized ovulation protocol," ***Journal of Dairy Science***, vol. 100, issue 8, 6753-6762. I participated in experimental design and sample collection. I managed and analyzed data, interpreted the results and drafted the manuscript. P.A. Dutra and T.C. Bruinjé assisted with sample collection and contributed to manuscript preparation. M.G. Colazo and S.T. Butler assisted with data analysis and manuscript preparation. D.J. Ambrose conceived the study, designed the experiment, assisted with sample collection and contributed to the writing and editing of the manuscript. He supervised the study and was the corresponding author.

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the corresponding author who participated in experimental design, assisted with data collection and offered editorial suggestions.

Dedications

To my father, Mr. Mohanathas, my mother, Mrs. Parameswary, and my wife Sobi, for their love, belief, and encouragement

To my friends for their continued support and motivation

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List of abbreviations

AFC	Antral Follicle Count
AGD	Ano-genital Distance
AMH	Anti- Müllerian Hormone
AI	Artificial Insemination
AUC	Area Under the Curve
BCS	Body Condition Score
BTA	*Bos taurus* Autosome
CI	Confidence Interval
CIDR	Controlled Intravaginal Drug Release
CL	Corpus Luteum
CLA	Commencement of Luteal Activity
CV	Coefficient of Variation
DIM	Days in Milk
DM	Dry Matter
DPP	Days Postpartum
DPR	Daughter Pregnancy Rate
EBI	Economic Breeding Index
EBV	Estimated Breeding Value
E2	Estradiol
FDR	False Discovery Rate
FG	Follicular Growth

FSH	Follicle Stimulating Hormone
FWE	Follicular Wave Emergence
GH	Growth Hormone
GnRH	Gonadotrophin Releasing Hormone
GCTA	Genome-wide Complex Trait Analysis
GWAS	Genome-wide Association Study
HD	High Density
IDE	Inseminated at Detected Estrus
IGF-1	Insulin like Growing Factor-1
IL	Interleukin
LH	Luteinizing Hormone
LPI	Lifetime Profit Index
LPS	Lipopolysaccharides
MAF	Minor Allele Frequency
ME	Mature-Equivalent
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
NRC	National Research Council
PAG	Pregnancy Associated Glycoprotein
PLOSS	Pregnancy Loss following first Artificial Insemination
P/AI	Pregnancy to first Artificial Insemination
P150	Pregnancy by 150 days postpartum
P250	Pregnancy by 250 days postpartum

P4	Progesterone
PGF2α	Prostaglandin F2α
QTL	Quantitative Trait Loci
r	Pearson Correlation of Coefficient
R^2	Coefficient of Determination
RDP	Rumen Degradable Protein
RFM	Retained Fetal Membranes
ROC	Receiver Operating Characteristics
RUP	Rumen Undegradable Protein
SEM	Standard Error of Mean
SD	Standard Deviation
SNP	Single Nucleotide Polymorphisms
TNF-α	Tumor Necrosis Factor-α
US	Transrectal Ultrasonography

Chapter 1. General introduction

Dairy farming plays a pivotal role in global agricultural production. According to the United Nations, the human population is estimated to increase from 7.6 billion in 2018 to 9.8 billion by 2050. The increase in human population means increasing demand for food commodities including milk and other dairy products. Milk production per cow has increased over the last six decades in order to meet the demand of increasing human population, and according to the Council on Dairy Cattle Breeding (https://www.uscdcb.com) more than half of that increase in production was attained through intense genetic selection for milk production. Dairy cows need to conceive and calve at desired calving interval to ensure the initiation of each new lactation cycle, thereby maintaining a longer herd life. Despite increasing milk production, fertility of dairy cows has been on a declining trend worldwide (Macmillan et al, 1996; Butler, 1998; Jorritsma and Jorritsma 2000; Roche et al., 2000; Royal et al., 2000; Bousquet et al. 2004) except for Nordic countries where fertility traits have been included in the national breeding program along with production and health related traits since 1972 (Berglund, 2008). Even though the decrease in dairy cow fertility is multifactorial, the genetic antagonism between milk production and fertility (Jason and Andreasson, 1981; Dematawewa and Berger, 1998; Hansen, 2000; Roxstorm et al., 2001; Kadri et al., 2014), and the exclusion of fertility traits from national selection indices, at least in part, may have contributed to the decline in fertility (Berglund, 2008).

In order to improve reproductive efficiency, along with implementation of better nutritional, postpartum health and reproductive management strategies, traditional fertility traits were also introduced in mid-2000's into genetic selection programs in several countries such as Canada, Ireland and the USA. Even though the incorporation of traditional fertility traits such as

daughter pregnancy rate in the USA has halted further decline in fertility among Holstein dairy cows (Garcia-Ruiz et al., 2016), the rate of genetic gain is slow due to low heritability, and fertility is still sub-optimal in the North American dairy industry. Phenotypic performance could evolve faster within few generations if the trait had high heritability. Therefore, identification of novel and physiologically important traits that are least-influenced by management and highly-related to reproductive performance is desirable for genetic selection (Chebel and Ribeiro 2016).

A reproductive trait that can be easily measured, less expensive, and has high variability, repeatability, heritability and association with fertility would be an ideal candidate trait for genetic selection to augment fertility in dairy cows. In addition, identification of genetic markers (single nucleotide polymorphisms; SNP) associated with phenotypic variations in the trait that are strongly associated with fertility would enable the incorporation of those SNP within genomic evaluations to accurately identify females with desired phenotypic performance to enhance fertility. In the context of novel reproductive traits, I aimed to study the aforesaid characteristics for gonadotropin releasing hormone induced luteinizing hormone (GnRH-induced LH) response, antral follicle count (AFC), circulating concentrations of anti-Mullerian hormone (AMH), insulin-like growth factor-1 (IGF-1), and ano-genital distance (AGD) in dairy cows.

The primary objectives of this thesis research were to characterize the distribution and variation, determine repeatability, estimate heritability, and test the association with fertility outcomes for the aforesaid five novel reproductive traits in dairy cows. The secondary objective was to identify genetic markers (SNP) associated with variations of three of these novel reproductive phenotypes (AMH, IGF-1, and AGD) through genome-wide association analysis, and to evaluate their functional relevance to fertility in dairy cows.

Chapter 2. Review of literature

2.1. Overview of the dairy industry

Dairy farming plays a pivotal role in the global food system. For example, an estimated 800 million tonnes of milk was produced in 2015, of which 680 million tonnes was cow milk produced from 260 million cows across the world (International Dairy Federation, 2016). In Canada, dairy is one of the top two agricultural sectors in seven out of ten provinces, and has about 0.9 million cows mainly managed under confinement systems in 11,683 dairy farms, 444 processing plants, 221,000 jobs and contributes approximately $ 20 billion a year to Canada's gross domestic product, with an annual milk production of 8.2 billion litres (Dairy Farmers of Canada, 2016) Whereas in Ireland, dairy is the biggest agricultural sector with 1.4 million cows, managed mainly under the pasture-based seasonal calving system in 18,000 dairy farms, and produce about 6.6 billion litres of milk per annum (Irish Farmers Association, 2016).

An increasing demand for dairy milk is currently evident as a result of rising human population. For example, annual consumption of dairy products (fresh milk equivalent basis) currently averages about 111.1 kg per person (International Dairy Federation, 2016) and is expected to increase to 119 kg per person by 2067 (Alexandratos and Bruinsma, 2012). The continued increment in milk production is attained through intense genetic selection for milk production alongside major improvements in the management of herd, nutrition, health, and reproductive practices (Lucy, 2001). The efficient way of dairy farming, however, is not only to meet the demand of growing human population by producing more milk, but also to secure both sustainability and profitability. In order to achieve these, dairy cows need to be fertile and have increased longevity.

2.2. Fertility of dairy cows

High milk production and optimum fertility and are vital parameters that determine profitability of dairy farms (Pryce et al. 2004; Ribeiro et al., 2012a; Galvao et al. 2013; Gobikrushanth et al., 2014). The rate of phenotypic gain in milk production per cow per year has been 193 kg for the United States, 131 kg for the Netherlands, 35 kg for New Zealand and 46 kg for Ireland between 1985 and 2003 (Dillon et al., 2006). For example, the average milk yield has nearly doubled from 6,619 to 12,662 kg in the 50 years from 1963 to 2013 in the US, and over 56% of that increase can be attributed to genetic changes (Council on Dairy Cattle Breeding, 2016). Despite the tremendous increase in milk production potential, fertility of dairy cows was on a declining trend, at least until the mid-2000's (Macmillan et al., 1996; Butler, 1998; Jorritsma and Jorritsma 2000; Roche, 2000; Royal et al., 2000; Bousquet et al. 2004). For example, Butler (1998) reported that conception rate declined from approximately 66 to 40% between 1951 and 1996, in US Holstein dairy cows. The decrease in fertility is multifactorial and attributable to intense genetic selection for milk production, inadequate dry matter intake, poor reproductive management and increased incidence of postpartum diseases (Lucy 2001).

It has been identified that modern dairy cows are experiencing greater incidence of anestrus and abnormal estrous cycles (de Vries and Veerkamp, 2000), lower circulating concentrations of progesterone and estradiol due to increased metabolic clearance rate associated with higher dry matter intake (Wiltbank et al., 2000; Sangsritavong et al., 2002), shorter duration and lesser intensity of estrus (Lopez et al., 2004), reduced conceptions rates (Butler, 1998), increased proportion of pregnancy losses (Diskin et al., 2016), and higher incidence of multiple ovulations and twinning (Fricke and Wiltbank, 1999; Wiltbank et al., 2006; Macmillan et al., 2018), and

thereby longer intervals from calving to conception, and between consecutive calvings. All of the aforementioned physiological defects contribute to an overall inefficiency of reproduction and thereby greater involuntary culling in dairy herds.

The impaired reproductive efficiency has hampered the profitability of dairy farms because reproductive inefficiency is the number one reason to cull dairy cows in Canada. Approximately, 25% of dairy cows are culled annually in Western Canada due to reproductive problems that is followed by 17, 14, and 11 % of culling due to mastitis, low milk production, and feet and leg problems, respectively (CanWest Dairy Herd Improvement, 2017). The total loss due to reproductive failure equates to about 2% of the gross production value or 10% of an average dairy farmer's income (Dijkhuizen et al., 1984). Based on information collected from United States Department of Agriculture (USDA) National Animal Health Reporting System, Bellows et al. (2002) reported the estimated costs of infertility to be $ 137 million per year for the US dairy industry, attributable to culling, replacement, labour, drugs, and veterinary costs. Consequently, one of the greatest challenges of reproductive biologists, nutritionists and geneticists is to gain an understanding of the underlying biology of the dairy cow that contributes to low fertility and develop strategies to improve fertility (Walsh et al., 2011).

2.3. Strategies to enhance fertility of dairy cows

2.3.1. Nutrition

Nutrition plays a key role in regulation of reproduction in dairy cows as well in other domestic animals. The effect of inadequate nutrition has shown to delay puberty (Kinder et al., 1987), prolong postpartum anovulatory period (Rhodes et al., 2003), reduce body condition score (Roche et al., 2009), and finally result in poor overall fertility outcomes (Buckley et al., 2013). In

the recent past, multiple reports have summarized the importance of different metabolites during early postpartum on subsequent fertility of dairy cows (Santos et al., 2008; Butler, 2014; Crowe et al., 2018; Salo, 2018).

The blood concentration of glucose is one of the key metabolites that controls reproductive function through its capacity to orchestrate changes in endocrine hormones such as insulin and insulin like growth factor-1 **(IGF-1)** (Lucy, 2008). Glucose causes release of insulin from pancreas and insulin partitions nutrients towards muscle and adipose tissue. Insulin stimulates the liver to release IGF-1 into circulation through upregulation of growth hormone **(GH)** receptors in liver (Butler et al., 2003). Insulin stimulates mitosis of cultured bovine granulosa and luteal cells, and thereby production of steroid hormones in-vitro (Gutierrez et al., 1997; Mamluk et al., 1999, respectively). In addition, insulin has been shown to increase steroidogenesis in response to gonadotropins in both in-vitro and in-vivo studies (Stewart et al., 1995; Butler et al., 2003). Cows with lower circulating insulin concentrations during early postpartum period reportedly suffer from delayed resumption of postpartum ovarian activity and normal ovarian cyclicity, and are at higher risk for cystic ovarian disease compared to cows with higher circulating insulin concentration (Vanholder et al., 2005). Later studies by Garnsworthy et al. (2008) demonstrated that maintaining adequate insulin-to-glucagon ratio in dairy cows at the start of the breeding period with a dietary starch concentration above 160 g/kg of dry matter and dietary fat below 44 g/kg of dry matter, had positive effects on ovarian function.

Acting synergistically with gonadotropins (Lucy et al., 1992; Spicer et al., 1993; Beam and Butler 1999) IGF-1exerts its endocrine effects on follicular growth, granulosa cell mitogenesis, granulosa and theca cell steroid production, and reproductive tract and endometrial gland secretions to support embryonic growth (Wathes et al., 1997; Robinson et al. 2000; Zulu et al.

2002; Pushpakumara et al., 2002; Fenwick et al., 2008). During the early postpartum period, cows are in a state of negative energy balance **(NEB)** due to high milk yield and low dry matter intake (Butler and Smith 1989), when the GH-IGF-1 axis is uncoupled due to down regulation of GH-receptors in liver (McGuire et al., 1995; Kobayashi et al., 1999). This in turn is associated with decreased IGF-1 and increased GH concentrations to promote the action of GH on lipolysis and gluconeogenesis to favor milk production (Lucy et al., 2001). As a result, reproductive functions were shown to be reduced in cows having low circulating IGF-1 than those having high circulating IGF-1 at early postpartum (Taylor et al., 2004; Patton et al., 2007; Green et al., 2012; Cummins et al., 2012a; Cardoso et al., 2013; Moore et al., 2014a; Macmillan et al., 2018). Therefore, feeding glucogenic diets to cows during the early postpartum period, to counteract the mechanisms related to NEB, were proposed (Gong et al., 2002) for greater fertility through increased glucose concentration in blood and thereby greater circulating insulin and IGF-1. It is noteworthy, however, high concentration of circulating insulin has detrimental effects on oocyte and embryo competence (Fouladi-Nashta et al., 2005), and stimulate enzymatic catabolism of progesterone in the liver (Lemley et al., 2008). Therefore, glucogenic diets may be of advantage when offered in the immediate postpartum period, while they should be avoided when cows are inseminated.

Since inadequate energy intake during early postpartum period results in NEB in dairy cows, feeding fat supplemented diets has become a strategy, to mitigate NEB during the transition to lactation (Curtis et al., 1985). Staples et al. (1998), however, suggested that positive effects of dietary fat on fertility of dairy cows are probably not only a result of improved energy status but also because dietary fats could affect the pituitary, ovarian and uterine function to enhance fertility. For example, cows fed diets supplemented with fat shown improved follicular growth, increased total number of follicles and the size of preovulatory follicle (Lucy et al., 1991; Lucy et al., 1993;

Beam and Butler 1997) probably through greater concentration of circulating LH (Palmquist and Weiss. 1994) compared to cows that fed a controlled diet. However, recent studies indicated that the type of fat has more selective effect on fertility than fat supplementation *per se* (Leroy et al., 2005; Ambrose et al., 2006; Petit and Twagiramungu 2006; Thangavelu et al., 2007; Santos et al., 2008; Colazo et al., 2009a; Hutchinson et al., 2012). In this regard, omega-6 fatty acids are believed to have pro-inflammatory, thus PGF-stimulating properties, rendering them of extra value during early postpartum. Whereas, omega-3 fatty acids can weaken this inflammatory potency, leading to a higher chance of survival of the embryo when supplemented during the breeding period through increasing CL lifespan and thereby maintenance of greater circulating progesterone concentration (Silvestre et al., 2011). A recent meta-analysis reported that including fats in the transition diet increases the pregnancy to first service by 27% in dairy cattle (Rodney et al., 2015). However, research results rarely provide a consensus on this topic, making fat-feeding strategies on oocyte and embryo quality an intriguing issue for debate (Crowe et al., 2018).

High protein diets are frequently fed to dairy cows to increase milk production. Several studies, however, have reported that increasing the percentage of crude protein in the diet could result in reduced fertility (Canfield et al., 1990; Elrod and Butler 1993; Rajala-Schultz et al., 2001; Thatcher et al., 2001; Tamminga, 2006). For example, feeding excess rumen degradable protein **(RDP)** has shown to delay the first ovulation or estrus, reduce the conception rate to first insemination, increase the number of days open and lower the overall conception rate in dairy cows (Tamminga, 2006). This decrease in fertility may result from (1) exacerbated NEB as the urea detoxification process in liver consumes additional energy (Westwood et al., 2000; Leroy et al., 2008), and (2) the deleterious effects of both ammonia and urea on both oocyte and embryo development (Chapa et al., 2001; Ocon and Hansen 2003; Rhoads et al., 2006). On the other hand,

feeding higher level of rumen undegradable protein **(RUP)** have shown to shorten the days to first estrus and calving-to-postpartum mating interval, and reduce services per conception in beef and dairy cows (Waterman et al., 2006; Aboozar et al., 2012; Rochijan et al., 2016). An increase in RUP may increase the supply of amino acid for intestinal absorption, which may improve the glucogenic potential of the supplement (Waterman et al., 2006) or contribute an essential AA such as methionine, which can improve ovarian function (Alonso et al., 2008). In this regard, others also reported that supplementation of rumen bypass methionine can improve reproductive performance of dairy cows (Ardalan et al., 2010; Titi et al., 2013). Furthermore, excess RUP has shown to stimulate the pancreas to increase insulin production (Sletmoen-Olson et al., 2000). Insulin affects ovarian tissues by enhancing LH receptor synthesis and actions of the pituitary through these receptors, and Kane et al. (2002) suggested that undegraded protein improves reproduction by mediating the synthesis of both LH and FSH.

Dietary protein is the one of the most important factors that determines milk nitrogen efficiency, urinary nitrogen losses, and consequently, ammonia emissions from dairy cow manure (Hristov and Giallongo 2014). Therefore, excessive supplementation of dietary protein above the requirements of cow might increase excretion of nitrogen in feces and urine (Weiss et al., 2009). This excreted nitrogen can cause environmental pollution in the forms of ammonia (Aneja et al., 2008) as the urea excreted in the urine immediately gets converted to ammonia by urease enzymes produced by the microorganisms present in feces and soil (Wright et al., 1998; Varel et al., 1999). Therefore, optimizing protein content of diets and synchronization of protein with energy is the best strategy to avoid ammonia pollution and reproductive problem in dairy cows (Salo, 2018).

2.3.2. Postpartum health

A healthy postpartum period of the dairy cow determines productive and reproductive responses during lactation and is therefore, a pivotal time in the production cycle of the cow. It is well known that impaired postpartum health has a detrimental effect on subsequent fertility of dairy cows. LeBlanc et al. (2006) reported that approximately 75% of postpartum diseases occur within the first month of lactation and Ribeiro et al. (2013) reported that 30% of dairy cows have at least one type of clinical disease within the first 21 d postpartum, and they represent 60 to 80% of all clinical cases occurring in lactating dairy cows. Some of the most commonly occurring postpartum disorders in dairy cows are calving related problems (14.6%), metritis (16.1%), clinical endometritis (20.8%), fever (21.0%), mastitis (12.2%), ketosis (10.4%), lameness (6.8%), digestive problem (2.8%) and pneumonia (2.0%) based on data from 5,719 cows across seven dairy herds in the US (Santos et al., 2010a). A recent study from our research group reported that the incidence of postpartum disorders was 7, 15, 9, 18, 1, 9, and 27% for retained fetal membranes **(RFM)**, metritis, milk fever, ketosis, displaced abomasum, fatty liver and mastitis, respectively, within 60 d postpartum based on data from 1096 cows in 11 dairy herds in Alberta (Colazo et al., 2017). Despite no impact on overall milk production (e.g. 305-d milk yield), cows diagnosed with health problems had delayed ovarian cyclicity within 60 d postpartum, reduced pregnancy to first artificial insemination **(P/AI)** and increased risk for pregnancy loss within first 60 d of gestation (Santos et al., 2010). Interestingly, Colazo et al. (2017) reported that cows with postpartum disorders had lowered milk yield (overall average of -290 kg) by 90 d in milk **(DIM)** compared to healthy cows. Thus, the effect of postpartum disorders is not only limited to fertility of dairy cows but also on milk production and thereby overall profitability of dairy farms (Liang 2013).

Retained fetal membranes, metritis and endometritis are considered uterine diseases, whereas, ketosis, milk fever, displaced abomasum, and fatty liver are considered metabolic diseases. Often, the placenta is expelled within 6h after parturition in dairy cows, but if it is not expelled up to 24h, it is considered as RFM (Sheldon et al., 2008). Even though the mechanisms behind RFM are not clear, abortion, dystocia, twin births, and immunological deficits in the expression of major histocompatibility complex molecules are considered as risk factors for RFM in dairy cows (Joosten et al., 1991). Retention of fetal membranes often leads to ascending uterine infection which in turn becomes a predisposing factor for uterine diseases such as metritis and endometritis. Uterine diseases and its physiological effects on subsequent fertility have been previously reviewed (Sheldon et al., 2006 and 2008). Several studies over the last two decades have clearly shown the negative relationship between uterine diseases and fertility in dairy cows (Opsomer et al., 2000; McDougall, 2001; LeBlanc et al., 2002; Kasimanickam et al., 2004; Gilbert et al., 2005; Galvao et al., 2009; Santos et al., 2010a; Dourey et al., 2011; Ribeiro et al., 2012b; Gobikrushanth et al., 2016). It has been reported that uterine infections during the early postpartum period perturb dominant follicle growth and normal corpus luteum **(CL)** function (Sheldon et al., 2002; Williams et al., 2007) despite having no effect on first postpartum follicle stimulating hormone **(FSH)** rise and the follicular wave emergence (Sheldon et al., 2002). Indeed, Savio et al., (1990) and Beam and Butler (1997) reported that the first postpartum dominant follicle is selected 10 to 12 d after calving in dairy cows irrespective of periparturient diseases or dietary deficiencies. Peter et al. (1989) reported that either intrauterine or intravenous infusion of lipopolysaccharide **(LPS)** blunted the estradiol-induced preovulatory LH surge and prevented ovulation in all treated dairy heifers. Indeed, LPS or various intermediary cytokines such as interleukin **(IL)**-1 or tumor necrosis factor **(TNF)**-α were shown to block GnRH secretion and the pituitary responsiveness to

GnRH pulses (Rivest et al., 1993; Battaglia et al., 2000; Williams et al., 2007). In summary, (1) RFM and uterine diseases are more common following parturition in dairy cattle and have detrimental effects on fertility, (2) microbial uterine infections stimulate a robust immune response disrupting normal reproductive physiology, and (3) increasing our knowledge about the interaction between the environment, uterine infection, immunity, and reproductive physiology is expected to improve prevention and treatment strategies for uterine diseases, thereby reducing infertility in dairy cows (Sheldon et al., 2008).

One of the consequences of having infectious and metabolic disease during early postpartum is that cows have reduced appetite and frequently lose body weight. Nutrient intake is the major driver of energy balance, and energy balance is associated with fertility in dairy cows as discussed previously (Butler, 2003). In fact, cows that lost more body condition (by > 0.5 units) had lower pregnancy rate compared to those lost less (≤ 0.5 units), maintain or gain body condition during early postpartum (Roche et al., 2007; Ribeiro et al., 2013; Chebel et al., 2018; Gobikrushanth et al., 2018b). Of note, we have recently reported that the optimum BCS at calving should be managed at 3.25 for primiparous cows and 3.0 for multiparous cows to promote BCS maintenance or allow only a moderate BCS loss to improve fertility without sacrificing milk yield (Gobikrushanth et al., 2018b).

In summary, events related to calving and the transition from late gestation to early lactation, such as dystocia and uterine diseases (McDougall, 2001; Sheldon et al., 2009), peripartum immune dysfunction (Kehrli et al., 1989; Hammon et al., 2006), endocrine and metabolic changes (Bauman and Currie, 1980), reductions in dry matter intake and nutrient imbalance (Drackley, 1999), and contaminated environment (LeBlanc et al., 2006), are major risk factors leading to the occurrence of health problems during early postpartum. Therefore, good

peripartum management including, but not limited to dry period, optimum BCS at calving, better calving management practices, cleanliness of the environment especially the calving pen, enhanced feed intake following calving to optimize immune function and minimize the severity of NEB, and early and accurate diagnosis and treatment of both infectious and metabolic diseases are critical factors to avoid infertility, thereby increasing the profitability of dairy farms.

2.3.3. Reproductive management tools and technologies

Reproductive management tools and technologies have been developed to identify cows in estrus and inseminate at optimal time, synchronize ovulation to facilitate fixed timed insemination without a need for estrus detection, and determine pregnancy at the earliest possible time to re-inseminate non-pregnant cows to enhance reproductive efficiency of dairy herds.

The detection of behavioural estrus continues to play a key role in the overall management of reproduction in both confinement and pasture-based dairy herds. However, detection of estrus remains a challenge in dairy herds. For example, the duration of estrus is reduced from 15 hours about four decades ago (Esslemont and Bryant, 1976) to 7 to 10 hours in the modern dairy cows (Lopez et al., 2004; Madureira et al., 2015). In a recent review, Fricke et al. (2014) clearly laid out the challenges of estrus detection in dairy farms as: greater proportion of cows with anovular conditions (Wiltbank et al., 2002); attenuation of the estrus behavior associated with increased milk production near the time of estrus resulting in shorter periods of estrus behavior (Lopez et al., 2004); few cows expressing standing estrus at any given time (Roelofs et al., 2005; Palmer et al., 2010); silent ovulations (Palmer et al., 2010; Ranasinghe et al., 2010; Valenza et al., 2012); and reduced expression of estrus owing to confinement housing systems (Palmer et al., 2010) with concrete flooring (Britt et al., 1986).

2.3.3.1. Technologies to improve estrus detection efficiency

The traditional way of estrus detection is through visual observation for 20 to 30 min, three to four times per day at equal intervals; however, this method is time consuming, labour intensive and could be ineffective, especially in larger dairy herds. In addition, economic cost analysis of improving the estrus detection rate by 20 to 30%, and assuming a 50% AI conception rate, resulted in an estimated annual benefit of $83 per cow (Pecsok et al., 1994). Thus, tools were developed to identify cows that exhibit characteristic primary estrus behaviour of standing to be mounted using pressure sensors such as scratch cards (e.g. Estrotect, USA), colour ampoules (e.g. Kamar, USA), and tail chalk or painting. However, there has been a considerable advancement in detection of estrus during the last decade including the introduction of activity monitors to measure secondary signs of estrus such as increased physical activity (Valenza et al., 2012).

Activity monitoring systems typically consist of three major components namely, (1) an activity tag containing pedometer or accelerometer attached to the leg, ear, or a collar on the cow's neck, (2) an antenna to receive data, and (3) a computer with software that allows the dairy farmer to enter information to the system and view activity data outputs. Senger (1994) suggested that the ideal electronic system to detect estrus would (1) provide for continuous surveillance and accurate identification of individual cows; (2) require minimal labour; and (3) accurately predict the timing of ovulation so that cows are inseminated at the appropriate time in relation to ovulation. Even though the activity monitoring systems clearly fulfill the first two requirements, the positive predictive values (proportion of positive that is true positive) were estimated to be 70, 84, and 90% with the use of Afitag (Afimilk, Kibbutz Afikim, Israel), Heatime-RuminAct (SCR Engineers, Netanya, Israel) or HeatPhone (Medria Solutions, Châteaubourg, France), and Heatime (SCR Engineers, Netanya, Israel), respectively, in dairy cows (Chanvallon et al., 2014; Madureira et al.,

2015). It is obvious that any estrus detection system will only detect cows that display estrus, and cows that do not display estrus (e.g. cows in anestrus) will go undetected, emphasizing the need for the refinement of existing technologies or development of new technologies.

The more recent technology of real-time in-line milk progesterone determination through a biochemical sensor, allows to identify cows in estrus and inseminate at optimal time (Herd Navigator™, DeLaval International, Tumba, Sweden). As progesterone is the key hormone that regulates reproductive cycles, frequent monitoring of in-line concentrations of progesterone in milk provides real-time physiological information of reproductive functions that not only predict the timing of AI but also cyclicity status during early postpartum and pregnancy after inseminations for each cow in the herd (Friggens et al., 2008; Saint-Dizier and Chastant-Maillard, 2012; Mottram, 2015; Yu and Maeda, 2017). Of note, recent studies from our laboratory have utilized these in-line milk progesterone data to characterize ovarian activity parameters and associated those with fertility outcomes in dairy cows (Bruinjé et al., 2017a,b). However, the acquiring of in-line milk progesterone system is expensive and is currently limited to only herds that have DeLaval milking system. It would be of more benefit if it is readily adaptable to automated milking systems such as robotic milking system in future.

2.3.3.2. Ovulation synchronization and timed-AI

Timed-AI protocols that are not dependent on estrus detection have been developed concurrently to accelerate the submission rates and thereby pregnancy rates in dairy herds to overcome the issues of poor estrus expression in modern dairy cows as well as to avoid increased labour cost associated with visual estrus detection. The development and applications of timed-AI

protocols have been extensively reviewed (Ambrose et al., 2010; Bisinotto et al., 2014; Colazo et al., 2014; Wiltbank and Pursley 2014).

The ovulation synchronization protocol so called Ovsynch (D0: Gonadotropin-releasing hormone **[GnRH]**, D7: Prostaglandin F2α **[PGF2α]**, D9: GnRH and D10: timed-AI) was first reported in 1995 by Pursley et al. The first GnRH is administered at random stage of the estrous cycle to induce ovulation in cows with functional dominant follicle and the initiation of synchronized follicular wave, PGF is to induce luteolysis, and the second GnRH at 48 h after PGF is to cause synchronized ovulation in order to inseminate all cows on a predicted time, such as at 16 h after the second GnRH of the Ovsynch. Later, it was shown that pregnancy to first AI was positively associated to ovulatory response to the first GnRH of the Ovsynch (Vasconcelos et al., 1999). Only 50 to 60% cows ovulate to the first GnRH of Ovsynch when administered during random stages of the estrous cycle (Bisinotto and Santos 2012; Ribeiro et al., 2012c) and only 68% cows were identified as fully synchronized at the end of the Ovsynch (Colazo et al., 2014); indicating the necessity of presynchronization prior to application of the Ovsynch. The most commonly used and adapted presynchronization methods are: (I) administration of two PGF injections 14 d apart with the second PGF given either 12 or 11 d prior to the initiation of Ovsynch (Presynch-Ovsynch protocol: Moreira et al., 2001; El-Zarkouny 2004; Galvao et al., 2007); (II) administration of PGF and GnRH injections 2 d apart, and the Ovsynch protocol started 6 or 7 d later (G6G protocol: Bello et al., 2006; Ribeiro et al., 2011); and (III) application of two Ovsynch protocols at 7 d interval (Double-Ovsynch: Souza et al., 2008; Herlihy et al., 2012). The major limitation of presynchronization only with PGF, is its ineffectiveness in anovular cows (Walsh et al., 2007; Santos et al., 2009); thus GnRH was included into presynchronization along with PGF injections. Overall, the application of any of the aforementioned presynchronization methods were

shown to result in greater P/AI compared to Ovsynch alone (Moreira et al., 2001; El-Zarkouny 2004; Bello et al., 2006; Ribeiro et al., 2011; Herlihy et al., 2012).

Despite presynchronization, 30% of cows lack a CL at initiation of Ovsynch and this 30% was encompassed of anovular cows and cyclic cows at proestrus, estrus or metestrus stages of their estrous cycle (Bisinotto et al., 2013). It is crucial to have an active CL at the initiation of Ovsynch to maintain a relatively high concentration of progesterone which is essential to avoid follicle aging and precocious maturation of oocyte, which may reduce P/AI and increase pregnancy losses in dairy cows (Savio et al., 1993; Revah et al., 1996; Sartori et al., 2002; Wiltbank et al., 2006). In this regard, later studies have shown that the presence of high P4 during the pre-ovulatory follicular growth is associated with lower LH pulses, greater concentration of follicular IGF-1, improved luteolytic responses, good embryo quality (Cerri et al., 2009; 2011a; 2011b) and thereby better fertility. In addition to missing a CL at initiation of Ovsynch, modern dairy cows even with a CL were known to have low circulating concentration of progesterone due to increased hepatic metabolism of steroid hormones associated with high dry matter intake and milk yield (Sangsritavong et al., 2002). As a result, protocols have been developed to supplement exogenous progesterone such as the use of controlled intravaginal drug release **(CIDR)** devices during the growth phase of dominant follicle between first GnRH and PGF within timed-AI programs (Lima et al., 2009; Denicol et al., 2012; Bisinotto et al., 2013; Colazo et al., 2013). For instance, in a meta-analysis, the application of CIDR within timed-AI programs increased the proportion of cows pregnant at 60 d post-AI by ~18% compared to untreated control cows (34 vs 30%, respectively; Bisinotto et al., 2014). In addition to supplementing exogenous progesterone to support the growth of dominant follicle, recent studies were undertaken to increase the length of proestrus to achieve optimum ovulatory follicle at second GnRH of Ovsynch to increase P/AI and reduce pregnancy

loss following first AI (Bridges et al., 2008; Santos et al., 2010b; Ribeiro et al., 2011, 2012c, 2012d). However, there is still substantial need for further research to improve the synchronization efficacy, simplicity, and practical application of these protocols (Wiltbank and Pursley, 2014).

2.3.3.3. Early diagnosis of non-pregnancy

Early identification of non-pregnant cows following inseminations improves reproductive efficiency and pregnancy rate by decreasing the interval between AI services and increasing AI service rate in dairy cows. In a recent review, Crowe et al. (2018) has summarized the methods of pregnancy detection in dairy cows using direct and indirect methods. Transrectal palpation (Cowie, 1948; Wisnicky and Cassida, 1948) and ultrasound scanning (Griffin and Ginther 1992) of the reproductive tract are the most widely used direct methods of pregnancy determination in the dairy industry. While transrectal palpation of the conceptus is possible from about 5 wk post-insemination, ultrasound scan facilitates the diagnosis of pregnancy at 28 d post-insemination or earlier (Curran et al., 1986; Kastelic et al., 1989). Even pregnancy diagnosis by 28 d post-insemination is considered too late to allow rebreeding of non-pregnant cows at the optimal time (e.g. 18 to 24 d post-insemination) as the normal estrous cycle is typically 18 to 24 d long in dairy cows (Forde et al., 2011).

Indirect methods for early pregnancy diagnosis use qualitative or quantitative measures of hormones or conceptus-specific substances in maternal body fluids as indirect indicators of the presence of a viable pregnancy (Crowe et al., 2018). The determination of progesterone concentration in milk (Nebel, 1988) and either circulating or milk concentrations of pregnancy associated glycoprotein **(PAG)** (Green et al., 2005) are indirect methods of pregnancy diagnosis in dairy cows. However, the accuracy of milk progesterone as a true pregnancy indicator is poor

due to non-pregnant cows with extended luteal phase, persisting CL, and those who have undergone early embryonic losses but continue to have greater circulating concentrations of progesterone (Crowe et al., 2018), as they will be flagged as pregnant for the test. In addition, Green et al., (2005) reported that PAG based pregnancy detection could result in a false positive diagnosis if it is measured too early after parturition as circulating PAG from the previous pregnancy can be detected in the maternal blood up to 6 wk postpartum. In this regard, Fricke et al. (2016) recently described that an ideal early pregnancy method should (1) have high diagnostic values, (2) be simple and less expensive, and (3) determine pregnancy status in real time by being a cow-side test to increase the accuracy of pregnancy detection and thereby facilitate rebreeding at the earliest possible to improve reproductive efficiency of dairy cows. Therefore, further improvements and refinements in these systems are required to increase the accuracy while keeping the cost of the test affordable.

2.3.4. Genetic selection for fertility traits

2.3.4.1. Milk production and fertility

Even though a wide variety of physiological and managerial factors contribute to poor reproductive performance, there is some evidence that the unfavorable genetic correlation with milk production (Jason and Andreasson, 1981; Macmillan et al., 1996; Dematawewa and Berger, 1998; Hansen, 2000; Roxstorm et al., 2001; Kadri et al., 2014) has led to a decline in reproduction in dairy cattle, at least in part due to reduced emphasis placed on fertility traits when selecting for higher milk production (Lucy 2001; Berglund, 2008). It is well known that Nordic countries are the only ones that have included fertility traits along with production and health related traits in their national breeding program since 1972, while the rest of the world primarily focused on

selecting for higher milk yield and conformational traits until up to mid-2000's (Berglund, 2008). As a consequence, the fertility of Nordic Red dairy cows has not been on a declining trend as observed for North American Holsteins. Interestingly, in a recent study, Kadri et al. (2014) showed that a section of bovine genome that simultaneously contributes to higher level of milk production and lower levels of fertility is missing only in few Nordic Red dairy cattle and present in a majority of animals. Consequently, genetic selection programs around the world have been broadened to include traditional fertility traits into national selection indices and focused on a multi-traits-based balanced selection over the last decade (Miglior, 2005).

It must be noted, however, that high milk production *per se* is not detrimental to fertility of dairy cows (LeBlanc, 2010). Several studies from Dr. Stephen Butler's research group (Cummins et al., 2012a, b; Moore et al., 2014a, b) have also demonstrated this by comparing Holstein Friesian cows with similar Holstein genetics (> 75%) and similar genetic merit for milk production (estimated breeding value **[EBV]** for milk yield between +200 to +900 kg), but either good (Fert+) or poor (Fert-) genetic merit for fertility (based on EBV for calving interval) managed under pasture based seasonal-calving system in Ireland. Overall, Fert+ cows had greater dry matter intake, BCS, better metabolic status, earlier resumption of cyclicity, superior uterine health during early postpartum and stronger estrus expression, less silent heats, less ovulation failure, greater luteal phase progesterone concentrations and high IGF-1 concentration at breeding period than Fert- cows, despite having similar genetic merit for milk production. These results suggest that the selection for fertility is possible without adversely affecting milk production.

2.3.4.2. Selection indices in North-America and Ireland

In Canada, daughter fertility sub index was introduced to the Lifetime Performance Index

(LPI) in 2007. Currently, LPI includes three sub-indexes (relative emphasis for Holstein breed are placed within parenthesis): production (40%), durability (40%) and health and fertility (20%), where the relative emphasis placed on the specific traits linked to survival and fertility are herd life (8%) and daughter fertility (13.4%), accounting for a total emphasis of 21.4% in relation to fertility in the LPI (Canadian Dairy Network, 2018). In Ireland, the Irish national breeding program introduced a multi-trait selection index called the Economic Breeding Index **(EBI)** in 2001. Since its introduction, the EBI has evolved to include six sub-indices: milk production (32%), fertility (35%), calving performance (10%), beef carcass (8%), maintenance (7%), management (4%), and health (4%) (Irish Cattle Breeding Federation, 2017), where the fertility sub-index is comprised of 2 traits: survival (11.6%) and calving interval (23.5%), accounting for a total emphasis of 35.1% for fertility, in the EBI. In the USA, the Net Merit Dollars **(NM$)** index included 3 sub-indices: production (43%), health (41%) and type traits (16%), where the relative emphasis placed on the specific traits within health sub-index linked to survival and fertility are cow livability (7%) and daughter pregnancy rate (7%), heifer conception rate (1%) and cow conception rate (2%), accounting for a total emphasis of 17% in relation to fertility in the NM$ (Council of Dairy Cattle Breeding, 2017). Notably, the Irish dairy industry has placed almost three time more emphasis on fertility traits even a decade ago than the Canadian dairy industry (30 vs. 12%, 2007).

2.3.4.3. Traditional fertility traits

Selecting for improved fertility by addition of fertility indices into the national selection indices as described above has not only halted nearly 40 years of a continuous decline in sire and dam EBV for fertility but also started to improve EBV gradually in the US dairy industry (Norman et al., 2009; Garcia-Ruiz et al. 2016). The rate of gain in EBV for traditional fertility traits (e.g.

days open, calving interval and number of services), however, has been slow due to their low heritability (Berry et al., 2014). Therefore, a conventional method of genetic selection is limited in its ability to improve the genetic progress (Berry et al. 2014; Chebel and Ribeiro 2016). In a recent meta-analysis of genetic parameters for female reproductive performance across 55 dairy studies reported that the heritability estimates were 0.02 for first service conception rate and number of services, 0.03 for calving interval, 0.04 for days open, and 0.05 for interval from calving to first service in dairy cows and 0.13 and 0.17 for age at first service and age at first calving, respectively, for dairy heifers (Berry et al., 2014). The low heritability of traditional reproductive traits may be due to the influence of a multitude of cow and managerial factors. For example, daughter pregnancy rate (**DPR**; heritability of 0.04) is the speed at which animals become pregnant, assuming a 60-day voluntary waiting period. However, DPR is highly affected by the actual voluntary waiting period adopted on-farm and use of fixed-time AI or natural services (Chebel and Ribeiro 2016). Despite low heritability, traditional fertility traits are still being included into the selection indices because the effect of additive genetic variation was shown to be substantial (Janson, 1980). While the inclusion of traditional fertility traits such as DPR in the USA to genetic selection played a pivotal role in halting further fertility decline to a certain extent (Garcia-Ruiz et al. 2016), it could not completely reverse the poor reproductive efficiency. Therefore, identification of novel and physiologically relevant fertility traits that are affected less by management and highly related to reproductive performance, for genetic selection, is desirable, to supplement or replace currently available traditional fertility traits.

2.3.4.4. Novel reproductive phenotypes

A fertility trait that can be easily measured in a large number of cows with less cost and has high variability, repeatability, heritability and associations with fertility could be an ideal candidate trait for selection to augment fertility in dairy cows. In addition, genomic selection has revolutionized dairy cattle breeding during the last decade and has the potential to increase genetic gain considerably by reducing generation intervals and increasing selection intensity (Peñagaricano et al., 2017). Furthermore, as the cost of genotyping decreases, the number of animals with genomic evaluations is expected to increase. Therefore, if genetic markers (single nucleotide polymorphisms; **SNP**) for fertility traits are identified, molecular breeding values could be more accurately estimated for each trait, enabling efficient genomic selection throughout the dairy industry.

The evaluation of endocrine (Berry et al., 2012; Tenghe, 2017; Häggman et al., 2018) and estrus-related traits (Ismael et al., 2015) as potential fertility traits for selection is of recent interest. Berry et al. (2012) reported a heritability estimate of 0.13, and identified numerous SNP on *Bos taurus* autosomes **(BTA)** 2 and 20 for commencement of luteal activity **(C-LA)** in primiparous Holstein Friesian cows. In a series of studies, Tenghe (2017) showed that several defined endocrine fertility traits (e.g. C-LA, proportion of cows with luteal activity between 25 and 60 d in milk, and interval from commencement of luteal activity to first service) were: (1) heritable (0.12, 0.12, and 0.11, respectively) and reasonably repeatable (0.29, 0.21, 0.15, respectively), (2) poorly correlated with milk production traits (0.01 to 0.07), and (3) associated with 17 quantitative trait loci **(QTL)** on BTA 2, 3, 8, 12, 15, 17, 23, and 25, identified based on SNP using genome-wide association study **(GWAS)**, in Holstein Friesian cows. More recently, Häggman et al. (2018) reported a

relatively greater heritability estimate of 0.19 and 0.33 for interval from calving to first estrus and C-LA, respectively, in Finnish Red dairy cattle. Ismael et al. (2015) studied the estrus-related traits derived from activity monitor and reported heritability estimates of 0.16 for interval from calving to first sign of high activity (i.e. somewhat similar to C-LA), 0.02 for estrus duration and 0.05 estrus strength. It is noteworthy that C-LA is generally based on frequent measuring of progesterone concentration from milk samples (Petersson et al., 2007), which is costly and logistically challenging to retrieve from a larger population of dairy cows. Nevertheless, endocrine traits such as luteinizing hormone **(LH)**, anti-Müllerian hormone **(AMH)**, IGF-1, and other novel traits such as antral follicle count **(AFC)** need further investigation in dairy cows.

2.3.4.5. Knowledge gaps

Luteinizing hormone has an important role in reproductive function; therefore, selecting cows with greater capacity for LH secretion could be a strategy to improve fertility in dairy cows. Previous studies reported high variability and poor and non-significant repeatability estimates for GnRH-induced LH responses in ram and ewe lambs (Tyrrell et al., 1980) and beef cows (Fajersson et al., 1999). Of note, these studies (Tyrrell et al., 1980; Fajersson et al., 1999) had few animals (< 20) and the negative effect of circulating progesterone on LH responses was not accounted for prior to the evaluation of repeatability estimates. In addition, high heritability (0.44) and inconsistent associations between high LH concentrations and fecundity have been reported in ewes (Haley et al., 1989). Similar conceptual studies evaluating the variability, repeatability and association with fertility of GnRH-induced LH responses, especially under minimal influence of progesterone, have not been conducted in dairy cows.

High variability and repeatability for other novel reproductive phenotypes such as AFC and AMH have been previously reported in cattle based on samples collected on the day of follicular wave emergence in between subsequent waves of the same or consequent estrous cycles (Burns et al., 2005; Ireland et al., 2008). However, the repeatability of AFC and AMH between unknown (random stage) and known (e.g., day of follicular wave emergence) stages of follicular growth has not been established yet in dairy cows. It will be beneficial if these traits could be determined at any stage of follicular growth to facilitate a more convenient sampling without the need for synchronization of estrous cycle and measure on a specific day to test associations with reproductive outcomes.

Regardless of high variability and repeatability reported for circulating AMH concentration in dairy cows (Rico et al., 2009; Monniaux et al., 2013; Ribeiro et al., 2014), the association with fertility outcomes remains unclear (Ribeiro et al., 2014; Baruselli et al., 2015; Jimenez-Krassel et al., 2015). If there is a positive association between circulating AMH and fertility in dairy cows, it would be beneficial to identify the optimum circulating AMH threshold predictive of P/AI in order to identify and selectively breed cows with high AMH to enhance fertility in dairy herds. In addition, genomic heritability for AMH and GWAS identifying SNP associated with phenotypic variation in circulating AMH are some novel aspects yet to be explored in dairy cows.

It has been well documented that circulating concentration of IGF-1 were highly variable, moderately heritable and positively associated with fertility in dairy cows (Velazquez et al., 2008). However, the sensitivity and specificity (diagnostic values) for optimum circulating IGF-1 threshold predictive of P/AI and SNP associated with phenotypic variation of circulating IGF-1 using GWAS remain to be explored in dairy cows. Identification of optimum circulating IGF-1 threshold predictive of P/AI may allow dairy farmers to manage low IGF-1 cows with enhanced

nutritional strategies or even to delay the first service to improve P/AI. Furthermore, the identification of SNP associated with phenotypic variation in circulating IGF-1 concentration, and the subsequent incorporation of these SNP within genomic evaluations may help to accurately identify females with high circulating IGF-1 to enhance fertility in dairy cows through genomic selection.

Apart from the aforementioned four reproductive phenotypes, ano-genital distance **(AGD)** has never been characterized as a potential fertility trait in dairy cows. In general, AGD has been defined as the distance from the center of the anus to the clitoris in females (Sathyanarayana et al., 2010), and known to be positively related to the degree of in-utero androgen exposure (Langman, 1975; Bowman et al., 2003; Macleod et al., 2010; Dean at al., 2012). Interestingly, prenatal exposure to excess androgen in female fetuses were shown to be associated with poor postnatal fertility outcomes in both polytocous (pigs, rodents, rabbits,) and monotocous (human) species (Drickamer et al., 1997; Zehr et al., 2001; Banszegi et al., 2012; Mendiola et al., 2012; Mira-Escolano et al., 2014; Wu et al., 2017). Similar to humans, dairy cows are also a monotocous species, known for having varying maternal concentrations of testosterone and androstenedione during gestation (Gaiani et al., 1984). However, phenotypic characterization of AGD and its relationship to fertility in dairy cows has never been investigated before. In addition, estimation of heritability for AGD and identification of SNP associated with phenotypic variation of AGD would be beneficial if AGD holds potential as a fertility trait in dairy cows.

Chapter 3. Characterization of the variability and repeatability of gonadotropin releasing hormone-induced luteinizing hormone responses in dairy cows within a synchronized ovulation protocol

3.1. Abstract

The primary objective was to determine the variability and repeatability of gonadotropin releasing hormone (GnRH)-induced luteinizing hormone (LH) responses. The secondary objective was to evaluate the associations among plasma LH, follicle stimulating hormone (FSH), estradiol (E2), and progesterone (P4) concentrations. One hundred lactating Holstein cows (35 primiparous, 65 multiparous) were initially subjected to a presynchronization protocol (d 0, PGF2α; d 3, GnRH)) followed 7 d later by Ovsynch (d 10, GnRH; d 17, PGF2α; 56 h later, GnRH)] and timed-AI 16 h after the last GnRH. Blood samples were collected immediately before the GnRH injection of presynchronization and the second GnRH of Ovsynch to determine plasma concentrations of LH, FSH and P4. A second blood sample was collected 2 h after each of the above GnRH injections to determine GnRH-induced LH and FSH concentrations. Plasma concentrations of E2 were also determined in samples collected immediately before the second GnRH of Ovsynch. Cows that (1) had higher LH concentrations at 0 h than at 2 h after GnRH, (2) were indicative of an ongoing spontaneous LH surge, (3) did not respond to GnRH and (4) had P4 ≥ 0.5 ng/mL at GnRH of presynchronization and the second GnRH of Ovsynch were excluded from the analysis. The variability (coefficient of variation) and repeatability (between animal variance / [within animal variance + between animal variance]) of GnRH-induced LH response were determined from samples collected 2 h after the GnRH of presynchronization and

the second GnRH of Ovsynch. The associations among plasma LH, FSH, E2 and P4 were determined at the second GnRH of Ovsynch.

Mean (± SEM) LH concentrations (ng/mL) before GnRH were 0.5 ± 0.04 and 0.6 ± 0.03, while mean LH concentrations 2 h after GnRH were 9.8 ± 1.0 and 12.1 ± 0.8 at GnRH of presynchronization and the second GnRH of Ovsynch, respectively. The variability of GnRH-induced LH was 76.1 and 52.1 % at GnRH of presynchronization and the second GnRH of Ovsynch, respectively. The repeatability estimate for GnRH-induced LH concentration between GnRH of presynchronization and Ovsynch assessments was 0.10. Plasma concentrations of LH were positively associated with FSH and E2 (r = 0.61 and 0.30, respectively) and negatively associated with P4 (r = - 0.46) at the second GnRH of Ovsynch. In summary, GnRH-induced LH responses were highly variable and unrepeatable, and LH concentrations were positively associated with FSH and E2 and negatively associated with P4.

Key words: luteinizing hormone, variability, repeatability, progesterone

3.2. Background

A functional hypothalamic-pituitary-gonadal-axis is essential for regulation of reproduction in both male and female mammals (Land, 1973). Gonadotropin releasing hormone **(GnRH)** is a decapeptide synthesized and released by GnRH neurons in the hypothalamus that induces the release of follicle stimulating hormone **(FSH)** and luteinizing hormone **(LH)** from the anterior pituitary gland through receptor-mediated mechanisms (Kaltenbach et al., 1974; Fink et al., 1988). Progesterone **(P4)** and estradiol **(E2)** regulate FSH and LH release through positive and negative feedback mechanisms that act on the hypothalamus, anterior pituitary or both (Goodman and Karsch., 1980; Karsch et al, 1987; Nett et al., 2002). While FSH is required for

follicular wave emergence (Adams et al., 1992), LH is essential for dominant follicle growth (Ginther et al., 2000), oocyte maturation (Hyttel et al., 1989), ovulation, corpus luteum development and synthesis of P4 (Tomac et al., 2011). These are critical events for establishment and maintenance of pregnancy in domestic animals (Spencer et al., 2004). Therefore, selecting cows with greater capacity for LH secretion under defined conditions could be a strategy to improve fertility in dairy cows.

A phenotype that has high variability, repeatability and heritability would be an ideal candidate for genetic selection. The variability and repeatability of other novel fertility traits such as anti-Müllerian hormone and antral follicle count (Burns et al., 2005; Ireland et al., 2008; Gobikrushanth et al., 2017a) and their association with fertility outcomes have been of recent interest to many researchers (Mossa et al., 2012; Ribeiro et al., 2014). However, the variability, repeatability and association with fertility under minimal influence of P4 have not been examined for GnRH-induced LH responses in dairy cows. Previous studies in rams and ewes (Haley et al., 1989) and beef cows (Webb et al., 1977; Williams et al., 1982; Williams and Stanko 1996; Fajersson et al., 1999) reported that GnRH-induced LH responses were variable between animals based on simple observations. However, none of the above studies quantified the variability using statistical analysis or were specific to lactating dairy cows. Endogenous LH release is pulsatile, resulting in a low correlation between repeated measures from the same animal (Haley et al., 1989); furthermore, measuring endogenous LH surge in large populations is impractical, making it an undesirable candidate for genetic selection. However, the induced LH surge response after exogenous GnRH administration may be a more useful endocrine parameter for investigating variability and repeatability. Previous studies reported a poor and non-significant repeatability for GnRH-induced LH responses when ram and ewe lambs (Tyrrell et al., 1980; n = 15 for each

sex) and beef cows (Fajersson et al., 1999; n = 18) were repeatedly challenged with exogenous GnRH treatments. However, small sample sizes and variable concentrations of P4 might have attributed to non-significant repeatability estimates. In addition, although high heritability (0.44) and associations between high LH concentrations and fecundity have been reported in ewes, the associations were inconsistent (Haley et al., 1989). Similar conceptual studies evaluating the association between GnRH-induced LH responses and fertility in lactating dairy cows are lacking.

The evaluation of the variability and repeatability of GnRH-induced LH response, and establishing its association with fertility may identify it as a fertility phenotype to be considered in future genomic selection in dairy cows. We hypothesized that cows have variable responses to GnRH injection even under low P4 environment, and those responses are repeatable. Therefore, our primary objective was to determine the variability and repeatability of GnRH-induced LH responses. The secondary objective was to evaluate the associations among plasma LH, FSH, E2, and P4 concentrations. In addition, the associations among LH response categories, FSH, E2, P4, and reproductive outcomes (i.e. ovulatory response, pregnancy to first AI [Pregnancy at 33-d post-AI] **(P/AI)**, pregnancy at 60-d post-AI and pregnancy loss **[PLOSS]**) were also examined.

3.3. Materials and methods

3.3.1. Animals and housing

The study was conducted at the Dairy Research and Technology Centre of the University of Alberta between November 2014 and September 2016. All the experimental procedures were approved by the University of Alberta's Animal Care and Use Committee for Livestock, and animals were cared for in accordance with the requirements of Canadian Council on Animal Care

(2009). One hundred lactating Holstein cows (35 primiparous, 65 multiparous) were initially enrolled in the study. Cows were individually fed a total mixed ration (primary ingredients were barley silage, alfalfa silage, alfalfa hay, and concentrates) and housed in tie-stalls and let out for approximately 2 h of exercise during weekdays. Diets were formulated according to **NRC** (National Research Council, 2001) to meet the requirements of a 650 kg lactating cow producing 45 kg of milk/d and cows had *ad libitum* access to water.

3.3.2. Reproductive management and blood sampling

Cows that were on average 52 (SD = 4.0; range = 45 to 59) DIM, were placed on a modified G6G protocol and subjected to timed-AI (Figure 1). In brief, the presynchronization protocol consisted of PGF2α (d 0; Estrumate; 500 μg, i.m; Merck Intervet Corp. Kirkland, QC, Canada) and GnRH (d 3; Fertiline; 100 μg gonadorelin acetate, i.m.; Vetoquinol N. A. Inc. Lavaltrie QC, Canada) administered 3d apart. The Ovsynch protocol was initiated 7 d after the GnRH injection of the presynchronization program, and involved i.m. injections of GnRH (d 10), PGF2α (d 17) and GnRH 56 h later, followed by timed-AI 16 to 20 h later (mean DIM = 72).

Transrectal ultrasonography (Aloka 500, Aloka Co Ltd., Tokyo, Japan) using a 7.5 MHz linear array transducer was first conducted at the time of the second GnRH of Ovsynch (~ 71 DIM) to confirm the presence of putative ovulatory follicle(s) (≥ 10 mm in diameter). Ovulation was confirmed on 73 DIM by the absence of the follicle(s) that had been detected at the previous ultrasound examination. Ovulatory response was defined as the proportion of cows that ovulated after the second GnRH of Ovsynch. Transrectal ultrasonography of uterine contents was performed 33 d post-AI and visualization of a viable embryo confirmed the pregnancy. Cows that were determined pregnant at 33 d post-AI were examined again at 60 d post-AI using transrectal ultrasonography, to reconfirm pregnancy. Pregnancies per AI (P/AI) at 33 d and pregnancy at 60

d post-AI were determined based on the proportion of cows pregnant at 33 and 60 d post-AI, respectively. When embryonic death occurred between 33 and 60 d post-AI, it was considered as a pregnancy loss.

Blood samples were collected from coccygeal blood vessel using evacuated Vacutainer® tubes containing sodium heparin as an anticoagulant (Becton Dickinson and Company, New Jersey, USA) immediately before (0 h) the GnRH of presynchronization and the second GnRH of Ovsynch to determine plasma concentrations of LH, FSH and P4 (ng/mL), and 2 h after each of the above GnRH treatments to determine plasma LH and FSH (ng/mL). The 2 h interval for collecting blood samples after GnRH administration to determine maximum pituitary responsiveness to GnRH was based on LH profiles in previous studies (Ambrose et al., 2005; Colazo et al., 2009b; Dias et al., 2010; Pulley et al., 2015). Plasma concentrations of E2 were determined from blood samples collected immediately before the second GnRH of Ovsynch. Samples were placed on ice upon collection and centrifuged at 1500 x *g* for 20 min at 4°C, plasma harvested and frozen at -20 °C until assayed for plasma LH, FSH, E2 and P4.

3.3.3. Determination of plasma concentration of LH, FSH, E2 and P4

Plasma concentrations of LH, FSH, E2 and P4 were determined at Endocrine Lab Services, University of Saskatchewan, Saskatoon, SK, Canada.

Plasma LH concentrations were determined in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) as described by Evans et al. (1994). All samples were analyzed in a single assay; the intra-assay coefficient of variation was 12.3 % for low reference samples (mean, 0.98 ng/mL) and 9.2% for high reference samples (mean, 1.70 ng/mL).

Plasma FSH concentrations were determined in duplicate using a double-antibody radioimmunoassay using NIDDK-anti-oFSH-1 primary antibody and expressed as USDA bovine

FSH-Il units as described by Evans et al., (1994). All samples were analyzed in a single assay; the intra-assay coefficient of variation was 10.1 % for low reference samples (mean, 0.27 ng/mL) and 4.5 % for high reference samples (mean, 3.17 ng/mL)

Plasma concentrations of E2 were determined after ether extraction using a radioimmunoassay procedure as originally described by Rawlings et al (1984). All samples were analyzed in a single assay; the intra-assay coefficient of variation was 13.7 % for low reference samples (mean, 2.8 pg/mL) and 12.1 % for high reference samples (mean, 9.1 pg/mL).

Plasma P4 concentrations were determined in duplicate using a commercial solid-phase radioimmunoassay kit (ImmuChem^{TM}; MP Biomedicals, LLC, Orangeburg, NY). All samples were analyzed in a single assay. The intra-assay coefficients of variations were 18.6 % for low- (mean, 1.2 ng/mL) and 11.6 % for high-reference samples (mean, 10.8 ng/mL), respectively.

3.3.4. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Cows were excluded from the analysis that fell within the following criteria: (1) LH concentrations were greater at 0 h than at 2 h after GnRH [n = 0 at GnRH of presynchronization; n = 2 at the second GnRH of Ovsynch]; (2) LH concentrations were indicative of an ongoing spontaneous LH surge at 0 h (≥ 1.0 ng/mL) even if LH concentration increased further at 2 h after GnRH injection [n = 7 at GnRH of presynchronization; n = 3 at the second GnRH of Ovsynch]; and (3) did not exhibit an increase in plasma LH in response to GnRH administration [n = 4 at GnRH of presynchronization; n = 4 at the second GnRH of Ovsynch]. A GnRH-induced LH response was considered to have occurred when the LH concentration at 2 h after GnRH exceeded the mean of the baseline by 2 standard deviations (Pulley et al., 2015).

The mean, standard error of mean **(± SEM)**, range and coefficient of variation for GnRH-induced LH was first determined in 89 cows at GnRH of presynchronization and 91 cows at the second GnRH of Ovsynch using MEANS procedure of SAS and later determined in cows that had plasma P4 < 0.5 ng/mL at GnRH of presynchronization (n = 60) and the second GnRH of Ovsynch (n = 70). The cutoff of < 0.5 ng/mL was chosen to simulate concentrations of P4 during estrus as suggested by Stevenson and Pulley (2016), and, in addition, this was the optimum P4 concentration to predict the probability of pregnancy using receiver operating characteristic **(ROC)** analysis in the current study (sensitivity 94.4 and specificity 27.4 %) and in previous studies (Colazo et al., 2017; Wilsdorf et al., 2016).

The repeatability (range 0 to 1, with 1 being the highest) was defined as the proportion of the total variance that attributed to between-animal variance, which was calculated as σ^2 between-animal / σ^2 between-animal + σ^2 within-animal. Variance components were estimated using ANOVA in Microsoft Excel 2016 and the repeatability for GnRH-induced LH responses between GnRH of presynchronization and the second GnRH of Ovsynch was calculated. Furthermore, the association between GnRH of presynchronization and the second GnRH of Ovsynch assessments for GnRH-induced LH response was determined by estimating the Pearson correlation of coefficient (r; ranges from - 1 to + 1 [where value 0, < 0 and > 0 indicates no association, negative association and a positive association, respectively]) using CORR procedure of SAS. These were conducted first in all 81 cows and later in a subset of cows (n = 45) that had plasma P4 concentrations < 0.5 ng/mL at both assessments.

The associations among plasma LH, FSH, E2 and P4 were determined first in all 91 cows and later in a subset of 70 cows that had P4 < 0.5 ng/mL at the second GnRH of Ovsynch by estimating the Pearson correlation of coefficient using CORR procedure of SAS. Moreover, the

linear regression among the aforementioned continuous variables was also tested using REG procedure of SAS and the regression line and equation were plotted using Microsoft Excel 2016.

Cows that had P4 concentration < 0.5 ng/mL at the second GnRH of Ovsynch (n = 70) were ranked based on plasma LH, from highest to lowest, and those in the top (n = 24) and bottom (n = 24) thirds were classified into HIGH- and LOW-LH categories. The associations among LH categories, parity and plasma concentrations of FSH, E2 and P4 were determined using MIXED procedure SAS. The associations among LH response categories, parity, ovulatory response to the second GnRH of Ovsynch, P/AI at 33 d post-AI, pregnancy at 60 d post-AI and PLOSS were tested using GLIMMIX procedure of SAS. The aforementioned continuous and binomial variables were initially modelled against LH category, parity and their interactions. As none of the interactions was significant, the final model only included LH category and parity. Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

3.4. Results

3.4.1. Variability and repeatability of GnRH-induced release of LH

Mean (± SEM) plasma concentrations of LH (ng/mL) before the GnRH of presynchronization (0.4 ± 0.03) did not differ ($P > 0.05$) from those preceding the second GnRH of Ovsynch (0.6 ± 0.03). Likewise, LH concentrations 2 h after GnRH of presynchronization (8.7 ± 0.7; range = 1.2 to 27.4 ng/mL) and Ovsynch (10.4 ± 0.7; range = 1.2 to 28.4 ng/mL) did not differ ($P > 0.05$). The variability of the GnRH-induced LH response was 78.7 and 63.1 % at GnRH of presynchronization and the second GnRH of Ovsynch assessments, respectively. When evaluated in cows that had P4 < 0.5 ng/mL, the variability values were 76.1 and 52.1 % at GnRH of presynchronization and the second GnRH of Ovsynch assessments and the mean (± SEM) LH

2 h after GnRH did not differ (P > 0.05) between presynchronization and Ovsynch assessments and were 9.8 ± 1.0 (range, 1.4 to 27.4 ng/mL) and 12.1 ± 0.8 (range, 1.2 to 28.4 ng/mL) at GnRH of presynchronization (n = 60) and the second GnRH of Ovsynch (n = 70; Figure 2a, b). The repeatability of GnRH-induced LH concentrations between presynchronization and Ovsynch assessments was low (0.16) yet significant when determined in all cows (n = 81; P = 0.02) and repeatability (0.10) was non-significant when determined only in cows that had P4 < 0.5 ng/mL (n = 45; P = 0.25). The estimated correlation coefficient for GnRH-induced LH between presynchronization and Ovsynch assessments was 0.24 (P = 0.03) when evaluated in all cows (n = 81; Figure 3a) and 0.15 (P = 0.33) when evaluated only in cows that had P4 < 0.5 ng/mL (n = 45; Figure 3b).

3.4.2. Associations among plasma LH concentration and FSH, E2 and P4

Plasma concentrations of LH were positively associated with FSH (r = 0.65; P<0.01; Figure 4a) and E2 (r = 0.35; P < 0.01; Figure 5a) in all 91 cows as well as in the 70 cows that had P4 < 0.5 ng/mL at the second GnRH of Ovsynch (r = 0.61 and 0.30; P < 0.01; Figure 4b and 5b, respectively). On the other hand, LH concentrations had a negative association with P4 when evaluated in all 91 cows (r = - 0.45; P < 0.01; Figure 6a) as well as in cows that had P4 ≥ 0.5 ng/mL (r = - 0.46; P = 0.03; Figure 6b). However, the association was very poor and non-significant in cows that had P4 < 0.5 ng/mL (r = - 0.07; P = 0.54; Figure 6c).

3.4.3. Associations among LH categories, parity, plasma LH, FSH, E2, P4, and reproductive outcomes

Cows that were categorized as HIGH-LH had greater (P < 0.01) mean plasma concentrations of FSH and a tendency (P = 0.09) for higher E2 than those categorized as LOW-

LH, but plasma P4 and the reproductive outcomes evaluated did not differ between HIGH-LH and LOW-LH categories (Table 1).

Primiparous cows had a tendency for lower P4 (mean ± SEM; 0.001 ± 0.01 vs. 0.03 ± 0.01 ng/mL; P = 0.06) and greater P/AI at 33 d post-AI (60.0 vs. 32.1 %; P = 0.06) than multiparous cows. However, mean plasma concentrations of LH, FSH and E2, and other reproductive outcomes (i.e., pregnancy at 60 d post-AI, ovulatory response and pregnancy loss) did not differ between primiparous and multiparous cows (Table 1).

3.5. Discussion

The variability for GnRH-induced LH responses was high at both presynchronization and Ovsynch assessments. To the best of our knowledge, this is the first study to report variability of GnRH-induced LH responses under minimal influences of P4 in lactating dairy cows. Progesterone had a negative association with LH response in the current study (Figure 6a and b), which has also been reported in previous studies (Colazo et al., 2008, Giordano et al., 2012, Stevenson and Pulley 2016). Elevated circulating P4 affects LH through a number of mechanisms: direct inhibition of LH release from the anterior pituitary gland (Schoenemann et al., 1985), downregulation of GnRH receptors in the pituitary gland thereby reducing pituitary responsiveness to GnRH and through inhibition of GnRH pulses from the hypothalamus (Nett et al., 2002). Therefore, we inferred that the high variability values observed for GnRH-induced LH responses (78.7 and 63.1 % at presynchronization and Ovsynch assessments, respectively) were negatively influenced by peripheral P4 concentrations at the time of GnRH administration. Notably, the between-animal-variability values were decreased, yet remained high (76.1 and 52.1 % at presynchronization and Ovsynch assessments, respectively) despite adjusting for the

possible suppressive influence of P4 on LH, by removing cows with P4 ≥ 0.5 ng/mL, indicating that a wide phenotypic variation exists for GnRH-induced LH responses in dairy cows, even after accounting for the possible effects of P4.

The repeatability of GnRH-induced LH concentration between presynchronization and Ovsynch assessments was low (0.16) when evaluated in all cows (P = 0.02). Our finding is in agreement with previous reports in small (Tyrrell et al., 1980) and large ruminants (Fajersson et al. 1999). Tyrrell et al. (1980) reported poor and non-significant repeatability estimates (0.10 to 0.20) for LH release when pre-pubertal ram and ewe lambs (n = 15 per sex) were repeatedly challenged with exogenous GnRH treatments for seven consecutive months starting from approximately 9 wk of age. Similarly, Fajersson et al. (1999) reported a non-significant range of correlation (r = - 0.21 to 0.50; P > 0.10) when beef cattle (n = 18) exhibiting phenotypically extreme LH responses (high and low; selected based on > 1 SD above the mean and > 1 SD below the mean, respectively) were subjected to exogenous GnRH injections (100 µg i.v.) at d 5 to 8 postpartum (after 2 consecutive calving events) and at 170 d of gestation. At these two distinct time points, cows would have had extreme differences in both energy status and circulating P4 concentrations. In the present study, we expected a greater repeatability for GnRH-induced LH responses by assessing cows at 8 and 10 weeks postpartum (assuming relatively similar energy states) and by eliminating the negative effect of circulating P4, but repeatability was further reduced (0.10). The GnRH-induced LH response and its repeatability could be negatively influenced by other factors such as low energy intake (Beal at al., 1978) and dietary long chain fatty acids (Salehi et al., 2015) in addition to negative energy balance during the early postpartum period (Leers-Sucheta at al., 1994). In the current study, possible influences of energy status, high P4 concentrations, and dietary fats on GnRH-induced LH responses were controlled and/or

avoided by evaluating GnRH-induced LH responses twice within a short interval (8 and 10 weeks), by eliminating cows that had P4 ≥ 0.5 ng/mL at GnRH, and by feeding similar diets. Indeed, the overall mean concentrations of GnRH-induced LH did not differ between GnRH of presynchronization and the second GnRH of Ovsynch assessments in the present study. Together, these results suggest that GnRH-induced LH responses have poor repeatability, even under conditions that have been standardized as much as practically possible.

The association between GnRH-induced LH response categories, reproductive hormones, and fertility, under controlled influences of P4 (< 0.5 ng/mL), has not been previously studied in dairy cows. Cows in the HIGH-LH category had greater concentrations of FSH and a tendency for higher E2 than cows in the LOW-LH category and this is evident from the positive associations among LH, FSH and E2 reported in the current study (Figures 4 and 5) as well as in previous studies (Foster et al., 1980, Nett et al., 2002, Stevenson and Pulley 2016). We expected that cows with a high LH response to GnRH treatment would have increased likelihood of ovulation and consequently improved reproductive outcomes compared to cows with a low LH response. However, the reproductive outcomes, such as P/AI and pregnancy at 60-d post-AI and PLOSS were similar between HIGH- and LOW-LH categories. Given that this experiment was not adequately powered to evaluate the association between GnRH-induced LH response and fertility outcomes, the results presented herein should be interpreted cautiously. A posteriori power analysis based on the actual difference of 12.5 % in ovulation response called for 93 animals per LH category. With only 24 animals per LH category in the current study, a difference of at least 45 % in ovulatory response was required to attain statistical significance.

In a study conceptually similar to ours, Haley et al., (1989) reported associations between GnRH-induced LH responsiveness and fertility in sheep. They classified ram lambs into high-

and low-responsive lines on the basis of mean concentration of LH determined at 10 wks of age following an i.v. injection of 5 μg GnRH. Thereafter, rams that were classified into high- and low- responsive lines were mated to ewes from the same lines, and progressively bred in a similar manner for several generations. After 8 male generations, the mean LH response for rams in the high-responsive line was 5 times greater than that of the low-responsive line (Haley et al., 1989). However, the associations between LH responsive lines and fertility were inconsistent. Ewes bred to high responder rams had higher ovulation rates in general than the low-responsive line during the first breeding season but not during the second breeding season. However, increases in the number of lambs born per ewe were not significant except during one generation.

In conclusion, despite high variability, the use of GnRH-induced LH response as a fertility phenotype for genetic selection remains questionable due to its poor repeatability. The association between GnRH-induced LH responses and fertility outcomes under minimal influences of P4 warrants further investigation in a larger population of dairy cows.

3.6. Acknowledgments

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Table 3. 1. Associations among LH categories, parity, plasma LH, FSH, estradiol, progesterone and reproductive outcomes in lactating dairy cows

	LH Category[1]			Parity		
	HIGH-LH (n = 24)	LOW-LH (n = 24)	P-Value	Primiparous (n = 16)	Multiparous (n = 32)	P-Value
Plasma concentration (mean [± SEM][2])						
LH (ng/mL)	18.9 ± 0.8[a]	5.6 ± 0.8[b]	< 0.01	12.3 ± 0.9	12.2 ± 0.6	0.90
FSH (ng/mL)	0.9 ± 0.05[a]	0.5 ± 0.05[b]	< 0.01	0.8 ± 0.06	0.7 ± 0.04	0.39
Estradiol (pg/mL)	2.6 ± 0.3[x]	1.8 ± 0.3[y]	0.09	2.0 ± 0.4	2.4 ± 0.3	0.49
Progesterone(ng/mL)	0.01 ± 0.01	0.02 ± 0.01	0.24	0.001 ± 0.01[x]	0.03 ± 0.01[y]	0.06
Ovulatory response (%)*[3]	95.8 (23/24)	83.3 (20/24)	0.17	93.7 (15/16)	87.5 (28/32)	0.44
Pregnancy at 33 d post-AI (%)*	43.5 (10/23)	40.0 (8/20)	0.43	60.0 (9/15)[x]	32.1 (9/28)[y]	0.06
Pregnancy at 60 d post-AI (%)*	34.8 (8/23)	30.0 (6/20)	0.43	46.7 (7/15)	25.0 (7/28)	0.11
Pregnancy losses between 33 and 60 d post-AI (%)*	20.0 (2/10)	25.0 (2/8)	0.80	22.2 (2/9)	22.2 (2/9)	1.00

[a,b] Different superscripts within the same row and category differ ($P < 0.05$); [x,y] tendency ($P > 0.05 \leq 0.10$)

[1]LH categories: cows that had P4 < 0.5 ng/mL at the second GnRH of Ovsynch (n = 70) were ranked by LH concentration, from highest to lowest, and those in the top and bottom thirds were designated as HIGH- and LOW-LH categories (n = 24 each)

[2]SEM: standard error of mean

[3]*Percentages reported for pregnancy at 33 and 60 d post-AI and pregnancy losses between 33 and 60 d post-AI were based on cows that ovulated

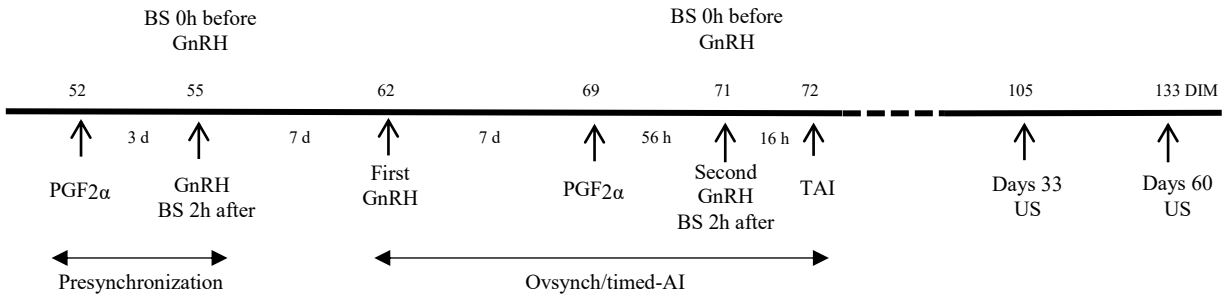

Figure 3. 1. Hundread lactating Holstein cows (35 primiparous, 65 multiparous) were subjected to a modified presynchronization-Ovsynch protocol [presynchronization (d 0, PGF2α; d 3, GnRH) followed 7 d later by Ovsynch-56 (d 0, GnRH; d 7, PGF2α; GnRH, 56 h later)] to receive first timed-AI (~ 72 DIM) 16 h after the last GnRH treatment.

All injections and timed-AI were performed in the morning (0800 h) except for the second GnRH of Ovsynch which was given in the afternoon (1600 h). Blood samples (BS) were collected immediately before (0 h) the GnRH of presynchronization and the second GnRH of Ovsynch to determine plasma concentrations of LH, FSH and P4 (ng/mL), and 2 h after each of the above GnRH injections to determine plasma LH and FSH (ng/mL). Plasma concentrations of E2 were determined from BS collected immediately before the second GnRH of Ovsynch. Transrectal ultrasonography (US) was first conducted at the time of the second GnRH of Ovsynch (~ 71 DIM) to confirm presence of putative ovulatory follicle(s). Ovulation was confirmed on 73 DIM by the absence of follicle(s) (≥ 10 mm in diameter) that had been detected at the previous US examination (not illustrated). Pregnancy was diagnosed at 33 d post-AI (P/AI) by US and cows diagnosed pregnant were reconfirmed at 60 d post-AI by US.

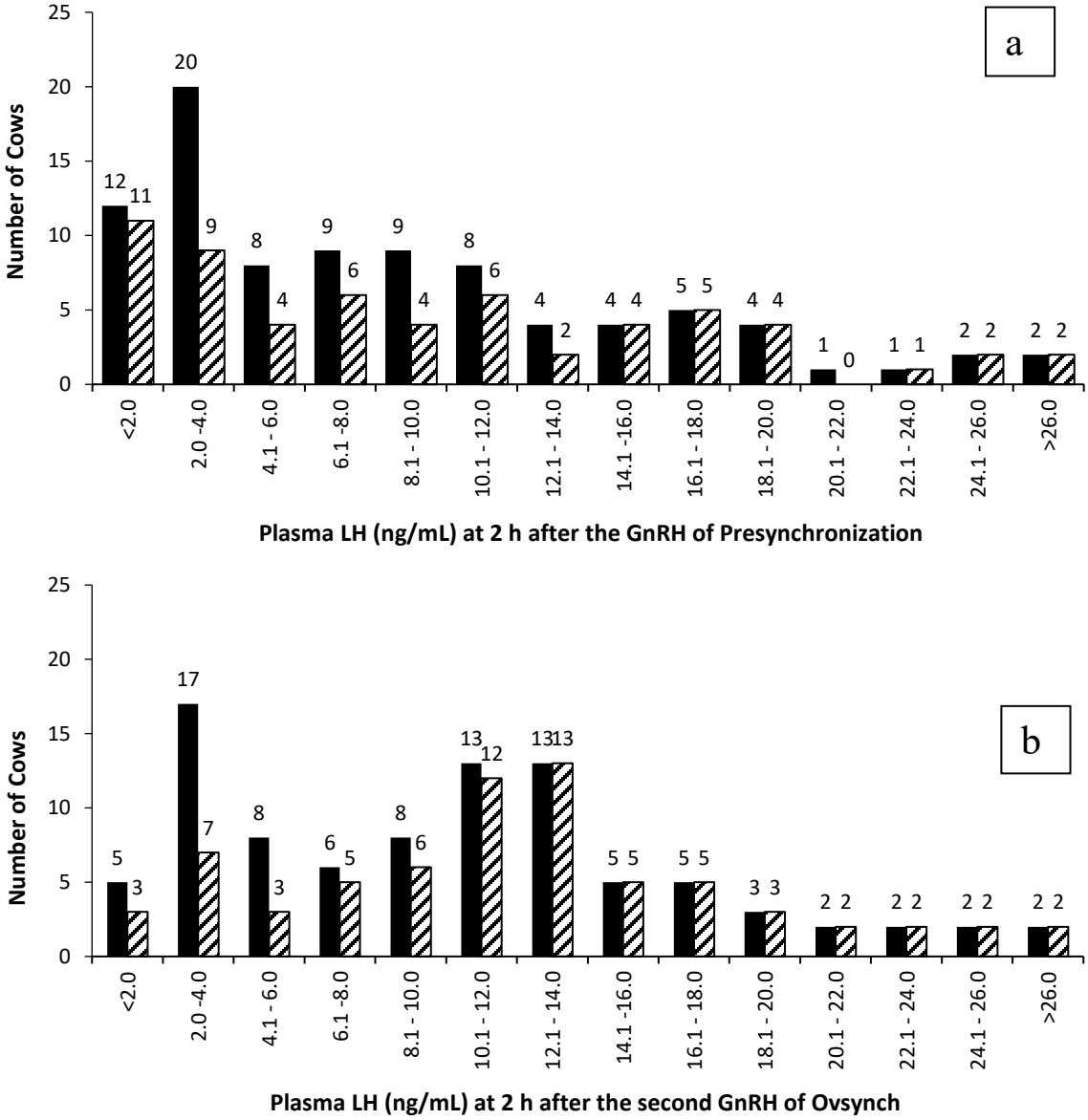

Figure 3. 2. The distribution of plasma LH concentrations (ng/mL) determined at 2 h after the GnRH of presynchronization (a) and the second GnRH of Ovsynch (b) in all cows (filled bars; n = 89 for presynchronization and 91 for Ovsynch assessments) and in cows that had plasma P4 < 0.5 ng/mL (hatched bars; n = 60 for presynchronization and 70 for Ovsynch assessments).

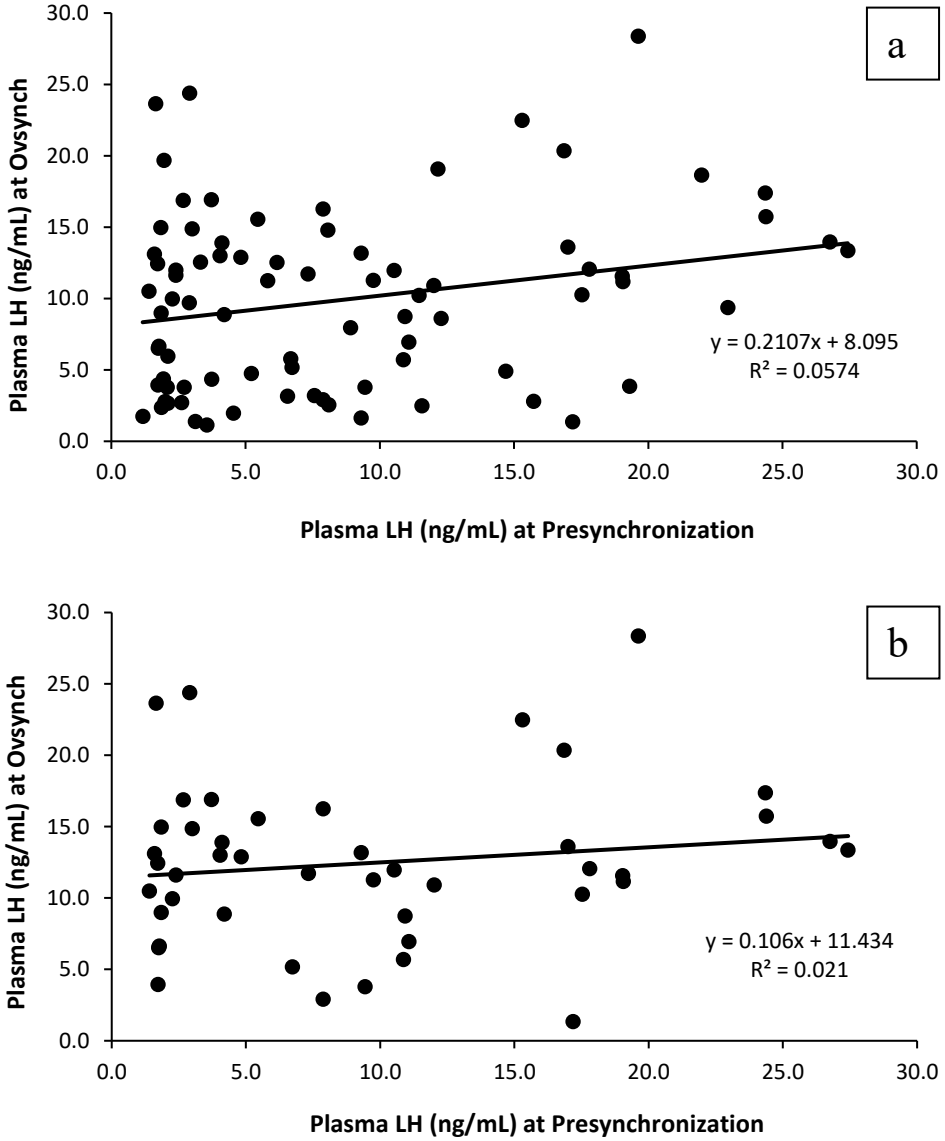

Figure 3. 3. Association between plasma LH (ng/mL) determined at 2 h after GnRH of presynchronization and the second GnRH of Ovsynch in all cows (a; n = 81; P = 0.03) and in cows that had P4 concentrations < 0.5 ng/mL at both GnRH of presynchronization and at the second GnRH of Ovsynch (b; n = 45; P = 0.33).

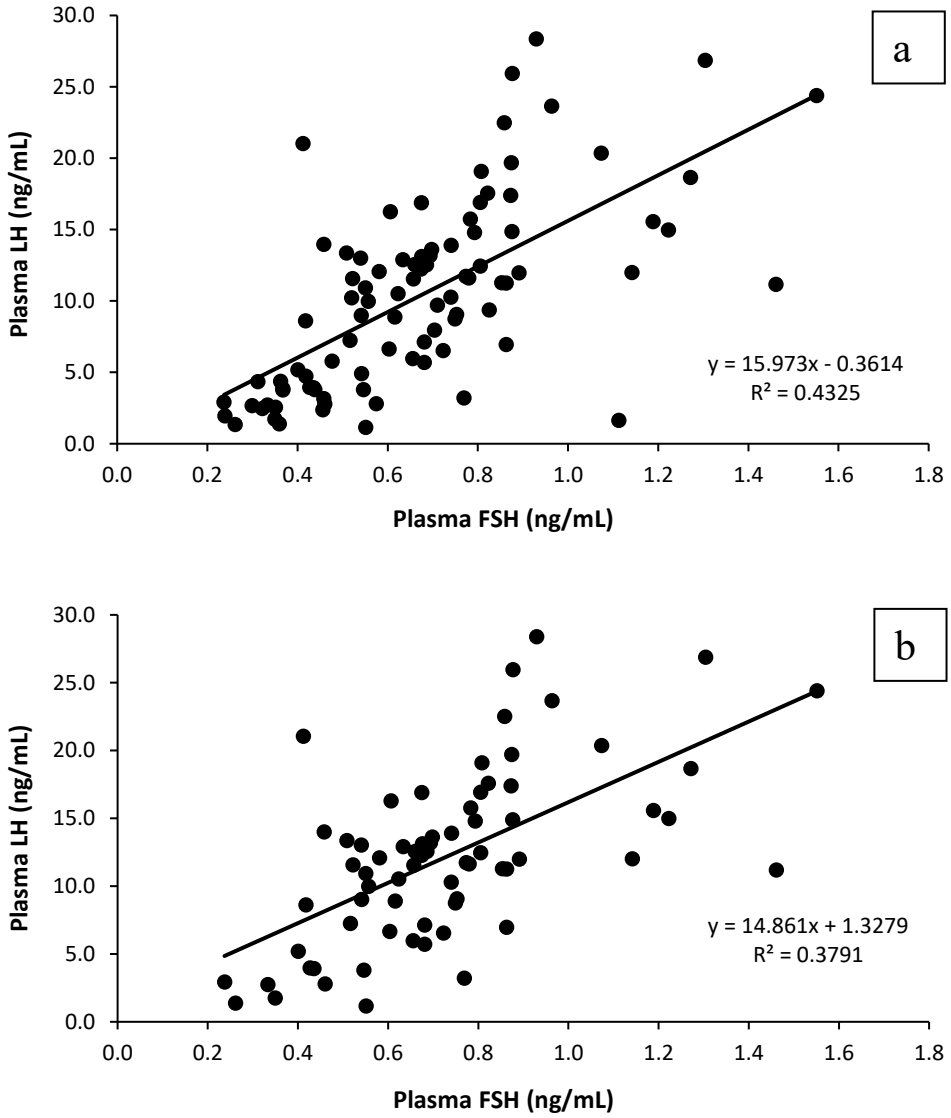

Figure 3. 4. Association between plasma FSH and LH (ng/mL) determined at 2 h after the second GnRH of Ovsynch in all cows (a; n = 91; P < 0.01) and in cows that had P4 < 0.5 ng/mL at the second GnRH of Ovsynch (b; n = 70; P < 0.01).

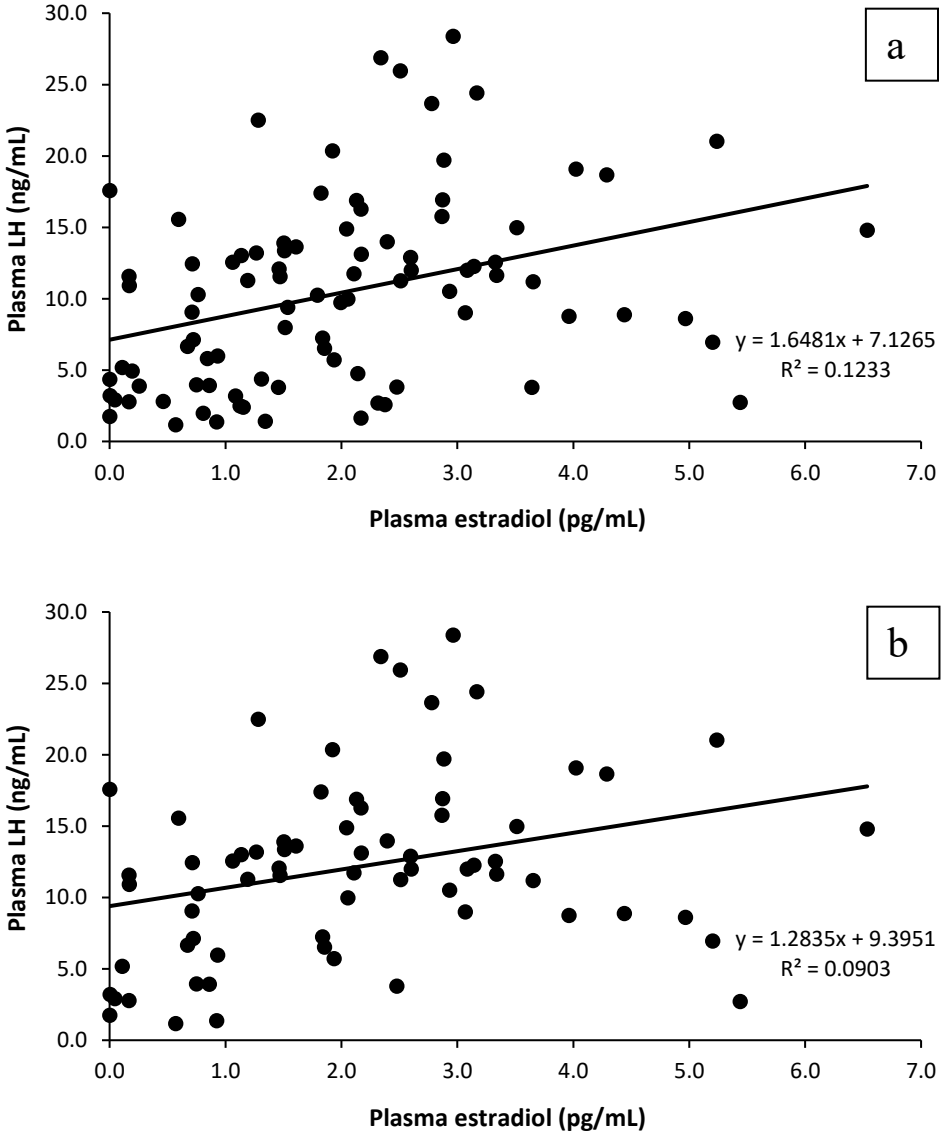

Figure 3. 5. Association between plasma estradiol (pg/mL) determined at 0 h before the second GnRH of Ovsynch and plasma LH (ng/mL) determined at 2 h after the second GnRH of Ovsynch in all cows (a; n = 91; $P < 0.01$) and in cows that had P4 < 0.5 ng/mL at the second GnRH of Ovsynch (b; n = 70; $P < 0.01$).

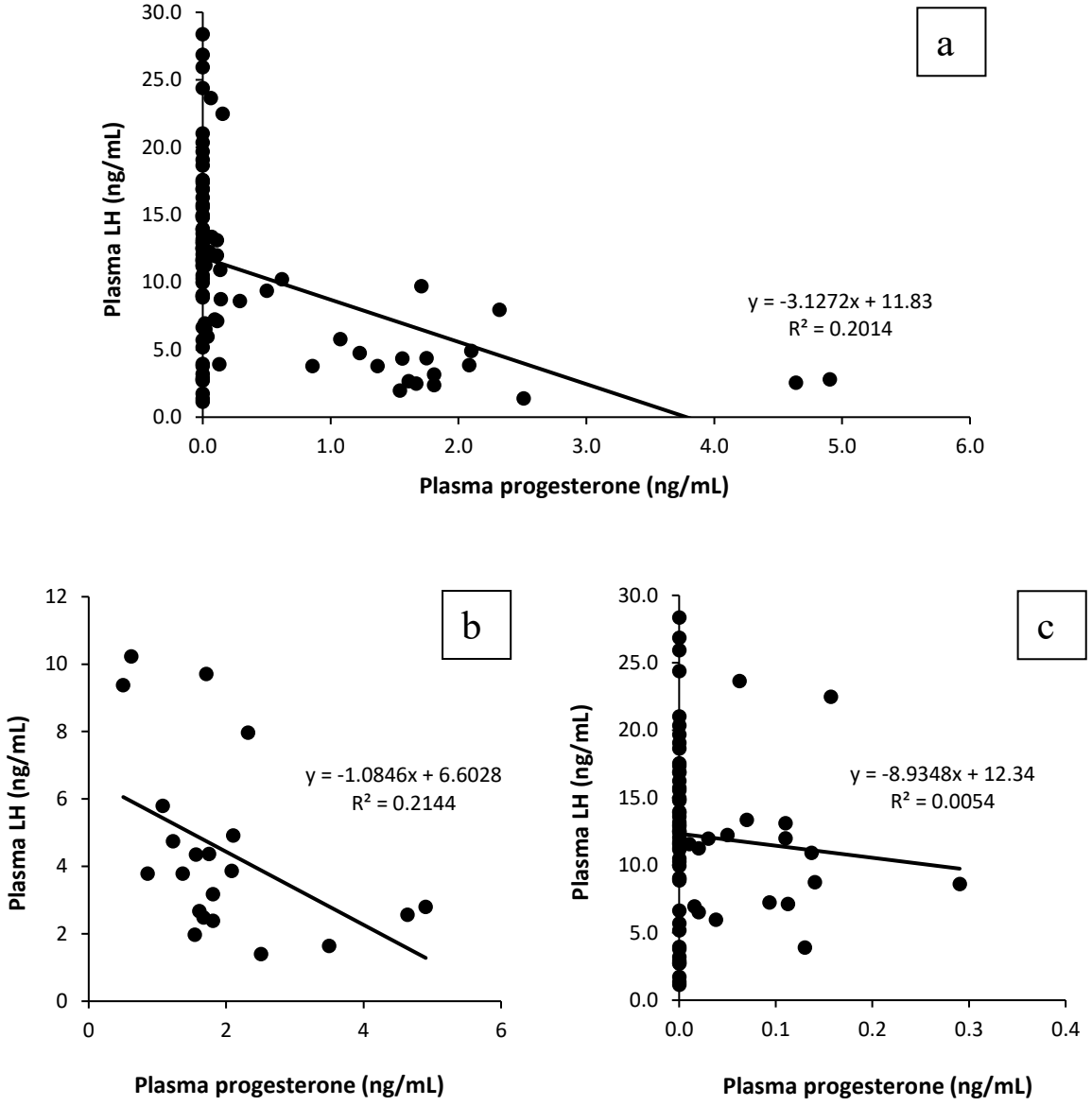

Figure 3. 6. Association between plasma progesterone (ng/mL) determined before (0 h) the second GnRH of Ovsynch and plasma LH (ng/mL) determined at 2 h after the second GnRH of Ovsynch in all cows (a; n = 91; P < 0.01), in cows that had P4 ≥ 0.5 ng/mL (b; n = 21; P = 0.03) and P4 < 0.5 ng/mL (c; n = 70; P = 0.54).

Chapter 4. Repeatability of antral follicle counts and anti-Müllerian hormone and their associations determined at an unknown stage of follicular growth and an expected day of follicular wave emergence in dairy cows

4.1. Abstract

The objectives were to determine (1) the repeatability of antral follicle counts (AFC) and anti-Müllerian hormone (AMH) concentrations at an unknown stage of follicular growth (FG) and at an expected day of follicular wave emergence (FWE) in dairy cows, and (2) the association between AFC and AMH at the above two stages. Transrectal ultrasound imaging of the ovaries and blood sample collection were performed on 91 lactating Holstein cows (33 primiparous, 58 multiparous) to determine AFC and serum concentrations of AMH at an unknown stage of FG (mean ± SEM; 14 ± 0.5 d postpartum) and an expected day of FWE (mean ± SEM; 73 ± 0.5 d postpartum, approximately 36 h after the 2nd GnRH of Ovsynch protocol). The AFC ranged from 10 to 53 (mean ± SEM; 26.1 ± 1.0) and 6 to 45 (mean ± SEM; 23.4 ± 1.0) at an unknown stage of FG and expected day of FWE, respectively (P = 0.02). Serum concentrations (pg/mL) of AMH ranged from 13.9 to 528.8 (mean ± SEM; 187.3 ± 13.1) and 38.2 to 774.1 (mean ± SEM; 218.7 ± 14.5) at an unknown stage of FG and expected day of FWE, respectively (P < 0.01). The repeatability estimates for AFC and serum AMH concentrations between an unknown stage of FG and the expected day of FWE were 0.37 and 0.73 (P < 0.01), respectively. The correlation between AFC and AMH was moderate at an unknown stage of FG (r =0.54; P < 0.01) and at an expected day of FWE (r = 0.59; P < 0.01). In summary, both AFC and AMH were repeatable when measured at an unknown stage of FG and an expected day of FWE, but the repeatability was

greater for AMH. Thus, if cows are tested at an unknown stage of FG, serum AMH may be a more reliable phenotype than AFC to test association with reproductive outcomes. Furthermore, AFC and AMH were moderately correlated at the two stages examined.

Key words: antral follicle count; anti-Müllerian hormone; repeatability; dairy cows

4.2. Background

Antral follicle count **(AFC)**, the total number of ultrasonically visible antral follicles, is a predictor of the number of morphologically healthy oocytes and follicles present in the ovaries (Ireland et al., 2008). Anti-Müllerian hormone **(AMH)**, a dimeric glycoprotein produced by granulosa cells of preantral and antral follicles (Monniaux et al., 2013), is a marker of the ovarian follicular reserve in cattle (Ireland et al., 2008; Rico et al., 2009). Several studies have reported that great variability exists among cattle for both AFC and AMH (Burns et al., 2005; Ireland et al., 2007; Ireland et al., 2008; Jimenez-Krassel et al., 2009; Rico et al., 2009; Mossa et al., 2010; Monniaux et al., 2013; Ribeiro et al., 2014). In addition, Burns et al. (2005) reported that the maximal number of antral follicles determined approximately at follicular wave emergence within the same estrous cycle or during consecutive estrous cycles had high repeatability (0.96 or 0.86, respectively) in a mixed population of nulliparous dairy heifers, non-lactating and lactating dairy cows. Similarly, Ireland et al. (2007) reported a repeatability of 0.84 for maximal number of antral follicles in beef heifers. The repeatability of plasma AMH concentrations in lactating dairy cows between d 7 and 15 following timed-AI was 0.90 (Ribeiro et al., 2014).

The associations among AFC, AMH and reproductive outcomes have been of recent interest to many researchers (Ribeiro et al., 2014; Mossa et al., 2012). In these studies, dairy cows were classified into categories (e.g. low and high) based on AFC or AMH determined on a

specific day of the estrous cycle, and their association with reproductive outcomes examined. Mossa et al. (2012) reported that cows with high AFC (≥ 25) at 4.6 d after estrus had 3.34 times greater odds of being pregnant at the end of breeding season compared to cows with low AFC (≤ 15). Ribeiro et al. (2014) reported that cows with high mean plasma AMH (631.0 pg/mL) at the initiation of Ovsynch/TAI program had lower pregnancy losses between 30 and 65 d of gestation (8.0 vs 16.7 %; P=0.03) than those that had low mean plasma AMH (85 pg/mL). From the perspective of using AFC and AMH as fertility markers for potential genetic selection, it is important to know whether these phenotypes should be determined at a specific day of follicular growth or could be determined at any random stage. It will be most advantageous if these phenotypes could be determined at any stage of follicular growth **(FG)**, even during the early postpartum period. Previous studies (Burns et al., 2005; Ireland et al., 2007; Ribeiro et al., 2014) were designed to test repeatability only between known stages of FG. Thus, the repeatability of AFC and AMH between unknown and known stages of FG in cattle remains to be established. If high repeatability estimates are established, it would enable the use of AFC and/or AMH determinations made at even unknown stages of FG to classify cows into different phenotypic groups and test associations with reproductive outcomes.

In addition to high variability (between-animal) and repeatability (within-animal) for both AFC and AMH, the correlation between AFC and AMH was high (Ireland et al., 2008; Rico et al., 2009) when small numbers of animals were evaluated. Whereas Ireland et al. (2008) reported a correlation of 0.88 between AMH and AFC in 16 crossbred beef heifers, Rico et al. (2009) used an even smaller population of only nine dairy cows and reported a correlation of 0.79. It is documented that correlation estimates would only stabilize with increasing sample size (Schonbrodt and Perugini 2013). In this regard, studies (Nardo et al., 2007; Goksedef et al., 2010;

Barbakadze et al., 2015)) using larger samples sizes (range, n = 112 to 141) in women reported a moderate correlation between AFC and AMH (range, r = 0.46 to 0.57). Therefore, determining the correlation between AMH and AFC in a larger population of lactating dairy cows is important.

The objectives of the present study were to determine (1) the repeatability of AFC and AMH between an unknown stage of FG and an expected day of follicular wave emergence **(FWE)**, and (2) the association between AFC (considering all follicles ≥2 mm diameter) and serum AMH at the above stages in lactating dairy cows.

4.3. Materials and methods

4.3.1. Animals and housing

The study was conducted at the Dairy Research and Technology Centre of the University of Alberta between November 2014 and February 2016. All the experimental procedures were approved by the Animal Care and Use Committee for Livestock at the University of Alberta, and animals were cared for in accordance with the requirements of Canadian Council on Animal Care (2009). One hundred lactating Holstein cows (35 primiparous, 65 multiparous) were initially enrolled in the study. Cows were individually housed and fed in tie-stalls and let out for approximately 2 h of exercise during weekdays. Diets were formulated according to National Research Council guidelines (2001) to meet the requirements of a 650 kg lactating cow producing 45.0 kg of milk/d and cows had *ad libitum* access to water.

4.3.2. Reproductive management

At approximately 52 d postpartum, all cows were placed on a modified G6G ovulation synchronization protocol and subjected to timed-AI at approximately 72 d postpartum. In brief, presynchronization consisted of PGF2α (Estrumate; 500 μg, i.m; Merck Intervet Corp. Kirkland,

QC, Canada) and GnRH (Fertiline; 100 μg, i.m.; Vetoquinol N. A. Inc. Lavaltrie QC, Canada) administered 3d apart, at approximately at 52 and 55 d postpartum, respectively. The Ovsynch/TAI protocol was initiated 7 d after the GnRH injection of the presynchronization program, and involved i.m. injections of GnRH (62 d postpartum), PGF2α (69 d postpartum) and GnRH 56 h later (71 d postpartum), followed by timed-AI 16 to 20 h later (72 d postpartum).

4.3.3. Transrectal ultrasonography and determination of AFC

To examine the repeatability of AFC, ovaries were subjected to transrectal ultrasonography (Aloka 500, Aloka Co Ltd., Tokyo, Japan) by the same individual using a 7.5 MHz linear array transducer on a fixed day postpartum but at an unknown stage of FG (14 ± 0.5 d postpartum) and again on the day after timed-AI (73 ± 0.5 d postpartum) coincident with the expected day of FWE. To determine AFC, both ovaries of each cow were scanned and the total number of antral follicles (≥ 2 mm in diameter) recorded. An additional ultrasound examination was conducted at the time of the last GnRH of Ovsynch (~ 71 d postpartum) to confirm presence of dominant follicle(s). Ovulation was confirmed on 73 d postpartum by the absence of the dominant (≥10 mm in diameter) follicle(s) that had been detected at the ultrasound exam on 71 d postpartum. Antral follicle count was determined only in 91 cows (33 primiparous and 58 multiparous) that were confirmed to have ovulated in response to the synchronization protocol.

4.3.4. Blood sampling and determination of AMH

Blood samples were collected at an unknown stage of FG and expected day of FWE from a coccygeal blood vessel using evacuated Vacutainer® glass serum tubes (Becton Dickinson and Company, New Jersey, USA). After collection, samples were left undisturbed for about 2 h to allow clot formation. Samples were then centrifuged at 1500 *x* g for 20 min at 4°C, serum harvested and frozen at -20 °C until assayed for AMH. Serum concentrations of AMH were

analyzed using a commercial chemiluminescence immunoassay (AnshLite Bovine AMH CLIA, Ansh Labs, Webster, TX) according to manufacturer instructions, as previously reported by Ribeiro et al. (2014). The intra- and inter-assay coefficients of variation were 2.25 and 5.23 %, respectively.

4.3.5. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The MEANS procedure of SAS was used for descriptive statistics such as range, mean, standard error of mean (SEM) and coefficient of variation (CV) for AFC and serum AMH concentrations. The differences for mean AFC and AMH between an unknown stage of FG and the expected day of FWE were tested using the TTEST procedure of SAS. The repeatability (range 0 to 1, with 1 being the highest) was defined as the proportion of the total variance that could be attributed to between-animal variance, which was determined as σ^2 between animal / σ^2 between animal + σ^2 within animal. Variance components were estimated using the MIXED procedure of SAS. The associations between AFC and AMH at an unknown stage of FG and expected day of FWE were determined using CORR procedure of SAS. Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

4.4. Results and discussion

The ranges (Table 1) for AFC determined at an unknown stage of FG and the expected day of FWE in the current study were similar to the ranges for maximal number of antral follicles reported in previous studies (11 to 54 [Burns et al., 2005], 9 to 45 [Ireland 2007], 7 to 50 [Ireland et al., 2008]). Likewise, the ranges for serum AMH concentrations reported in the current study (Table 1) were similar to those previously reported for serum AMH of ~ 40 to 400 pg/mL in

crossbred beef heifers (Ireland et al., 2008) and plasma AMH of 25 to 359 pg/mL in dairy cows (Rico et al., 2009). Although Ribeiro et al. (2014) reported that plasma AMH concentrations varied from 10 to 3198 pg/mL in lactating dairy cattle (n = 1237), very few cows (< 3%) had AMH concentrations exceeding 700 pg/mL. The aforementioned findings, along with the moderate and high variability reported for AFC and AMH in the current study (Figures 1 and 2), illustrate the wide phenotypic variation for these two traits in dairy cattle.

The mean AFC was greater when determined at an unknown stage of FG than at the expected day of FWE; conversely, mean serum AMH was lower at an unknown stage of FG than at the expected day of FWE (Table 1). Previous studies have shown that neither negative energy balance (Beam and Butler 1997) nor postpartum uterine infections (Williams et al., 2007; Broomfield et al., 2013; Gobikrushanth et al., 2016) affect ovarian follicular development during the early postpartum period in dairy cows. Beam and Butler (1997) reported that a wave of follicular development occurred in all cows within the first two weeks postpartum in association with elevated FSH, and the numbers of follicles determined were unrelated to energy balance or metabolic hormones. Moreover, high uterine pathogen growth density at 7 d postpartum did not affect the number of first wave follicles in cows (Williams et al., 2007), and development of preantral and antral follicles was not negatively affected in bovine ovarian cortical tissues treated with lipopolysaccharides (10µg/mL) *ex vivo* (Broomfield et al., 2013). In addition, in a recent study, we (Gobikrushanth et al., 2016) concluded that postpartum ovarian follicular dynamics were not affected by different categories of endometritis. Monniaux et al. (2013) reported that the mean AMH concentrations did not differ and remained at ~ 100 pg/mL when measured at 8, 18, 28, 38 and 48 d postpartum despite dramatic changes in blood non-esterified fatty acid concentrations (~ 1000 to 500 µmol/L) indicating that neither days postpartum nor negative

energy balance (as indicated by high non-esterified fatty acid concentrations) influenced AMH production. Therefore, while a mixed population of both growing and atretic follicles of varying sizes (small, medium and large) was likely present at an unknown stage of FG contributing to the high mean AFC at that time, a more functionally-homogenous population of growing (small and medium) follicles may have been present at the expected day of FWE. Such a scenario would explain the lower mean AMH concentration at the unknown stage of FG vs. higher AMH at the expected day of FWE despite having incongruent AFC. Rico et al. (2009) reported that both plasma and follicular fluid AMH concentrations were greater in cows with more small- and medium-sized antral follicles (3-7 mm diameter) than in those with large follicles (> 7 mm in diameter). Based on the above finding, we speculate that the high AMH concentrations determined at the expected day of FWE was due to AMH produced by a relatively larger number of small and medium sized antral follicles that were active at the time of follicular wave emergence than at unknown stage of FG.

To the best of our knowledge, this is the first report on the repeatability of AFC or serum AMH concentrations between an unknown stage of FG and the expected day of FWE in dairy cattle. The repeatability of AFC reported in the current study (0.37) was lower than previously reported. Higher repeatability of 0.95 and 0.84 was reported when AFC was determined approximately at wave emergence within the same estrous cycle or during consecutive estrous cycles (Burns et al., 2005; Ireland et al., 2007). The lower repeatability for AFC in the current study was likely due to determination of AFC at random stages of follicular growth during early postpartum (14 ± 0.5 d postpartum). Conversely, Burns et al. (2005) and Ireland et al. (2007) selected time points for repeatability estimates when they could measure maximal numbers of antral follicles (approximately at follicular wave emergence). In contrast to AFC, serum AMH

concentrations had higher repeatability (0.73). Although repeatability for serum AMH concentrations was greater than for AFC, the repeatability for serum AMH in the current study was lower than in previous reports. For example, the repeatability for plasma AMH was 0.87 between 4 and 5 d before estrus (day of first FSH treatment) and the day of estrus in superovulated cows (Rico et al., 2009), and was 0.90 when determined between d 7 and 15 of a controlled estrous cycle (d 0: day of last GnRH of Ovsynch/timed-AI; [Ribeiro et al., 2014]). Furthermore, Martinez et al. (2016) recently reported that AFC classification based on single transrectal ultrasonography at random stages of the estrous cycle during first 4 weeks of breeding season had no association with fertility measurements in grazing dairy cows. Therefore, based on the poor repeatability estimate obtained in the current study for AFC, and the above finding (Martinez et al., 2016), we conclude that AFC determined at an unknown stage of FG is less reliable to classify cows into categories of AFC and test associations with reproductive outcomes.

The associations between AFC and serum AMH concentrations determined either at an unknown stage of FG or expected day of FWE were moderate (r = 0.54 and 0.59, respectively; Figure 3) and comparable to reports in women (r = 0.54 [Nardo et al., 2007], 0.47 [Goksedef et al., 2010], 0.57 [Barbakadze et al., 2015]). However, the correlation reported in the present study is lower than previously reported (Ireland et al., 2008; Rico et al., 2009) in cattle (r = 0.88 and 0.79, respectively). Ireland et al. (2008) determined the correlation between AFC (≥ 3 mm in diameter) and serum AMH concentrations in 16 crossbred beef heifers, whereas Rico et al. (2009) reported the correlation between follicles of 3 to 7 mm and plasma AMH in nine Prim'Holstein cows (a French Friesian breed). On the other hand, we have reported the correlation between AFC (≥ 2 mm) and serum AMH in a relatively larger population of 91 cows. The correlation between AFC and AMH was 0.71 when evaluated in 54 women (de Vet et al., 2002) compared

to a lower average correlation of 0.52 when evaluated in a larger sample size (~ 130 women) in each of three studies (Nardo et al., 2007; Goksedef et al., 2010; Barbakadze et al., 2015) supporting the need for larger sample sizes to obtain a reliable correlation estimate (Schonbrodt and Perugini 2013). Therefore, in addition to possible influences of breed differences, the smaller sample sizes used in the previous studies (Ireland et al., 2008; Rico et al., 2009) likely contributed to higher correlations between AFC and AMH than in the current study.

In summary, both AFC and AMH were repeatable when determined at an unknown stage of FG and expected day of FWE, but repeatability was greater for AMH than for AFC. Therefore, if dairy cows are screened at an unknown stage of FG, peripheral concentrations of AMH may be a more reliable phenotype than AFC to test association with reproductive outcomes. Furthermore, AFC and AMH were moderately correlated regardless of follicular stages.

4.5. Acknowledgments

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Table 4. 1. The descriptive statistics of AFC (≥ 2 mm in diameter) and serum AMH concentrations (pg/mL) determined at an unknown stage of follicular growth (FG) and expected day of follicular emergence (FWE) in 91 dairy cows

	AFC (n) at an unknown stage of FG	AFC (n) at expected day of FWE	AMH (pg/mL) at an unknown stage of FG	AMH (pg/mL) at expected day of FWE
Range	10 – 53	6 – 45	13.9 – 528.8	38.2 – 774.1
Mean ± SEM	26 ± 1^{a}	23 ± 1^{b}	187.3 ± 13.1^{a}	218.7 ± 14.5^{b}
Variability (CV %)	37.4	42.5	66.9	63.4

[a,b] Different superscripts within the same row and category differ ($P < 0.05$)
AFC: antral follicle count
AMH: anti- Müllerian hormone
SEM: standard error of mean
CV: coefficient of variation

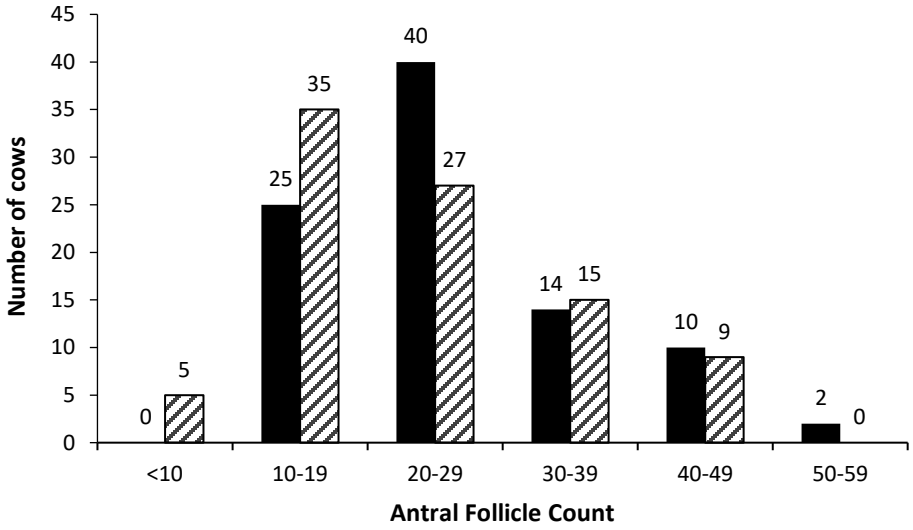

Figure 4. 1. The distribution of AFC (≥ 2 mm in diameter) determined at an unknown stage of follicular growth (FG; filled bars) and on the expected day of follicular wave emergence (FWE; hatched bars) in lactating dairy cows.

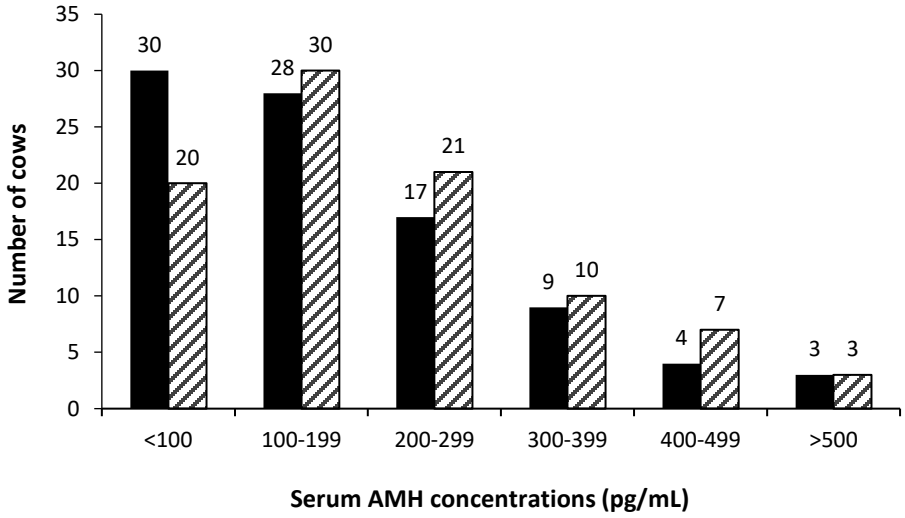

Figure 4. 2. The distribution of serum AMH (pg/mL) determined at an unknown stage of follicular growth (FG; filled bars) and on the expected day of follicular wave emergence (FWE; hatched bars) in lactating dairy cows.

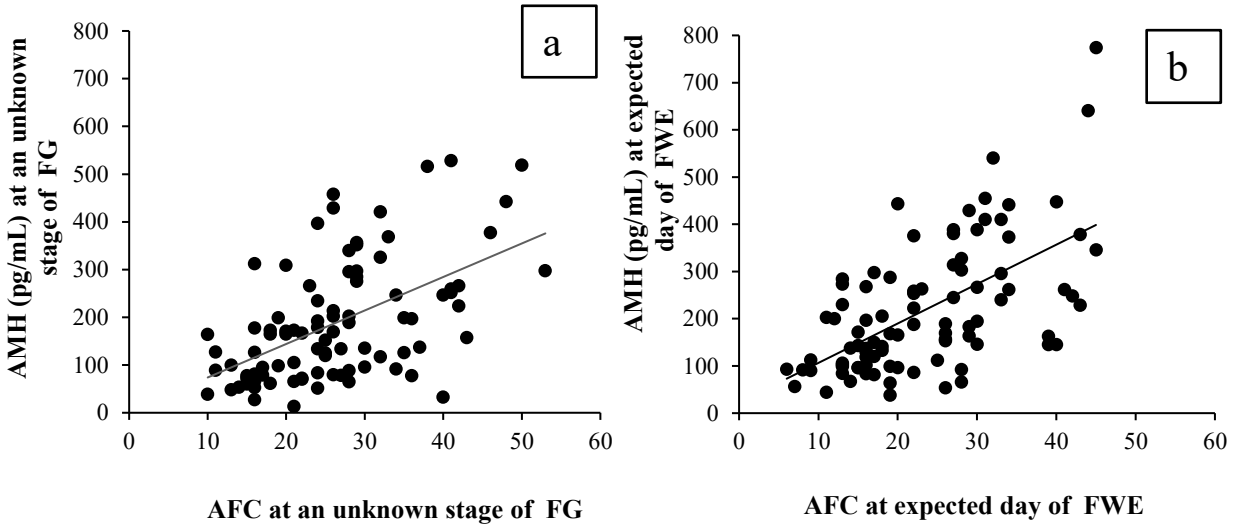

Figure 4. 3. The association between AFC (≥ 2 mm in diameter) and AMH determined at an unknown stage of follicular growth (FG; a) and on the expected day of follicular emergence (FWE; b) in 91 lactating dairy cows (r = 0.54 and 0.59, respectively; $P < 0.01$).

Chapter 5. The relationship between serum anti-Müllerian hormone (AMH) concentrations and fertility, and genome wide associations for AMH in Holstein cows

5.1. Abstract

The objectives of this study were to (1) evaluate factors associated with variation in circulating anti-Müllerian hormone (AMH) concentrations, (2) establish an optimum AMH threshold predictive of pregnancy to first artificial insemination (P/AI), (3) examine the relationship between AMH and fertility (P/AI, pregnancy loss between 30 and 60 d post-AI [PLOSS] and pregnancy risk up to 250 d postpartum), and (4) identify quantitative trait loci (QTL) associated with phenotypic variation of AMH concentrations in dairy cows. Serum AMH concentrations (pg/mL) were determined at 7 ± 2.4 d postpartum in 647 lactating Holstein cows (213 primiparous, 434 multiparous) from one research and six commercial dairy herds in Alberta, Canada. Of these, 589 cows were genotyped on the 26 K Bovine Bead Chip and subsequently imputed to the Illumina Bovine High Density Bead Chip for genome wide association analysis for variation in serum AMH concentrations. Factors associated with variation in serum AMH concentrations and the relationship between categories of AMH and aforementioned fertility outcomes were evaluated only in a subset of 460 cows that had a complete data set available.

The overall mean (± standard error of the mean), median, minimum and maximum AMH concentrations were 191.1 ± 6.3, 151.7, 13.9 and 1,879.0 pg/mL, respectively. The AMH concentrations were not associated with herd, pre-calving BCS, postpartum week and season of sampling; the lactation number, however, had a quadratic relationship with serum AMH concentrations (116.2, 204.9 204.5 and 157.9 pg/mL for first, second, third and ≥fourth lactation, respectively). The optimum AMH threshold predictive of P/AI could not be established, as the

receiver operating characteristic curve analysis model was non-significant. Categories of AMH (low [<83.0 pg/mL; n=92], intermediate [≥83.0 to ≤285.0 pg/mL; n=276] and high [>285.0 pg/mL; n=92] based on lowest 20%, intermediate 60%, and highest 20% serum AMH) had no associations with P/AI (34, 43 and 40%), PLOSS (20, 12 and 8%) or pregnancy risk up to 250 d postpartum. One candidate gene associated with AMH production (*AMH* gene on *Bos taurus* autosome [BTA] 7) and four candidate genes related to embryo development (*SCAI* and *PPP6C* genes on BTA 11 and *FGF18* and *EEF2K* genes on BTA 20 and 25, respectively) were in linkage disequilibrium with single nucleotide polymorphisms associated with phenotypic variation in serum AMH in dairy cows.

Key words: fertility traits, genomic heritability, pregnancy loss, superovulation

5.2. Background

A trait that has high variability, repeatability, heritability and associations with fertility would be an ideal candidate for genetic selection to augment reproductive performance in dairy cows. The use of circulating anti-Müllerian hormone (**AMH**) concentrations as a potential fertility trait in cattle has been of recent interest to many researchers (Ribeiro et al., 2014; Baruselli et al., 2015; Jimenez-Krassel et al., 2015; Gobikrushanth et al., 2017). Anti-Müllerian hormone, a dimeric glycoprotein produced by granulosa cells of growing preantral and antral follicles (La Marca and Volpe. 2006), is a marker of ovarian follicular reserve in cattle (Rico et al., 2009; Monniaux et al., 2013). A positive correlation between circulating AMH and antral follicle counts (**AFC**) in cattle has also been reported (Ireland et al., 2008; Rico et al., 2009; Gobikrushanth et al., 2017).

In cattle, AMH concentrations were highly variable among animals but were quite

repeatable within an animal (Rico et al., 2009; Monniaux et al., 2013; Ribeiro et al., 2014; Gobikrushanth et al., 2017). In cattle, maternal nutritional status, species, breed, and lactation number were all identified as potential factors associated with phenotypic variation in AMH concentrations (Mossa et al., 2013; Batista et al., 2014; Ribeiro et al., 2014). Interestingly, however, the associations between circulating AMH concentrations and reproductive outcomes remain unclear (Ribeiro et al., 2014; Baruselli et al., 2015; Jimenez-Krassel et al., 2015). Hence, additional studies are warranted to further elucidate the factors that affect circulating AMH concentrations, and the relationship between circulating AMH and phenotypic fertility performance.

An optimum circulating AMH threshold predictive of pregnancy to first AI (**P/AI**), heritability of AMH, and genome wide association studies (**GWAS**) identifying single nucleotide polymorphisms (**SNP**) associated with phenotypic variation in AMH concentrations are novel aspects yet to be explored in dairy cows. If a positive association exists between AMH and reproductive outcomes and an optimum AMH threshold could be established, it will assist dairy producers to make economically beneficial decisions by selectively breeding cows with high AMH to improve reproductive efficiency of dairy herds. In addition, identification of genetic makers (SNP) associated with variation in circulating AMH concentrations would potentially help to identify, and preselect at birth, future elite genetic merit donors with greater embryo production potential for use in multiple ovulation embryo transfer programs in dairy cattle.

Therefore, the primary objectives of the current study were to (1) evaluate the factors associated with variation in circulating AMH concentrations, (2) establish an optimum AMH threshold that has predictive value for P/AI, (3) examine the relationship among categories of AMH and fertility (P/AI, pregnancy loss between 30 and 60-d post-AI [**PLOSS**] and pregnancy

risk up to 250 d postpartum), and (4) identify Quantitative trait loci **(QTL)** associated with phenotypic variation in AMH concentrations in dairy cows.

5.3. Materials and methods

5.3.1. Animals and management

This study was conducted in one research herd (Dairy Research and Technology Centre, University of Alberta) and six commercial dairy herds located in Alberta, between November 2014 and 2015. Animals were housed and cared for in accordance with the requirements of Canadian Council on Animal Care (2009). Cows were fed once daily a total mixed ration (primary ingredients were barley or corn silage, alfalfa silage, alfalfa hay, and concentrates) and had *ad libitum* access to potable water. Cows from four herds were subjected to GnRH-based synchronization protocols and inseminated without estrus detection for first and subsequent AIs (timed-AI; **TAI**), whereas, cows from three other herds were inseminated at detected estrus (**IDE**).

5.3.2. Blood sampling and determination of serum concentrations of AMH

Blood samples were collected at (mean±SD) 7 ± 2.4 d postpartum from 647 lactating Holstein cows (213 primiparous, 434 multiparous) from a coccygeal blood vessel using evacuated Vacutainer® (Becton Dickinson and Company, New Jersey, USA) into clot-activator tubes (serum tubes) for AMH determination and anti-coagulant K_2 EDTA coated tubes (EDTA tubes) for genotyping. After collection, serum tubes were left undisturbed for about 2 h to allow clot formation and were then centrifuged at 1500 *x* g for 20 min at 4°C, serum harvested and frozen at -20 °C until assayed for AMH. The EDTA tubes were frozen at -20 °C until processed for genotyping. Serum concentrations (pg/mL) of AMH were analyzed at Ansh Labs (Webster, TX,

USA) using the Ansh Labs Bovine AMH enzyme-linked immunosorbent assay. The assay has an analytical measurable range of 13.5 to 2240 pg/mL. The AMH assay limit of detection is 11 pg/mL, and the both intra- and inter-assay coefficients of variation (**CV**) were <5%.

5.3.3. Determination of body condition score (BCS), reproductive measurements and milk yield for the current lactation

The BCS were determined between one and two weeks before the estimated calving date (hereafter referred to as pre-calving BCS) and again between four and six weeks after calving (hereafter referred to as post-calving BCS) on a 1-5 scale system measured in increments of 0.25 units (1 = thin, 5 = fat) as previously described (Edmonson et al., 1989). The reproductive data and 305-d mature-equivalent (**ME**) milk yield were retrieved using DairyComp 305 herd management software (CanWest DHI, Guelph, Ontario, Canada).

5.3.4. Genotyping, quality control and imputation

DNA extraction was performed on blood samples drawn from a subset of 589 lactating Holstein cows using the Qiagen BioSprint 96 DNA (3840) Kit blood and tissue protocol (Qiagen, Toronto, ON). DNA samples were normalized and genotyped according to the Illumina Infinium Ultra protocol (Illumina, San Diego, CA) and markers were scored on the Bovine Geneseek Genomic Profiler 26K Beadchip (Neogen Inc, Lincoln, NE) at Delta Genomics (Edmonton, AB, Canada). The beadchips were scanned using the Illumina HiScan (Illumina) and the raw data was processed and exported using Genome Studio 2.0 software according to the Genome Studio Framework User Guide (Illumina) based on selection criteria of at least 95% animal call rate.

Genotype quality control was performed using PLINK v1.09 (Purcell et al., 2007). All single nucleotide polymorphisms (**SNP**) with an unknown, Y chromosomal or mitochondrial position were excluded. In addition, SNPs with a call rate <90%, minor allele frequency **(MAF)**

<0.01 and that deviated significantly from Hardy-Weinberg equilibrium ($P \leq 10^{-6}$) were removed. All animals had a genotype call rate >90% and 19,896 SNP remaining after edits. To increase the density of the SNP panel for GWAS, imputation to the Illumina Bovine high density **(HD)** beadchip was undertaken using FImpute2 (Sargolzaei et al., 2014). Imputation was completed using a two-step approach whereby the 589 animals were first imputed to the Illumina BovineSNP50 chip using a reference population of 3,532 Irish Holstein-Friesian BovineSNP50 genotyped animals, and subsequently imputed to HD density using a reference population of 974 Irish Holstein-Friesian HD genotyped animals. After imputation, all 589 cows had 636,471 SNP with a MAF >0.01 for analysis.

5.3.5. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The descriptive statistics and normality for serum AMH concentrations (pg/mL) were first determined in all 647 cows using the UNIVARIATE procedure.

To identify factors associated with serum AMH concentrations (dependent continuous variable), a multivariable model including herd (A, B, C, D, E, F and G), lactation number (first, second, third and ≥fourth lactations), pre-calving BCS (categorized as high and low-BCS; >3.00 and ≤3.00, respectively), week postpartum (first and second week) and season of blood sampling (fall, spring, summer and winter) were used to explain the variability observed in serum AMH concentration by analysis of variance (ANOVA) method using the MIXED procedure in a subset of 460 cows that had a complete data available on the aforesaid explanatory variables studied. In addition, contrast statements were constructed to test linear, quadratic and cubic effects of lactation number on serum AMH concentrations. Data pertaining to serum AMH concentrations were first transformed by Box-Cox data transformation to meet the assumptions for a Gaussian

distribution. The serum concentrations of AMH (transformed values) were initially modelled against all of the aforementioned categorical variables and their interactions. As none of the interactions was significant, the final model only had the categorical variables modelled against serum AMH concentrations, and the differences in means were tested using the Tukey-Kramer multiple means comparison test. The reported results are back transformed for ease of interpretation.

The optimum AMH threshold predictable of P/AI, including sensitivity and specificity, was first determined in 563 cows that had P/AI data available using the receiver operating characteristic **(ROC)** curve analysis. A priori power analysis based on type I error of 0.05, power of 0.80 and an allocation ratio of 1:2 (assuming only 1/3 of cows become pregnant to the first AI) to obtain an area under the curve of 0.60 called for only 219 cows in total to attain statistical significance. The ROC curves analyze sensitivity and 1 − specificity. Sensitivity is the proportion of cows above the optimum AMH threshold diagnosed as pregnant to first AI, and specificity is the proportion of cows below the optimum AMH threshold diagnosed as not pregnant to first AI. The optimum AMH threshold was chosen based on the highest Youden's J statistic index. The significance of the optimum AMH threshold was determined based on the area under the curve **(AUC)**, where the AUC ranged from 0.50 to 1.00, with AUC of 0.50 considered non-informative and the AUC of 1.00 considered perfect as previously described (Swets, 1988).

A subset of 460 cows that had a complete data set available for both reproductive outcomes and all explanatory variables were ranked across herds in an ascending order by serum AMH concentrations and grouped into low-AMH (lowest 20% values; n=92; mean=56.6; range=13.9 to 81.7 pg/mL), intermediate-AMH (intermediate 60% values; n=276; mean=164.6; range=83.8 to 284.9 pg/mL), or high-AMH (highest 20% values; n=92; mean=420.1;

range=287.4 to 1879.0 pg/mL) categories. Cows were ranked across herds as there was no overall effect of herd on variation in serum AMH concentrations, and one of the objectives of the study was to establish a common cutoff across herds. In addition, the categories were chosen to mimic the proposed importance of ovarian reserve for fertility (Ireland et al., 2011) and to take an approach similar to that of Ribeiro et al. (2014).

The associations among categories of serum AMH (low-AMH, intermediate-AMH and high-AMH), type of AI (IDE and TAI), herd (A, B, C, D, E, F and G), parity (primiparous and multiparous), pre-calving BCS (categorized as high and low-BCS; >3.00 and ≤3.00, respectively), post-calving BCS (categorized as high and low-BCS; >2.75 and ≤2.75, respectively) and 305-d ME milk yield (categorized as high and low-ME milk yield based on mean 305-d ME milk yield within each herd) and P/AI or PLOSS were analyzed using the GLIMMIX procedure, while the model specifications included a binomial distribution and logit function, and an option to retrieve odds ratios and their confidence intervals. The P/AI or PLOSS was initially modelled against all of the aforementioned categorical variables and their interactions. As none of the interactions was significant, the final model included only the categorical variables and tested against P/AI or PLOSS. The mean 305-d ME milk yield was 12,197, 11,349, 13,686, 9,858, 11,031, 11,502 and 12,387 kg for herds A to G, respectively, and the overall mean milk production for all cows was 11,092 kg.

The differences in intervals from calving to pregnancy risk up to 250 d postpartum between categories of serum AMH were evaluated using the Kaplan-Meier Survival analysis (LIFETEST procedure). The results from Kaplan-Meier survival analysis were confirmed by a Cox proportional hazard model (PHREG procedure). Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

5.3.6. Genome-wide association study and estimation of genomic heritability for AMH

Whole genome association analysis was performed in genome-wide complex trait analysis **(GCTA)** (Yang et al., 2011) using a mixed linear model based association analysis based on the leave-one-chromosome-out method (Yang et al., 2014), across all 589 genotyped animals. This approach accounts for population substructure and relatedness through the construction of genomic relationship matrixes. The following model was used for analysis; $y = \mu + bx + g^{-} + e$, where y is the box-cox transformed AMH dependent variable, μ is the overall mean, b is a vector of fixed effects including herd and the additive effect of the candidate SNP tested for association, x is the incidence matrix for the parameters b, g^{-} is the accumulated polygenic effect of all SNP except those on the chromosome where the candidate SNP is located and e is the residual. False discovery rate **(FDR)** control was performed using the Benjamini-Hochberg method using a FDR of 0.05. Gene search was done using Ensembl (http://ensembl.org/) and NCBI map viewer (http://www.ncbi.nlm.nih.gov/mapview/) on the UMD 3.1 genome build. The relative roles of nearest candidate genes were searched using both Bovine Genome Database (http://bovinegenome.org/) and Human Genome Database (http://www.genecards.org/). In addition, the proportion of phenotypic variance accounted for by all SNP (SNP-based heritability / genomic heritability) was estimated using the genomic restricted maximum likelihood approach in GCTA (Yang et al., 2010). To ensure the genomic heritability estimate was not inflated due to close familial relationships, all animals with an estimated coefficient of relatedness of >0.125 were removed (n=391) prior to estimation.

5.4. Results and discussion

The overall mean (±SEM), median, minimum and maximum serum AMH concentrations were 191.1 ± 6.3, 151.7, 13.9 and 1,879.0 pg/mL, respectively. The circulating concentrations of AMH were highly variable among cows in the current study (Figure 1) as reported in previous studies (Monniaux et al., 2013; Ribeiro et al., 2014; Gobikrushanth et al., 2017). The variation in circulating AMH were reported to be associated with maternal nutritional status, species, breed and lactation number in cattle (Mossa et al., 2013; Batista et al., 2014; Ribeiro et al., 2014). In this regard, Mossa et al. (2013) showed that female calves of dams that had been nutritionally-restricted from 11 d before AI up to day 110 of gestation had lower circulating AMH concentrations from 4 mo to 1.8 yr of age compared to control calves born to mothers that had no nutritional restriction imposed during gestation (~180 vs. 280 pg/mL). Batista et al. (2014) reported that *Bos indicus* heifers had greater circulating AMH than *Bos taurus* heifers (930 vs. 300 pg/mL). Moreover, Ribeiro et al. (2014) found that Jersey and Holstein x Jersey crossbred cows had greater plasma AMH concentrations than Holstein cows (337 and 298 vs. 264 pg/mL) and that 2nd and 3rd lactation cows (irrespective of breed) had higher plasma AMH than those of 1st and 4th lactation (342 and 328 vs. 257 and 273 pg/mL, respectively).

In the present study, none of the non-genetic factors evaluated were associated with serum concentrations of AMH in dairy cows (Figure 2). Although there was no overall herd effect on the variation in serum AMH concentrations (Figure 2a; P=0.25), it was greater for herd F compared to herd B (P=0.03), but the exact reason underpinning this difference is unknown. It is plausible, however, that genetic variation among cows both within and between herds for circulating AMH might have been a contributory factor. A quadratic relationship between serum AMH concentrations and lactation number was observed in the current study (P<0.01; Figure

2b). This is consistent with previous observations of circulating AMH and lactation number in dairy cows (Ribeiro et al., 2014) or between AFC and age in beef heifers and dairy cows (Cushman et al., 2009 and Mossa et al., 2012, respectively). Collectively, these results support the hypothesis that circulating concentrations of AMH increase with greater ovarian follicular recruitment up to 5 yr of age (or third parity) and then decline following gradual depletion of the ovarian reserve. In addition, we found that pre-calving BCS, postpartum week or season of blood sampling did not influence serum AMH concentrations in dairy cows (Figure 2 c, d and e). Monniaux et al. (2013) reported that AMH concentrations were quite stable when repeatedly measured in a same set of cows at 8, 18, 28, 38 and 48 d postpartum, despite marked changes in energy balance status. Therefore, for a given species, breed or lactation number, circulating AMH concentrations do not appear to be influenced by some of the common explanatory variables used in reproductive studies such as BCS, week postpartum and season.

As mentioned elsewhere, if a positive association exists between AMH and reproductive outcomes and an optimum AMH threshold could be established, it will assist dairy producers to make economically beneficial decisions by selectively breeding cows with high AMH to improve reproductive efficiency of dairy herds. Therefore, we proposed to identify the optimum circulating AMH threshold that was predictive of P/AI. Using ROC curve analysis, however, the association between serum AMH and P/AI was not significant (P=0.72; n=563); consequently, the optimum circulating AMH threshold, including its sensitivity and specificity that could predict P/AI was not established. A previous report by Baruselli et al. (2015) also failed to detect any association between circulating AMH and age at conception in 528 nulliparous heifers or the interval from calving to conception in 223 Nelore cows (*Bos indicus)*. Collectively, these findings suggest that the use of circulating AMH as a predictor of fertility outcomes in cattle seems

unrealistic.

The role of AMH on reproductive function is poorly understood, and therefore the exact mechanisms linking AMH to fertility, if any, are unknown. A recent review by Dewaily et al. (2014) suggested that AMH plays two major functions in women: (a) inhibition of follicular growth from the primordial follicle reserve, averting premature exhaustion of the ovarian follicular reserve; and (b) reduction in the responsiveness to FSH of preantral and small antral follicles, restricting ovarian follicular growth. Nevertheless, the relative importance of these specific functions of AMH on fertility is not clear. Ribeiro et al. (2014) was the first to report that cows (a combination of Holstein, Jersey, and Holstein x Jersey crossbreds) with low (≤140.0), intermediate (>140.0 to ≤450.0) and high (>451.0) plasma AMH concentrations (pg/mL) did not differ in P/AI following a timed-AI event; however, there was some evidence that cows in the low AMH category had greater embryo mortality after timed-AI, and had lower likelihood of pregnancy establishment after subsequent spontaneous estrus (AI and natural service). Moreover, Jimenez-Krassel et al. (2015) found no association between serum AMH concentrations (categorized into quartiles) in 11 to 15 month old Holstein heifers and subsequent measures of reproductive success as heifers and as lactating cows (conception rate at first AI, days open, calving interval, and services per conception). Of note, however, Jimenez-Krassel et al. (2015) reported that the total percentage of lactating cows that became pregnant across parities one to three combined was significantly lower for cows in the first quartile compared to those in the second or third quartiles, but was not different from cows in the fourth quartile. In the present study, P/AI (34, 43 and 40%; P=0.37) and pregnancy risk up to 250 d postpartum were similar among low-, intermediate- and high-AMH cows subjected either timed-AI or IDE (Table 1 and Figure 3, respectively). Although no statistical significance was detected, cows IDE that had low

serum AMH concentrations had P/AI that was 8-10% lower than cows with intermediate or high serum AMH concentrations (24, 32 and 34%; P=0.57). Therefore, future studies should focus on the evaluation of circulating AMH and fertility in a larger population of dairy cows inseminated at detected estrus to further test the favorable relationship between circulating AMH concentrations and fertility reported herein and by others (Ribeiro et al., 2014; Jimenez-Krassel et al., 2015).

Similar to the lack of associations reported between serum AMH categories and pregnancy establishment (i.e. P/AI or pregnancy risk up to 250 d postpartum), PLOSS also not differ between low-, intermediate- and high-AMH categories (20 vs. 12 and 8 %, respectively, P=0.38). This was likely because only a small proportion of cows that became pregnant to first AI contributed towards PLOSS data under each AMH categories (low-AMH = [6/30], intermediate-AMH = [14/113] and high-AMH = [3/36]). In this regard, Ribeiro et al. (2014) found significantly greater pregnancy losses in cows with low plasma AMH concentrations compared with cows with intermediate or high plasma AMH concentrations (17 vs. 9 and 8 %, respectively) and suggested that the greater pregnancy losses observed for cows with low circulating AMH concentration may be at least in part explained by the low-AFC (presumed to have low AMH) - low-progesterone model initially proposed by Jimenez-Krassel et al. (2009). Therefore, the preliminary results on the association between AMH and embryo survival in the current and previous (Ribeiro et al., 2014) studies, and the molecular mechanisms underlying this association warrants further investigation in dairy cows.

The genomic heritability of AMH, and a GWAS identifying potential QTL associated with phenotypic variation in AMH concentrations are novel aspects yet to be explored in dairy cows. A preliminary study by Nawaz et al. (2017) recently estimated a genomic heritability of

0.36 for circulating AMH concentrations using 2,914 Holstein dairy heifers. A slightly greater genomic heritability of 0.46 (SE=0.31) was estimated in the present study; however, caution should be taken when interpreting such a result due to large standard error and small sample size (n=198) used in the heritability estimation after excluding 391 cows that had close familial relationships out of 589 cows genotyped. Interestingly, the moderate heritability estimate reported for circulating AMH in the current study and that of Nawaz et al. (2017) were quite comparable to the heritability estimate of 0.31 reported for AFC in dairy cows (Walsh et al., 2014). In total 670 SNPs across twelve *Bos taurus* autosomes (BTA) were significantly associated with variation in serum AMH concentration after adjustment for multiple testing (Figure 5). The list of nearest candidate genes identified for each significant lead SNP from their respective chromosomes and the major proteins encoded by those candidate genes are listed in Table 2. Only candidate genes that were related to AMH and fertility are discussed below. The strongest association ($p=1.58x10^{-8}$; $q=6.92x10^{-5}$) on BTA 7 (rs43505499) was located 15.51kb up-stream of the *AMH* gene, suggesting that variants within *AMH* or perhaps regulatory regions of this gene are associated with the variability in circulating AMH concentrations. This is further substantiated by the significant association of the up-stream *AMH* variant rs43505519 ($p=1.49x10^{-7}$; $q=4.27x10^{-4}$). Further fine mapping of this genomic region is required to identify the causal variants impacting AMH production. In the interim, however, this QTL could be potentially used to identify heifers and cows as potential donors for future superovulation programs.

The strongest associations with AMH variation were located on BTA 11, where 513 SNPs within a 14Mb region remained significantly associated after adjustment for multiple testing. Several putative candidate genes were identified within close proximity (<200kb) of the strongest

association (rs109286956) including *SCAI* and *PPP6C*. Indeed, three of the five strongest associations on BTA11 (rs109629605, rs109469337, and rs109162401) were located within regulatory regions of *PPP6C,* which has been recently described as indispensable for mouse embryogenesis after implantation; mice lacking the Ppp6c phosphatase domain showed clear developmental defects after implantation and no viable pups were born (Ogah et al., 2016). In addition, *SCAI* deficiency has also been associated with subfertility in mice, whereby *SCAI* deficient mice were found to contain few or no developing primary follicles, and showed a seven-fold reduction in fertility rates in comparison to controls (Hansen et al., 2016).

Furthermore, the candidate genes *FGF18* and *EEF2K* identified on BTA 20 and 25, respectively, have also been related to embryonic development in mice (Ohbayashi et al., 2002; Chu et al., 2014). The strongest association on BTA 20, rs110249317 (at 3.1 Mb) was an intronic variant within the candidate gene *FGF18*. This gene encodes the member of the fibroblast growth factor family that is known to be involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion (Ornitz and Itoh. 2015). Ohbayashi et al. (2002) reported that deletion of the *FGF18* gene affected skeletal development in mice embryos by reducing the proliferation of osteogenic mesenchymal cells and terminal differentiation of osteoblasts in the calvarial bone. Similarly, the lead SNP rs137098518 (at 20.1 Mb) on BTA 25 is an intronic variant within the candidate gene *EEF2K*, and encodes a highly conserved protein kinase enzyme, which plays a key role in the calmodulin-mediated signaling pathway that links activation of cell surface receptors to cell division (Berchtold and Villalobo. 2014). Deletion of the gene *EEF2K* in mice has been shown to result in reduced ovarian apoptosis, with consequent accumulation of aberrant follicles and defective oocytes at advanced reproductive age (Chu et al., 2014). Perhaps, numerically fewer pregnancy

losses observed in cows with high circulating AMH in the present study could be at least partially explained by the functions of above four candidate genes that are in linkage disequilibrium with genetic markers identified for variation in circulating AMH concentration. However, these genomic associations would require validation in dairy cows. In addition, it is important we recognize that fertility is a highly polygenic trait under the control of thousands of SNP effects (Minozzi et al., 2013) and these candidate genes are some of many that contribute marginally to the phenotypic variance.

In summary, we identified four main results: (1) serum AMH concentrations were not associated with herd, pre-calving BCS, postpartum week and season of sampling, but lactation number had a quadratic relationship with serum AMH concentrations; (2) an optimum circulating AMH threshold predictive of P/AI could not be established; (3) categories of serum AMH had no associations with P/AI, PLOSS or pregnancy risk up to 250 d postpartum, and (4) one candidate gene associated with AMH production (*AMH* gene on BTA 7) and four candidate genes related to embryo development (*SCAI* and *PPP6C* genes on BTA 11 and *FGF18* and *EEF2K* genes on BTA 20 and 25, respectively) were in linkage disequilibrium with SNPs associated with phenotypic variation in serum AMH in dairy cows.

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Table 5. 1. Associations among categories of serum anti-Müllerian hormone (AMH), type of AI, herd, parity, pre- and post-calving body condition score, 305-d mature-equivalent (ME) milk yield and pregnancy to first AI (P/AI) in a subset of 460 Holstein cows

Variable	Least square means for Pregnancy to first AI, % (no./no.)	Odds ratio estimates	95% CI	*P*-value
[1]AMH category				
High-AMH	40.2 (36/92)	1.29	0.67-2.47	0.37
Intermediate-AMH	43.2 (113/276)	1.46	0.86-2.49	
Low-AMH	34.2 (30/92)	Reference		
[2]Type of AI				
IDE	38.4 (63/164)	0.97	0.65-1.43	0.87
TAI	39.2 (116/296)	Reference		
Herd				
A	48.0 (40/80)	0.72	0.20-2.61	0.16
B	39.4 (37/99)	0.51	0.14-1.82	
C	20.0 (3/15)	0.19	0.03-1.12	
D	37.0 (54/146)	0.51	0.14-1.84	
E	29.0 (20/69)	0.33	0.09-1.20	
F	48.7 (19/39)	0.70	0.18-2.70	
G	50.0 (6/12)	Reference		
Parity				
Multiparous	33.4 (106/308)	0.61	0.39-0.95	0.03
Primiparous	45.2 (73/152)	Reference		
[3]Pre-calving BCS				
High (>3.00)	40.2 (78/194)	0.93	0.55-1.56	0.79
Low (≤3.00)	38.0 (101/266)	Reference		
[4]Post-calving BCS				
High (>2.75)	46.7 (85/185)	1.54	0.93-2.56	0.09
Low (≤2.75)	34.2 (94/275)	Reference		
[5]305-d ME milk yield (kg)				
High (>11,092)	33.3 (79/237)	0.77	0.50-1.17	0.21
Low (≤11,092)	44.8 (100/223)	Reference		

[1]AMH category: The mean and range of serum AMH concentrations (pg/mL) for cows grouped into low- (mean=56.6; range=13.9 to 81.7), intermediate- (mean=164.6; range=83.8 to 284.9), and high-AMH (mean=421.4; range=287.4 to 1879.0) categories.
[2]Type of AI; Cows were inseminated at detected estrus (IDE) or timed-AI (TAI) following synchronization of ovulation.
[3 and 4]: Categories of pre-calving BCS (determined between one and two weeks before calving) and post-calving BCS (determined between four and six weeks after calving) were based on pre-calving BCS of 3.00 and post-calving BCS of 2.75. [5]Cows were categorized as High or Low if 305-d ME milk yield was > or ≤ the mean within each herd, respectively. Overall mean across herds was 11,092 kg

Table 5. 2. Genomic regions associated with anti-Müllerian hormone production after adjustment for multiple testing. The strongest association within each region is identified as well as the nearest putative candidate gene

[1]BTA	Position ([2]Mb)	[3]Number of SNP/s	Lead SNP	Lead SNP ([4]bp)	[5]P	[6]Q	Minor allele frequency	Number of genes within [7]QTL	Name of the nearest candidate gene	Major protein encoded by candidate gene
3	121.191-121.238	4	rs41723399	121192377	4.18×10^{-5}	0.04	0.28	2	*ING5*	Tumor suppressor protein
5	112.865-112.865	1	rs110707003	112864693	4.43×10^{-5}	0.05	0.45	1	*EP300*	E1A Binding Protein P300
7	22.489-23.190	44	rs43505499	22681472	1.58×10^{-8}	6.92×10^{-5}	0.26	21	*AMH*	AMH
9	16.548-16.548	1	rs42748550	16547693	3.77×10^{-5}	0.04	0.17	-	-	-
11	88.859-102.944	513	rs109286956	95849270	1.33×10^{-15}	8.14×10^{-10}	0.14	213	*PPP6C*	Catalytic subunit of protein phosphatase
12	23.363-23.375	2	rs133561787	23363138	2.65×10^{-5}	0.03	0.05	1	*FREM2*	FRAS1 Related Extracellular Matrix Protein 2
18	6.331-6.338	3	rs109461605	6334071	3.65×10^{-5}	0.04	0.24	-	-	-
20	2.947-3.188	6	rs110249317	3188231	5.96×10^{-6}	0.01	0.38	5	*FGF18*	Member of the fibroblast growth factor
20	61.871-67.981	80	rs109765164	65483549	8.30×10^{-7}	0.002	0.36	21	*MTRR*	Member of the ferredoxin-NADP(+) reductase family of electron transferases
21	42.438-45.293	6	rs133903040	42443958	4.97×10^{-6}	0.009	0.34	5	*NUBPL*	Member of the Mrp/NBP35 ATP-binding proteins family
22	53.203-54.030	6	rs43364068	53203469	7.70×10^{-6}	0.01	0.11	22	*PTH1R*	Member of the G-protein coupled receptor family 2
24	26.493-26.985	2	rs135359222	26493099	7.88×10^{-6}	0.01	0.31	-	*DSC3*	Calcium-dependent glycoprotein
25	20.147-20.147	1	rs137098518	20146996	2.74×10^{-5}	0.03	0.41	1	*EEF2K*	Highly conserved protein kinase

[1]BTA: *Bos taurus* autosome
[2]Mb: Mega base
[3]Number of SNP/s: Number of significant single nucleotide polymorphisms within the region after adjusting for multiple testing (false discovery rate <0.05)
[4]bp: Base pairs
[5]P: unadjusted *P*-value of the most strongly associated SNP within the region
[6]Q: adjusted *P*-value of the most strongly associated SNP within the region
[7]QTL: Quantitative trait loci

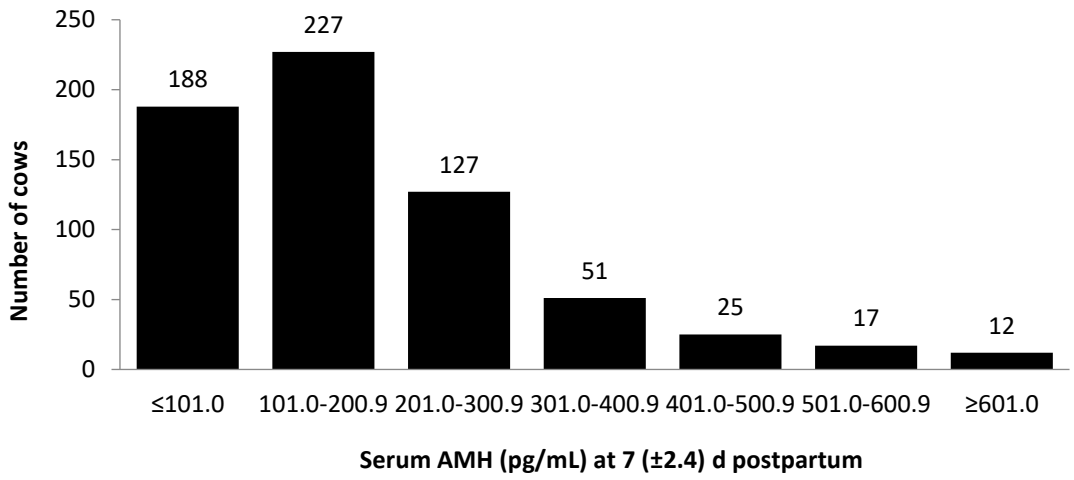

Figure 5. 1. The distribution of serum AMH concentrations at 7 (±2.4) d postpartum in all 647 lactating Holstein cows. The lowest and highest serum AMH concentrations were 13.9 and 1879.0 pg/mL, respectively.

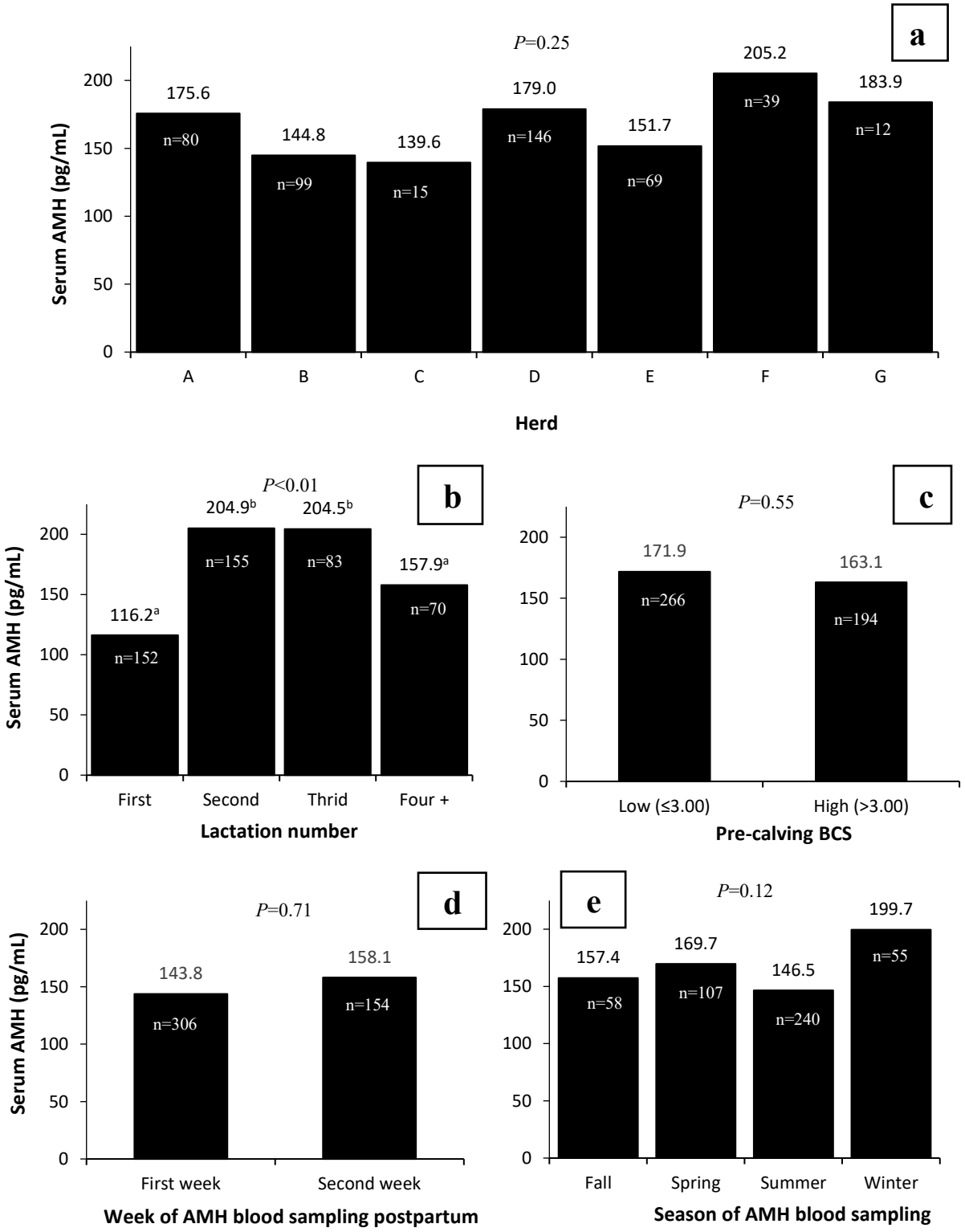

Figure 5. 2. The associations among herd, lactation number, pre-calving BCS, week postpartum, season and serum AMH concentrations at 7 (±2.4) d postpartum in a subset of 460 lactating Holstein cows (A, B, C, D, and E, respectively). The serum concentrations of AMH (transformed values) were back transformed for ease of interpretation.

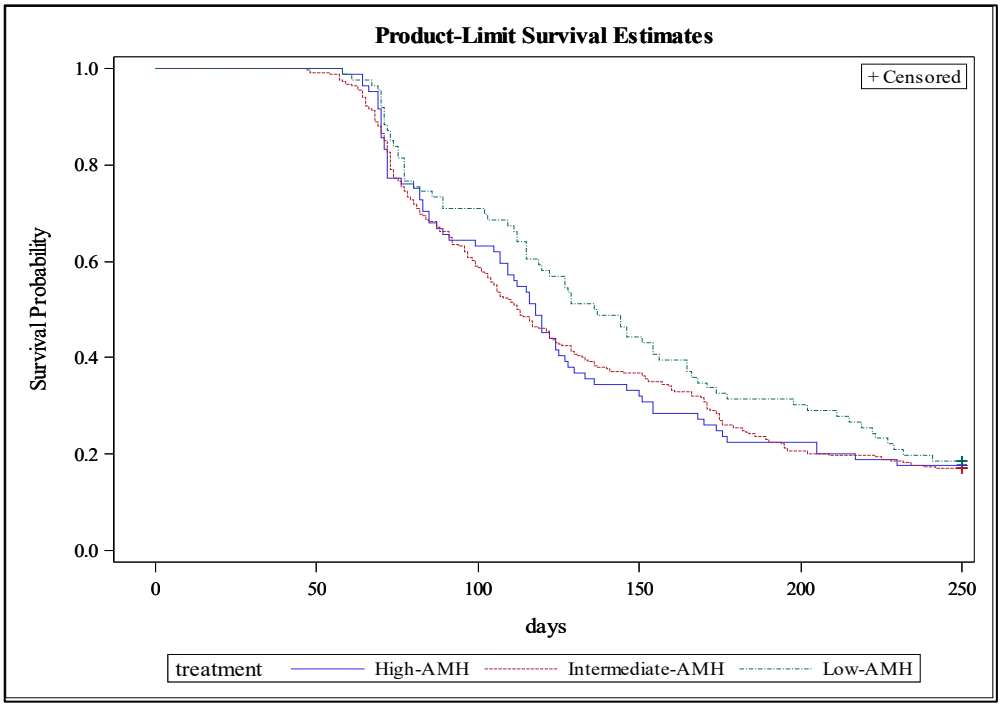

Figure 5. 3. Kaplan-Meier survival curve illustrating the probability of pregnancy risk up to 250 d postpartum based on anti-Müllerian hormone (AMH) concentrations (pg/mL) in serum categorized as low- (mean=56.6; range=13.9 to 81.7; n=92), intermediate- (mean=164.6; range=83.8 to 284.9; n=276), or high-AMH categories (mean=421.4; range=287.4 to 1879.0; n=92) in a subset of 460 lactating Holstein cows. The overall likelihood of pregnancy by 250 d postpartum did not differ between categories of AMH (P=0.44). The hazard of pregnancy risk up to 250 d postpartum was 1.18 [confidence interval; 0.85-1.65; P=0.33]) for cows in the high-AMH category than for those in low-AMH category and 1.19 [confidence interval; 0.91-1.56; P=0.0.21]) for cows in the intermediate-AMH category than for those in low-AMH category.

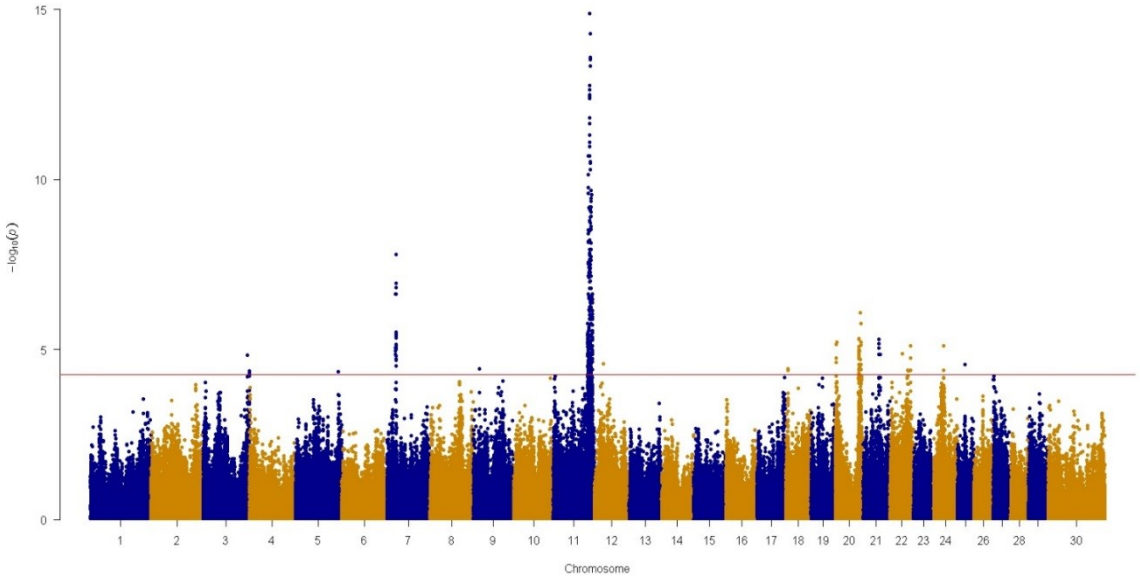

Figure 5. 4. Manhattan plot of the genome-wide association P-values for transformed serum AMH concentrations across 589 genotyped lactating Holstein cows. The red horizontal line indicates significant single nucleotide polymorphisms (SNP) identified across twelve Bos taurus autosomes (BTA 3, 5, 7, 9, 11, 12, 18, 20, 21, 22, 24 and 25) after adjusting for false discovery rate of <0.05.

Chapter 6. The relationship between serum insulin-like growth factor-1 (IGF-1) concentration and reproductive performance, and genome-wide associations for serum IGF-1 in Holstein cows

6.1. Abstract

The objectives of this study were to determine (1) factors associated with serum concentration of insulin-like growth factor-1 (IGF-1), (2) the relationship between serum IGF-1 concentration during the first week postpartum and ovarian cyclicity status by 35 d postpartum (DPP), (3) an optimum serum IGF-1 concentration threshold predictive of pregnancy to first artificial insemination (P/AI) including its diagnostic values, (4) the associations among categories of serum IGF-1 concentration and reproductive outcomes (P/AI and pregnancy risk up to 150 and 250 DPP), and (5) single nucleotide polymorphisms (SNP) associated with phenotypic variation in serum IGF-1 concentration in dairy cows. Serum IGF-1 concentration was determined at 7 (±2.4) DPP in 647 lactating Holstein cows (213 primiparous, 434 multiparous) from seven herds in Alberta, Canada.

The overall mean (±standard error of the mean), median, minimum and maximum serum IGF-1 concentrations during the first week postpartum were 37.8 (±1.23), 31.0, 20.0 and 225.0 ng/mL, respectively. Herd, age, parity, pre-calving body condition score and season of blood sampling were all identified as factors associated with serum IGF-1 concentrations. Although serum IGF-1 concentration during the first week postpartum had no association with ovarian cyclicity status by 35 DPP in primiparous cows, it was greater in cyclic than in acyclic multiparous cows (32.2 vs. 27.4 ng/mL, respectively). The optimum serum IGF-1 thresholds predictive of P/AI were 85.0 ng/mL (sensitivity: 31.9; specificity: 89.1%) and 31.0 ng/mL

(sensitivity: 45.5; specificity 66.9%) for primiparous and multiparous cows, respectively. When cows were grouped into either high or low IGF-1 categories (> or ≤85.0 ng/mL for primiparous cows and > or ≤31.0 ng/mL for multiparous cows, respectively) primiparous cows with high IGF-1 had 4.43 times greater odds of P/AI and a tendency for higher pregnancy risk up to 150 DPP than those with low IGF-1, but not up to 250 DPP. Likewise, multiparous cows with high IGF-1 had 1.61 times greater odds of P/AI than those with low IGF-1. Pregnancy risk up to 150 and 250 DPP, however, did not differ between IGF-1 categories in multiparous cows. Moreover, 37 SNPs across ten *Bos taurus* autosomes were associated with variation in serum IGF-1 concentration, and four previously identified candidate genes related to fertility that were in linkage disequilibrium with some of these SNP were also identified.

Key words: fertility traits, genome-wide association study, reproductive efficiency

6.2. Background

The insulin-like growth factor-1 **(IGF-1)** is a low molecular weight peptide mainly produced by liver (Lund et al., 1986), and controls the growth and differentiation of different cell types in the body through activation of cell cycle (Rechler and Nissley 1990). Hence, it plays a key role in the control of postnatal growth, mammary gland development, lactation and reproduction in dairy cows (Lammers et al., 1999; Jiang and Lucy, 2001; Renaville et al., 2002; Butler 2003).

During the early postpartum period, cows are in a state of negative energy balance **(NEB)** due to high milk yield and low dry matter intake (Butler and Smith 1989), where the growth hormone **(GH)**-IGF-1 axis is uncoupled due to down regulation of GH-receptors in liver (McGuire et al., 1995; Kobayashi et al., 1999). This in turn is associated with decreased IGF-1

and increased GH concentrations to promote the action of GH on lipolysis and gluconeogenesis to favor the milk production (Lucy et al., 2001). Insulin-like growth factor-1 is also essential for reproduction by acting synergistically with gonadotropins (Lucy et al., 1992; Spicer et al., 1993; Beam and Butler 1999). Therefore, partitioning of nutrients mainly towards milk production, indicated by high NEB /low IGF-1 concentration jeopardizes the fertility of dairy cows (Butler 2000).

As a potential fertility trait, circulating concentration of IGF-1 has been identified as having high variability, moderate heritability and positive associations with fertility (see review by Velazquez et al., 2008). Zulu et al. (2002) and Moyes (2004) reported that postpartum concentrations of circulating IGF-1 were highly variable among dairy cows. While 18 to 48% of this variation was attributable to genetic variation (Grochowska et al., 2001; Stirling et al., 2008; Hayhurst et al., 2009), the remaining variation was influenced by other factors such as postpartum nutrient intake, body weight and body condition score **(BCS)** at calving, and parity in cattle (Ciccioli et al., 2003; Pushpakumara et al., 2003; Wathes et al., 2003, respectively). Previous studies also reported that cows with high circulating IGF-1 concentrations postpartum had greater pregnancy to first AI **(P/AI)** than those with low circulating IGF-1 (Pushpakumara et al., 2003; Taylor et al., 2004; Kawashima et al., 2007; Patton et al., 2007; Falkenberg et al., 2008).

Although previous studies reported a positive association between circulating IGF-1 concentration and fertility, most of them were primarily based on multiparous cows only (Lucy et al., 1992; Beam and Butler 1997; Pushpakumara et al., 2003; Kawashima et al., 2007; Patton et al., 2007; Falkenberg et al., 2008). Taylor et al. (2004) used a combination of 177 primiparous and 142 multiparous cows to evaluate the association between circulating IGF-1 concentration and P/AI; however, the sensitivity and specificity (diagnostic values) for optimum IGF-1

threshold predictive of P/AI were not reported. Falkenberg et al. (2008) reported that determining circulating IGF-1 concentration at early postpartum (1, 4, 10, 20 or 40 d postpartum **[DPP]**) had a very limited diagnostic value in predicting P/AI in multiparous cows but such evaluations are unknown in primiparous cows.

Recent evidence suggest that despite similar milk yield, cows with high genetic merit for fertility were able to maintain better body condition and had greater circulating IGF-1 concentration than cows with poor genetic merit for fertility (Cummins et al., 2012; Moore et al., 2014). This finding suggests the existence of potential genetic differences for circulating IGF-1 among cows that are genetically divergent in fertility. Consequently, the identification of single nucleotide polymorphisms **(SNP)** associated with phenotypic variation in circulating IGF-1 concentrations and the subsequent incorporation of these SNP within genomic evaluations may help to accurately identify females with high circulating IGF-1 to enhance fertility in dairy cows. The main hypotheses of the present study were that the threshold serum IGF-1 concentration predictive of P/AI will differ between primiparous and multiparous cows, and SNPs associated with phenotypic variation in serum IGF-1 will be identified.

Our objectives were to determine (1) the factors associated with serum concentration of IGF-1, (2) the association between serum IGF-1 concentration during the first week postpartum and ovarian cyclicity status by 35 DPP, (3) an optimum serum IGF-1 threshold predictive of P/AI and its diagnostic values for primiparous and multiparous cows, (4) the associations among categories of serum IGF-1 concentration and reproductive outcomes (P/AI and pregnancy risk up to 150 and 250 DPP) for primiparous and multiparous cows, and (5) SNP associated with phenotypic variation in serum IGF-1 concentrations through a genome-wide association study **(GWAS)**.

6.3. Materials and methods

6.3.1. Animals and management

This study was conducted in the Dairy Research and Technology Centre, University of Alberta and six commercial dairy herds located in Alberta, between November 2014 and November 2015. Animals were housed and cared for in accordance with the requirements of Canadian Council on Animal Care (2009). Cows were fed a total mixed ration (primary ingredients were barley or corn silage, alfalfa silage, alfalfa hay, and concentrates) formulated according to NRC (2001) and had *ad libitum* access to potable water. Cows from four herds were subjected to GnRH-based synchronization protocols and inseminated without estrus detection for the first and subsequent AIs (timed-AI; **TAI**), whereas, cows from three herds were inseminated at detected estrus (**IDE**).

6.3.2. Blood sampling and determination of serum concentration of IGF-1

Blood samples were collected at (mean±SD) 7 ± 2.4 DPP (hereafter referred to as during the first week postpartum) from 647 lactating Holstein cows (213 primiparous, 434 multiparous) from a coccygeal blood vessel using evacuated Vacutainer® (Becton Dickinson and Company, New Jersey, USA) clot-activator tubes (serum tubes) for serum IGF-1 determination and anti-coagulant K_2 EDTA coated tubes (EDTA tubes) for genotyping. Blood samples were collected after morning milking when cows had just been offered fresh feed. After collection, serum tubes were left undisturbed for about 2 h to allow clot formation, centrifuged at 1500 *x* g for 20 min at 4°C and the serum was harvested and frozen at -20 °C until assayed for IGF-1. The EDTA tubes containing whole blood were frozen at -20 °C until processed for genotyping. Serum concentrations (ng/mL) of IGF-1 were determined at Endocrine Lab Services, University of Saskatchewan, Saskatoon, SK, Canada using a solid phase, enzyme-labelled chemi-luminescent

immunometric assay (IMMULITE, Siemens, Tarrytown, NY, USA) in singlicate, with every fifth sample run in duplicate, as previously described (Elmlinger et al., 2005). The samples were diluted 1:10 with the pre-treatment solution provided with the kit. The intra-assay coefficient of variation was 7.8% for low reference samples (mean, 75.0 ng/mL) and 7.2% for high reference samples (mean, 236.5 ng/mL) and the sensitivity of the assay was 20 ng/ml. When an IGF-1 concentration fell below the assay sensitivity (<20.0 ng/mL), it was considered as 10.0 ng/mL.

6.3.3. Determination of age, body condition score (BCS), ovarian cyclicity status, reproductive measurements and 305-d mature-equivalent milk yield

The age (yr) of the cow was calculated by subtracting the date of birth from the date of serum IGF-1 determination. The BCS was determined between one and two weeks before to the estimated calving date (hereafter referred to as pre-calving BCS) and again between four and six weeks after calving (hereafter referred to as post-calving BCS) on a 1-5 scale system measured in increments of 0.25 units (1 = thin, 5 = fat) as previously described (Edmonson et al., 1989). The ovarian cyclicity status (cyclic; presence of at least one corpus luteum and acyclic: absence of a corpus luteum) was determined between 28 and 35 DPP (hereafter referred to as by 35 DPP) using transrectal ultrasonography. The data on reproductive outcomes and 305-d mature-equivalent **(ME)** milk yield were retrieved at the end of the production cycle using DairyComp 305 herd management software (Valley Agricultural Software, Tulare, CA, USA).

6.3.4. Genotyping, quality control and imputation

DNA extraction was performed on blood samples drawn from a subset of 589 lactating Holstein cows using the Qiagen BioSprint 96 DNA (3840) Kit blood and tissue protocol (Qiagen, Toronto, ON). DNA samples were normalized and genotyped according to the Illumina Infinium Ultra protocol (Illumina, San Diego, CA) and markers were scored on the Bovine Geneseek

Genomic Profiler 26K Beadchip (Neogen Inc, Lincoln, NE) at Delta Genomics (Edmonton, AB, Canada). The beadchips were scanned using the Illumina HiScan (Illumina) and the raw data was processed and exported using Genome Studio 2.0 software according to the Genome Studio Framework User Guide (Illumina) based on selection criteria of at least 95% animal call rate.

Genotype quality control was performed using PLINK v1.09 (Purcell et al., 2007). After excluding SNP with an unknown, Y chromosomal or mitochondrial position, SNP with a call rate <90%, minor allele frequency **(MAF)** <0.01 and those that deviated significantly from Hardy-Weinberg equilibrium ($P \leq 10^{-6}$) were also removed. All animals had a genotype call rate >90% and 19,896 SNP remained after edits. To increase the density of SNP panel for GWAS, imputation to the Illumina Bovine High Density **(HD)** beadchip was performed using FImpute2 (Sargolzaei et al., 2014). Imputation was completed using a two-step approach whereby the 589 animals were first imputed to the Illumina BovineSNP50 chip using a reference population of 3,532 Irish Holstein-Friesian BovineSNP50 genotyped animals, and subsequently imputed to HD density using a reference population of 974 Irish Holstein-Friesian HD genotyped animals. After imputation, all 589 cows had 636,471 SNP with a MAF >0.01 for analysis.

6.3.5. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The descriptive statistics and normality for serum IGF-1 concentration were first determined in all 647 cows (213 primiparous, 434 multiparous) using the UNIVARIATE procedure.

The differences in serum IGF-1 concentration (dependent continuous variable) among herd (A, B, C, D, E, F and G), age (2, 3, 4 and ≥5 yr), parity (primiparous and multiparous), pre-calving BCS (categorized as high and low BCS; >3.00 and ≤3.00, respectively) and season of blood sampling (fall [September, October and November], spring [March, April and May],

summer [June, July and August] and winter [December, January and February]) were determined in a subset of 460 cows (152 primiparous cows and 308 multiparous cows) that had complete data available on all explanatory variables studied. The non-parametric Kruskal-Wallis test was employed because of the non-normal distribution for serum IGF-1 concentration. Pairwise comparisons for levels within each significant factor were performed using the Dunn-Bonferroni post-hoc test.

The proportion of cows cyclic by 35 DPP for primiparous (n = 152) and multiparous (n = 308) cows was compared using chi-squared analysis using the FREQ procedure in the subset of 460 cows. The differences in serum IGF-1 concentration during the first week postpartum in cows stratified by ovarian cyclicity status (cyclic vs. acyclic) by 35 DPP determined within primiparous (n = 152) and multiparous (n = 308) cows by the non-parametric Kruskal-Wallis test. The results from the non-parametric Kruskal-Wallis test were based on Wilcoxon scores (rank sums) estimated for the variable serum IGF-1; however, the mean values for serum IGF-1 concentration were reported in the related figures (Figure 1 and 2) for ease of interpretation.

Out of 647 cows enrolled, the relationship between P/AI (binomial outcome) and serum IGF-1 concentration (predictor continuous variable) during the first week postpartum was first evaluated, separately for primiparous (n = 186) and multiparous (n = 377) cows that had P/AI data (n = 573) available, by logistic regression analysis using the LOGISTIC procedure. The serum IGF-1 concentration predictive of P/AI, including specificity and sensitivity, was determined using receiver operating characteristic **(ROC)** curve analysis separately for primiparous and multiparous cows. The ROC curves analyze sensitivity and 1 − specificity. Sensitivity is the proportion of cows above the optimum IGF-1 threshold diagnosed pregnant to first AI, and specificity is the proportion of cows below the optimum IGF-1 threshold diagnosed

as not pregnant to first AI. The optimum serum IGF-1 concentration threshold was chosen based on the highest Youden's J statistic index. The significance of the optimum serum IGF-1 concentration threshold was determined based on the area under the curve **(AUC)**, where the AUC ranged from 0.50 to 1.00, with AUC of 0.50 considered non-informative and the AUC of 1.00 considered perfect as previously described (Swets, 1988). Thereafter, cows were classified into either high or low IGF-1 categories (>or≤), separately for primiparous and multiparous cows, based on the optimum serum IGF-1 concentration threshold determined by the ROC curve analysis.

The association among categories of serum IGF-1 (high and low IGF-1), herd (A, B, C, D, E, F and G), type of AI (IDE and TAI), ovarian cyclicity status by 35 DPP (cyclic and acyclic), pre-calving BCS (categorized as high and low BCS; >3.00 and ≤3.00, respectively), post-calving BCS (categorized as high and low BCS; >2.75 and ≤2.75, respectively), season of IGF-1 blood sampling (fall [September, October and November], spring [March, April and May], summer [June, July and August] and winter [December, January and February]), 305-d ME milk yield (categorized as high and low 305-d ME milk yield; based on the mean 305-d ME milk yield within each herd) and P/AI was analyzed, separately for primiparous (n = 152) and multiparous (n = 308) cows, using the GLIMMIX procedure in the subset of 460 cows that had complete data available on the explanatory variables studied. The model specifications included a binomial distribution and logit function, and an option to retrieve odds ratios and their confidence limits. The P/AI was initially modelled against all of the aforementioned categorical variables and their interactions. As none of the interactions were significant, they were removed from the final model.

The differences in intervals from calving to pregnancy risk up to 150 and 250 DPP between categories of serum IGF-1, separately for primiparous (n = 152) and multiparous (n = 308) cows, were evaluated using the Kaplan-Meier survival analysis (LIFETEST procedure). The results from Kaplan-Meier survival analysis were confirmed by a cox proportional hazard model (PHREG procedure). Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

6.3.6. Genome-wide association study for circulating IGF-1 concentration

Whole genome association analysis was performed in genome-wide complex trait analysis (Yang et al., 2011) using a mixed linear model based association based on the leave-one-chromosome-out method (Yang et al., 2014). The following model was used; $y = \mu + bx + g^{-} + e$, where y is the IGF-1 category classified separately for primiparous and multiparous cows as previously described, μ is the overall mean, b is a vector of fixed effects including herd, parity, pre-calving BCS, season of blood sampling and the additive effect of the candidate **SNP** tested for association, x is the incidence matrix for the parameters b, g^{-} is the accumulated polygenic effect of all SNP except those on the chromosome where the candidate SNP is located and e is the residual. False discovery rate (FDR) control was performed using the Benjamini-Hochberg method using a FDR of 0.05. Gene search was completed using Ensembl (http://ensembl.org/) and NCBI map viewer (http://www.ncbi.nlm.nih.gov/genome/gdv/) on the University of Maryland assembly of *Bos taurus* 3.1 genome build (UMD 3.1, College Park, MD). The relative roles of nearest candidate genes were searched using Bovine Genome Database (http://bovinegenome.org/).

6.4. Results and discussion

The overall mean (±SEM), median, minimum and maximum serum IGF-1 concentrations during the first week postpartum were 37.8 (±1.23), 31.0, 20.0 and 225.0 ng/mL. To the best of our knowledge, this is the first study to examine the relationship between several risk factors (e.g. herd, age, parity, BCS and season of blood sampling for IGF-1) and serum IGF-1 concentration in a single large experiment comprising both primiparous and multiparous cows (Figure 1). It has been previously reported that circulating IGF-1 concentrations were significantly greater in primiparous than multiparous cows (Wathes et al., 2003; Taylor et al., 2004; Grimard et al., 2013). Similarly, the results from the current study indicated that circulating concentration of IGF-1 gradually declined with age (Figure 1b), and was approximately two-fold greater in primiparous cows than in multiparous cows (Figure 1c). The above findings collectively indicate the requirement for high circulating IGF-1 in young cows to support their body growth until they reach physical maturity (Kerr et al., 1991).

In the current study, serum IGF-1 concentration during the first week postpartum was greater in cows with high pre-calving BCS than in cows with low pre-calving BCS (Figure 1d), which is in agreement with previous reports (Pushpakumara et al., 2003; Meikle et al., 2004; Cummins et al., 2012). Interestingly, serum concentration of IGF-1 was greater in samples collected during winter than in the other seasons (Figure 1e). High serum IGF-1 concentration reported during winter in the current study supports the initial observation of Spicer et al. (1990) who reported an inverse relationship between ambient temperature and circulating IGF-1 in 11 lactating multiparous cows. It is plausible that high energy requirements in winter to meet increased metabolic rate (Young 1981) stimulated greater dry matter intake and thereby resulted in greater secretion of IGF-1 during winter than rest of the seasons. The serum concentration of

IGF-1 was quite similar among herds except for herd B (Figure 1a). The greater IGF-1 concentration observed for herd B compared with other herds was likely due to approximately 50% of cows from this herd being sampled during winter months. In addition to the risk factors studied (e.g. herd, age, parity, BCS and season of blood sampling), the differences among cows in their feed intake and energy status on the day of blood sampling for serum IGF-1 may also have contributed to the variation observed in serum IGF-1 concentration during the first week postpartum in the current study.

Overall, 57% of the cows were cyclic by 35 DPP, which agrees with a recent report from our lab (Bruinjé et al., 2017) wherein 53% of dairy cows had commenced their ovarian cyclicity by 35 DPP based on progesterone concentrations determined through an automated in-line milk analysis system. A higher proportion of multiparous cows tended to be cyclic by 35 DPP than primiparous cows (60 vs. 52%; $P = 0.07$), despite primiparous cows having two-fold greater concentrations of serum IGF-1 than multiparous cows. Insulin-like growth factor-1 acts synergistically with gonadotropins to promote early postpartum ovarian follicular growth and ovulation in dairy cows (Lucy et al., 1992; Spicer et al., 1993; Beam and Butler 1997). For example, circulating IGF-1 concentrations were reported to be 40 to 50% greater during first two weeks postpartum in multiparous cows having an ovulatory dominant follicle compared with cows that had a non-ovulatory dominant follicle (Beam and Butler 1997). Similarly, Patton et al. (2007) showed that circulating concentrations of IGF-1 during first two weeks postpartum were negatively associated with commencement of luteal activity in multiparous cows. Although serum IGF-1 concentration determined during the first week postpartum was not associated with ovarian cyclicity status by 35 DPP in primiparous cows (cyclic vs. acyclic; 62.1 vs. 60.8 ng/mL), it was greater in cyclic than acyclic multiparous cows (32.2 vs. 27.4 ng/mL; Figure 2). The lack

of association reported herein between circulating IGF-1 concentration and ovarian cyclicity status in primiparous cows might be attributable to partitioning of nutrients mainly towards growth and milk production than supporting other biological functions (Wathes et al., 2007).

Taylor et al. (2004) suggested that multiparous cows with circulating IGF-1 concentration < 25.0 ng/mL were less likely to conceive to first AI, and therefore it is cost-effective to delay the first service in those cows until the circulating IGF-1 has increased to a concentration ≥ 50 ng/mL. Those authors did not, however, report the sensitivity and specificity of using 25.0 ng/mL as an optimum threshold predictive of P/AI. In contrast, Falkenberg et al. (2008) concluded that determining circulating IGF-1 concentration either at 1, 4, 10, 20 or 40 d postpartum has very limited diagnostic value to predict P/AI in multiparous cows. Hence, the optimum IGF-1 concentration threshold that is predictive of P/AI, including its diagnostic values, is unknown in primiparous cows. In the current study, the optimum serum IGF-1 threshold predictive of P/AI was 85.0 ng/mL for primiparous cows (Figure 3a) and 31.0 ng/mL for multiparous cows (Figure 3b). The optimum serum IGF-1 threshold established for multiparous cows in the present study (31.0 ng/mL) was comparable to 25.0 ng/mL threshold reported by Taylor et al. (2004). Primiparous cows, however, required greater serum IGF-1 threshold concentration (~50.0 ng/mL more) for optimal prediction of P/AI than multiparous cows. Nevertheless, the sensitivities of these optimum serum IGF-1 concentration thresholds were moderate in both primiparous and multiparous cows (31.9 and 45.5 %, respectively) despite relatively higher specificities (89.1 and 66.9%, respectively). Therefore, the routine measurement of early postpartum circulating IGF-1 concentrations to predict P/AI and thereby to manage low IGF-1 cows with enhanced nutritional strategies or even to delay the first service to improve P/AI seems neither practical nor economically justifiable in either primiparous or multiparous cows.

Positive associations between circulating IGF-1 concentrations and P/AI have been reported in lactating dairy cows (Pushpakumara et al., 2003; Taylor et al., 2004; Patton et al., 2007; Falkenberg et al., 2008). In a recent study, Grimard et al. (2013) reported that the likelihood of fertility (proportion of cows pregnant to first and second AI; n = 32) tended to increase with increasing circulating IGF-1 concentration in dairy cows. However, that report did not statistically quantify the magnitude of increment, and did not account for parity. In the current study, for every one-unit (ng/mL) increase in serum IGF-1 concentration, the odds of estimated probability of P/AI increased by 1.1 and 0.9% for primiparous and multiparous cows, respectively (Figure 4a and b). Moreover, primiparous and multiparous cows in the high IGF-1 category had 4.43 and 1.61 times greater odds of P/AI than cows in the low IGF-1 category (Table 1 and 2). These findings collectively indicate a positive association between high circulating IGF-1 concentration and increased P/AI in dairy cows. The effect of IGF-1 on early pregnancy outcomes may be mediated by its endocrine effects on follicular growth, granulosa cell mitogenesis, granulosa and thecal cell steroid production and reproductive tract and endometrial gland secretions to support embryonic growth (Wathes et al., 1997; Robinson et al. 2000; Zulu et al. 2002; Pushpakumara et al., 2002; Fenwick et al., 2008).

Even though the likelihood of pregnancy by 150 DPP was greater in the high IGF-1 category than in low IGF-1 category within primiparous cows, it did not differ between IGF-1 categories within multiparous (Figure 5). The likelihood of pregnancy by 250 DPP did not differ between high and low IGF-1 categories within both primiparous (hazard ratio: 1.40 [confidence interval; 0.93-2.10; $P = 0.11$]) and multiparous cows (hazard ratio: 0.98 [confidence interval; 0.75-1.27; $P = 0.87$]). Although, Falkenberg et al. (2008) found no association between circulating concentration of IGF-1 at 1, 4 or 10 DPP and proportion of multiparous cows pregnant

within 200 DPP, they reported that the proportion of multiparous cows pregnant within 200 DPP was smaller for those with low circulating IGF-1 concentration than those with high circulating IGF-1 concentration at 40 DPP. These results collectively indicate that IGF-1 concentration based on a single blood sample collected during the first week postpartum has no predictive value on reproductive performance beyond first service in dairy cows.

Although GWAS evaluating the relationship between SNP in the *IGF-1* gene and fertility is recent interest (Mullen et al., 2011; Nicolini et al., 2013), studies unravelling SNP associated with phenotypic variation in circulating IGF-1 concentrations are lacking in dairy cows. In the current study, no SNP remained significantly associated with serum IGF-1 concentration after adjustment for multiple testing. However, 37 SNP of suggestive significance in this study ($P<0.0001$) were identified across ten *Bos taurus* autosomes (BTA; 1, 2, 4, 6, 8, 9, 16, 23 and 26; Figure 6). Genomic regions associated with serum IGF-1 concentration and putative candidate genes within 250 kb up and downstream of the strongest association were listed in Table 3. Of these 37 SNP, those that were in linkage disequilibrium with previously identified candidate genes related to fertility were discussed below. It is noteworthy, however, that no SNP were identified to be in linkage disequilibrium with the candidate gene *IGF-1* from BTA 5, and this was likely because the regulation of circulating IGF-1 concentrations may not be primarily controlled by the *IGF-1* gene. For example, Schneider et al., (2013) studied the effects of GH receptor (GHR) AluI polymorphism on the reproductive performance of Holstein cows and reported that cows with two (-/-) GHR AluI alleles had the highest serum IGF-1 concentration and shortest calving to conception interval compared to those cows with either one (-/+) or zero (+/+) allele for GHR AluI polymorphism.

We identified four candidate genes (*ARHGEF26, HGF, GC and PTPRK*) that were in linkage disequilibrium with SNP related to phenotypic variation in serum IGF-1 which have also been previously reported to influence fertility in mice (Uehara et al., 1995) or cattle (Huang et al., 2010; Minten et al., 2013; Killeen et al., 2014). None of these genes, however, is known to regulate serum IGF-1 concentrations in dairy cows. Thus, the results presented herein should be interpreted with caution. The candidate gene *ARHGEF26* is located within 215 kb up-stream of the lead SNP rs110526526 (at 114.2 Mb) on BTA 1 and encodes the member of the rho guanine nucleotide exchange factor family. In a recent study, Killeen et al. (2014) reported *ARHGEF* as one amongst several differentially expressed endometrial genes during the mid-luteal phase of the estrous cycle between beef heifers ranked as either high or low fertility, and identified as functionally related to cell morphology, inflammatory response and lipid metabolism using the ingenuity pathway analysis. The candidate gene *HGF* (hepatic growth factor) is located 105 kb down-stream of the lead SNP rs42716293 (at 39.1 Mb) on BTA 4, and is involved in cell growth, cell motility and morphogenesis in numerous cell and tissue types. In this regard, Uehara et al. (1995) showed that homozygous mutant mice embryos for *HGF* gene had severely impaired placentas with markedly reduced numbers of labyrinthine trophoblast cells, and died before birth.

Moreover, the candidate gene *GC* (Group specific component) is located 217 kb up-stream of the lead SNP rs108983037 (at 88.6 Mb) on BTA 6. The protein encoded by this gene belongs to albumin gene family and binds to vitamin D and its plasma metabolites to transport them to target tissues (Cooke and Haddad 1989), where vitamin D has been shown to be essential for endometrial functions and pregnancy success in humans and animal models (Luk et al., 2012). In a recent study, Minten et al. (2013) demonstrated that the gene *GC* was differentially expressed in the endometrium of infertile, sub-fertile and highly fertile beef heifers at d 14 of the estrous

cycle. Finally, the lead SNP rs109274083 on BTA 9 is an intronic variant within the candidate gene *PTPRK,* which encodes the member of the protein tyrosine phosphatase family and is known to regulate a variety of cellular processes including cell growth and differentiation. Huang et al. (2010) showed that *PTPRK* was down regulated by at least four-fold in in-vitro derived degenerative embryos compared to blastocysts in cattle. Perhaps, greater P/AI reported for cows with high circulating IGF-1could at least partially supported by the roles of aforementioned candidate genes that are in genomic regions identified for variation in circulating IGF-1 concentrations in addition to the direct effect of circulating IGF-1 on fertility. Moreover, it is important to recognize that fertility is a highly polygenic trait under the control of thousands of SNP effects (Minozzi et al., 2013) and these candidate genes are some of many that contribute marginally to the phenotypic variance.

In summary, (1) serum IGF-1 concentration during the first week postpartum was associated with herd, age, parity, BCS at pre-calving and season of blood sampling, (2) cyclic multiparous cows had greater serum IGF-1 concentration during the first week postpartum compared with acyclic multiparous cows, (3) optimum serum IGF-1 thresholds predictive of P/AI, separately for primiparous and multiparous cows, were identified; however, the reliability of IGF-1 as a predictor of P/AI is low due to its moderate sensitivity, (4) primiparous and multiparous cows with high circulating IGF-1 concentration had greater P/AI than those with low circulating IGF-1, and (5) 37 SNP associated with phenotypic variation in serum IGF-1 concentrations across ten chromosomes (BTA; 1, 2, 4, 6, 8, 9, 16, 23, 25 and 26), and four previously identified candidate genes related to fertility that were in linkage disequilibrium with some of these SNP were identified. Despite identification of SNP, the collective findings suggest that a single measurement of serum IGF-1 concentration during the first week postpartum might

not be an accurate predictor of fertility given the many factors that influence reproductive success in dairy cows.

6.5. Acknowledgments

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Table 6. 1. The associations among categories of insulin-like growth factor (IGF-1), herd, type of AI, ovarian cyclicity status, pre- and post-calving body condition score (BCS), season, 305-d mature-equivalent (ME) milk yield and pregnancy to first insemination in 152 primiparous cows

Variable	Pregnancy to first AI, % (no./no.)	Odds ratio estimates	95 % CI	*P*-value
[1]IGF-1 category (ng/mL)				
High-IGF-1 (>85.0)	74.3 (28/38)	4.43	(1.51-12.96)	<0.01
Low-IGF-1(≤85.0)	44.2 (45/114)	Reference		
Herd				
A	60.9 (14/23)	-	-	0.10
B	45.7 (16/35)	-	-	
C	0.0 (0/5)	-	-	
D	56.6 (30/53)	-	-	
E	26.3 (5/19)	-	-	
F	44.4 (8/15)	-	-	
G	0.0 (0/2)	Reference		
[2]Type of AI				
IDE	40.4 (19/47)	0.64	(0.32-1.29)	0.21
TAI	51.4 (54/105)	Reference		
[3]Ovarian cyclicity status				
Cyclic	46.8 (37/79)	1.22	(0.48-3.11)	0.67
Acyclic	49.3 (36/73)	Reference		
[4]Pre-calving BCS				
High (>3.00)	42.4 (28/66)	0.59	(0.19-1.83)	0.35
Low (≤3.00)	52.3 (45/86)	Reference		
[5]Post-calving BCS				
High (>2.75)	52.1 (37/71)	2.27	(0.74-6.97)	0.15
Low (≤2.75)	44.4 (36/81)	Reference		
[6]Season				
Fall	53.8 (7/13)	1.91	(0.18-19.9)	0.78
Spring	52.6 (20/38)	0.73	(0.13-4.24)	
Summer	45.1 (37/82)	0.92	(0.17-5.03)	
Winter	47.3 (9/19)	Reference		
[7]305-d ME milk yield (kg)				
High (>10,179)	31.6 (24/76)	0.21	(0.09-0.51)	<0.01
Low (≤10,179)	64.5 (49/76)	Reference		

[1]IGF-1 categories were based on the optimum serum IGF-1 threshold (85.0 ng/mL) predictive of pregnancy to first AI (P/AI) in primiparous cows determined using receiver operating characteristic curve analysis (Figure 8.4a)
[2]Type of AI; cows were inseminated at detected estrus (IDE) or timed-AI (TAI) following synchronization of ovulation
[3]Ovarian cyclicity status by 35 DPP; cows were determined as cyclic; presence of at least one corpus luteum and acyclic: absence of a corpus luteum by 35 DPP using transrectal ultrasonography
[4,5]Categories of pre-calving BCS (determined between one and two weeks before calving) and post-calving BCS (determined between four and six weeks after calving) were based on pre-calving BCS of 3.00 and post-calving BCS of 2.75
[6]Season of IGF-1 blood sampling; categorized as (fall [September, October and November], spring [March, April and May], summer [June, July and August] and winter [December, January and February])
[7]Cows were categorized as high or low if 305-d ME milk yield was > or ≤ the mean within each herd, respectively. Overall mean across herds was 10,179 kg.

Table 6. 2. The associations among categories of insulin-like growth factor (IGF-1), herd, type of AI, ovarian cyclicity status, pre- and post-calving body condition score (BCS), 305-d mature-equivalent (ME) milk yield and pregnancy to first insemination (P/AI) in 308 multiparous cows

Variable	Pregnancy to 1st AI, % (no./no.)	Odds ratio estimates	95 % CI	*P*-value
[1]IGF-1 category (ng/mL)				
High-IGF-1 (>31.0)	40.9 (52/127)	1.61	(`0.95-2.73)	0.08
Low-IGF-1(≤31.0)	29.8 (54/181)	Reference		
Herd				
A	45.6 (26/57)	0.50	(0.12-2.15)	0.25
B	32.8 (21/64)	0.20	(0.04-1.08)	
C	30.0 (3/10)	0.30	(0.04-2.19)	
D	25.8 (24/93)	0.27	(0.06-1.28)	
E	30.0 (15/50)	0.26	(0.06-1.09)	
F	45.8 (11/24)	0.58	(0.12-2.85)	
G	60.0 (6/10)	Reference		
[2]Type of AI				
IDE	37.6 (44/117)	1.25	(0.77-2.03)	0.36
TAI	32.5 (62/191)	Reference		
[3]Ovarian cyclicity status				
Cyclic	37.5 (69/184)	1.13	(0.63-2.02)	0.68
Acyclic	29.8 (37/124)	Reference		
[4]Pre-calving BCS				
High (>3.00)	39.1 (50/128)	0.97	(0.51-1.86)	0.93
Low (≤3.00)	31.1 (56/180)	Reference		
[5]Post-calving BCS				
High (>2.75)	42.1 (48/114)	1.28	(0.67-2.43)	0.46
Low (≤2.75)	29.9 (58/194)	Reference		
[6]Season				
Fall	40.0 (18/45)	0.55	(0.13-2.33)	0.55
Spring	27.5 (19/69)	0.42	(0.13-1.40)	
Summer	34.1 (54/158)	0.53	(0.16-1.77)	
Winter	41.6 (15/36)	Reference		
[6]305-d ME milk yield (kg)				
High (>11,542)	36.9 (55/149)	1.09	(0.63-1.87)	0.76
Low (≤11,542)	32.1 (51/159)	Reference		

[1]IGF-1 categories were based on the optimum serum IGF-1 threshold (31.0 ng/mL) that tended to predict pregnancy to first AI (P/AI) in multiparous cows using receiver operating characteristic curve analysis (Figure 8.4b)

[2]Type of AI; cows were inseminated at detected estrus (IDE) or timed-AI (TAI) following synchronization of ovulation

[3]Ovarian cyclicity status by 35 DPP; cows were determined as cyclic; presence of at least one corpus luteum and acyclic: absence of a corpus luteum by 35 DPP using transrectal ultrasonography

[4,5]Categories of pre-calving BCS (determined between one and two weeks before calving) and post-calving BCS (determined between four and six weeks after calving) were based on pre-calving BCS of 3.00 and post-calving BCS of 2.75

[6]Season of IGF-1 blood sampling; categorized as (fall [September, October and November], spring [March, April and May], summer [June, July and August] and winter [December, January and February])

[7]Cows were categorized as high or low if 305-d ME milk yield was > or ≤ the mean within each herd, respectively. Overall mean across herds was 11,542 kg.

Table 6. 3. Genomic regions associated with serum insulin-like growth factor (IGF-1) concentration in dairy cows. The strongest association within each region is identified as well as the nearest putative candidate genes

[1]BTA	Position ([2]Mb)	[3]Number of SNP/s	Lead SNP	Lead SNP ([4]bp)	[5]*P*	Minor allele frequency	Genes within 250kb of the strongest association
1	114.647-118.703	2	rs110526526	114,646,540	5.28×10^{-5}	0.20	*ARHGEF26, ENSBTAG00000011631*
2	59.488-59.596	7	rs42530109	59,591,867	6.38×10^{-6}	0.02	*HNMT, ENSBTAG00000039437, THSD7B*
4	39.011-39.011	1	rs42716293	39,011,023	6.78×10^{-5}	0.41	*CACNA2D1, HGF*
6	88.865-89.036	5	rs108983037	88,913,092	6.89×10^{-6}	0.41	*GC,NPFFR2, ADAMTS3*
8	77.827-78.202	9	rs134959907	78,183,232	2.83×10^{-5}	0.24	*FRMD3, IDNK, UBQLN1, GKAP1, KIF27*
9	56.349-56.349	1	rs132755904	56,349,339	7.28×10^{-5}	*0.48*	*ENSBTAG00000033083*
9	67.365-67.397	6	rs109274083	67,377,964	8.38×10^{-5}	0.44	*PTPRK*
16	2.611-2.611	1	rs109145522	2,610,719	8.18×10^{-5}	0.40	*NFASC, CNTN2, TMEM81, RBBP5DSTYK, TMCC2*
23	27.219-27.219	1	rs135963713	27,219,241	9.60×10^{-5}	0.23	*AGPAT1, PRRT1, FKBPL, TNXB, ENSBTAG00000006864, NELFE, STX19, DXO, SKIV2L, C2, EHMT2, ZBTB12, NEU1, HSPA1L, LSM2, MSH5, VWA7, VARS, CLIC1, LY6G6D*
25	25.740-25.740	1	rs41574560	25,740,992	2.84×10^{-5}	0.45	*KIAA0556, GSG1L, XPO6*
26	29.082-29.208	2	rs42718552	29,082,643	2.67×10^{-5}	0.09	-
26	50.522-50.522	1	rs137415315	50,522,157	2.71×10^{-5}	0.05	*SYCE1, CYP2E1, ENSBTAG00000005267, ENSBTAG00000026185, ENSBTAG00000010461, ENSBTAG00000007248*

[1]BTA: *Bos taurus* autosome
[2]Mb: Mega base
[3]Number of SNP/s: Number of significant single nucleotide polymorphisms within the region after adjusting for multiple testing
[4]bp: Base pairs
[5]*P*: Unadjusted *P*-value of the most strongly associated SNP within the region.

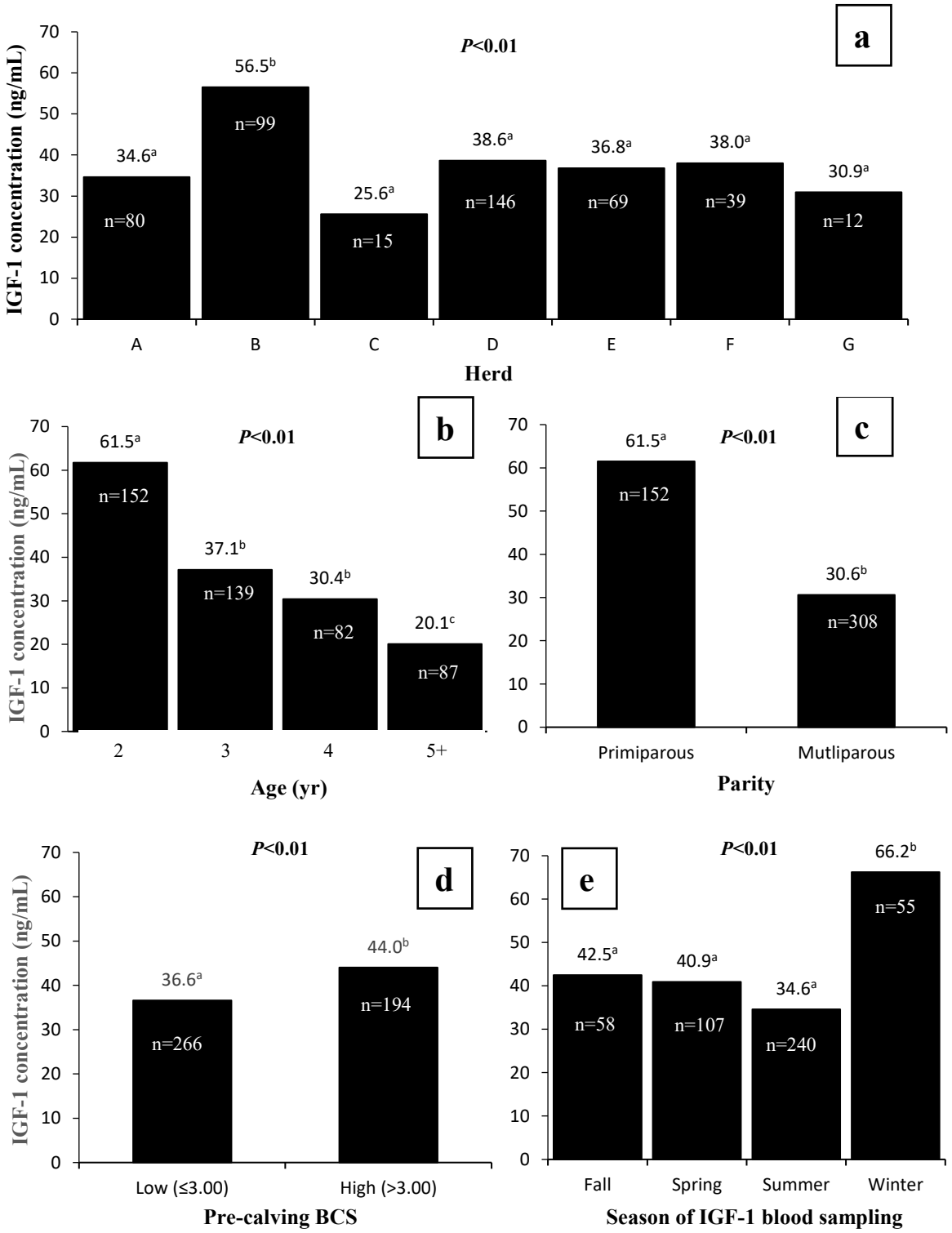

Figure 6. 1. Mean concentrations of serum IGF-1 (ng/mL) at 7 (±2.4) d postpartum among herd (a), age (b), parity (c), pre-calving body condition score [BCS] (d), and season of IGF-1 blood sampling (e) in a subset of 460 lactating Holstein cows. Different superscripts within the same category differ (a,b,c; P<0.05).

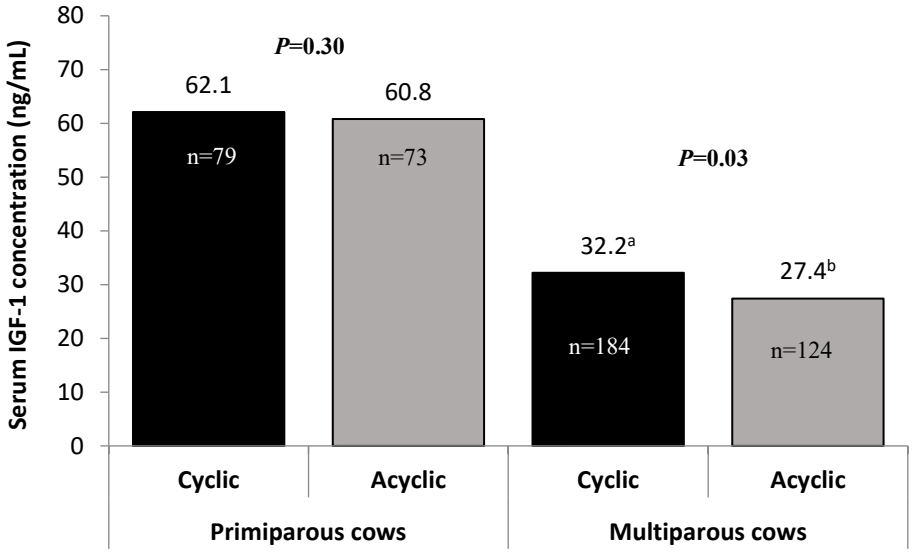

Figure 6. 2. Mean serum concentrations of IGF-1 (ng/mL) at 7 (±2.4) d postpartum stratified by cyclicity status (black bar = cyclic and grey bar = acyclic) determined by ultrasonography (based on presence of a CL) at 35 d postpartum in primiparous (n = 152) and multiparous (n = 308) cows. Different superscripts within the same category differed (a,b; P<0.05).

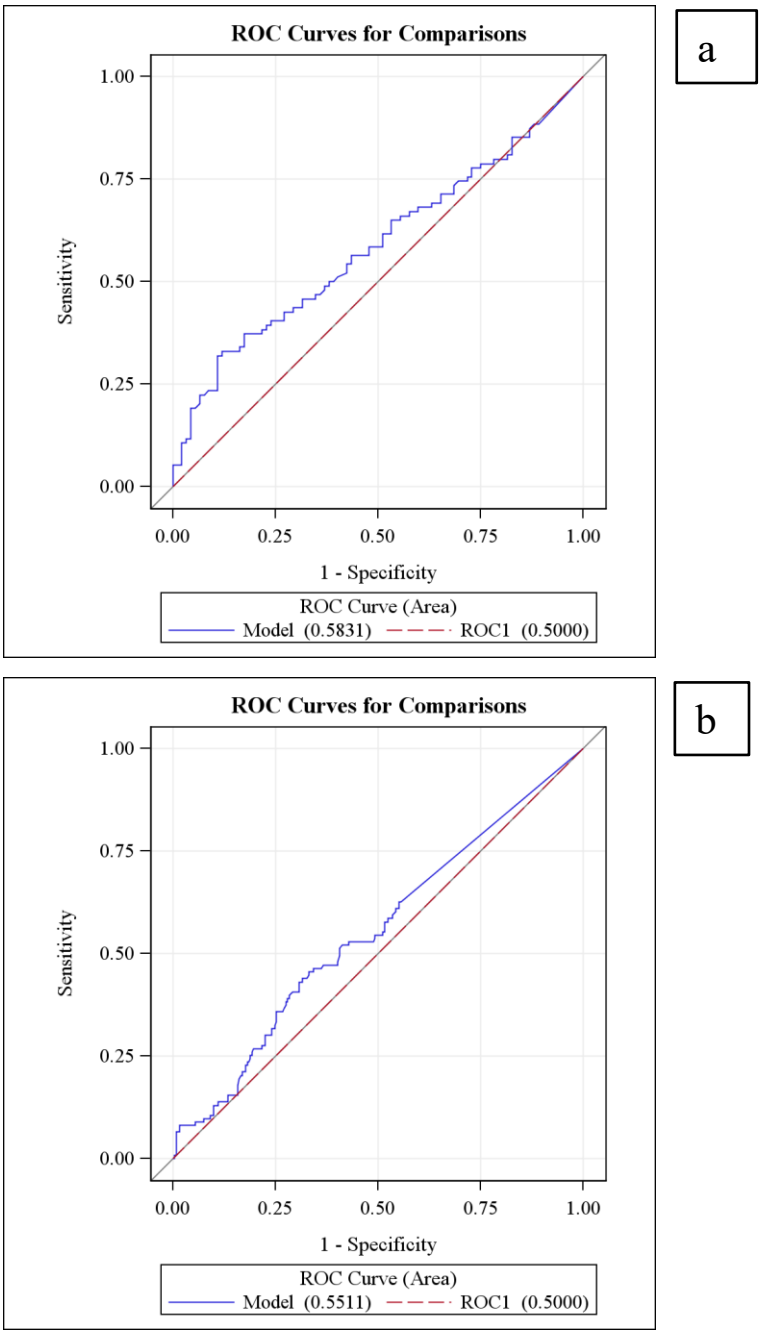

Figure 6. 3. The receiver operating characteristic (ROC) curve analysis for serum IGF-1 concentration at 7 (±2.4) d postpartum predictive of P/AI in primiparous (a; n = 186) and multiparous (b; n = 377) cows. The optimum serum IGF-1 concentration threshold predictive of P/AI was 85.0 ng/mL for primiparous cows (sensitivity: 31.9 and specificity of 89.1%; P = 0.04) and 31.0 ng/mL for multiparous cows (sensitivity: 45.5 and specificity of 66.9 %; P = 0.09).

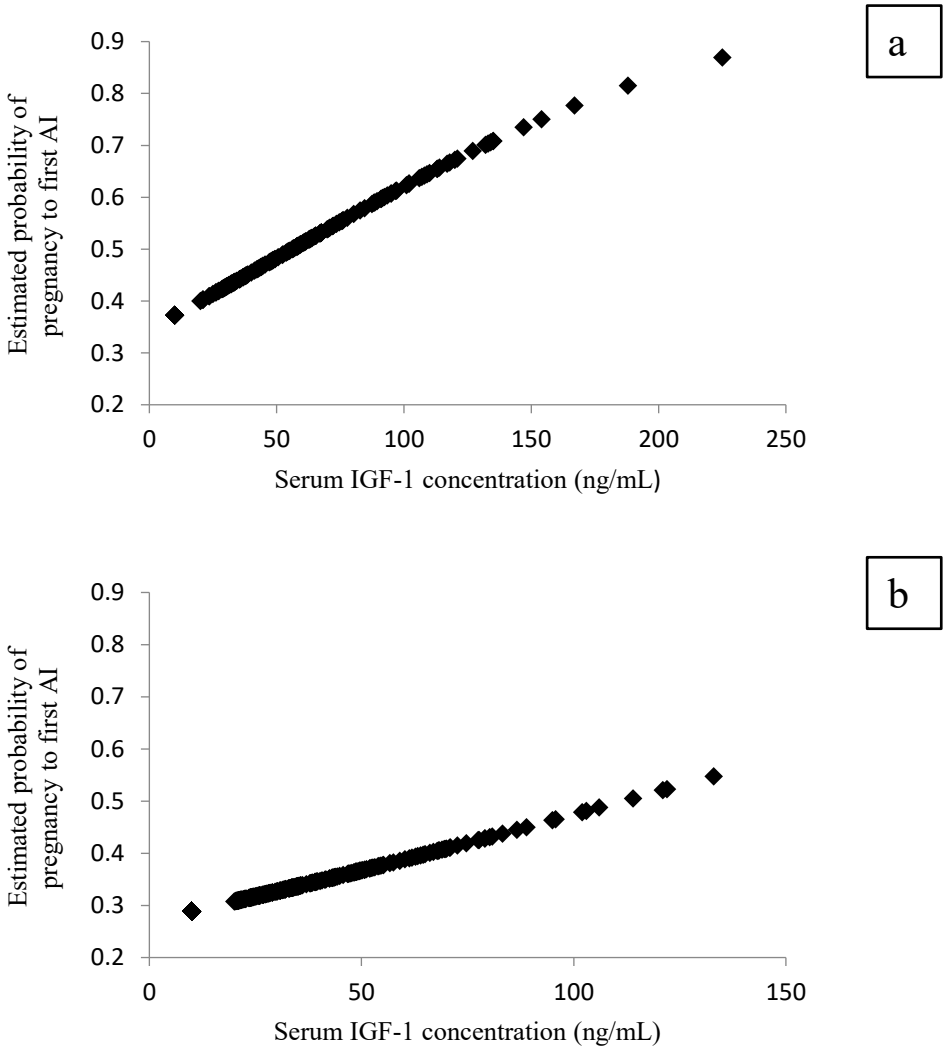

Figure 6. 4. The odds of estimated probability of pregnancy to first AI (P/AI) plotted against serum IGF-1 concentrations at 7 (±2.4) d postpartum in primiparous (a; n = 186) and multiparous (b; n = 377) cows. For every one-unit (ng/mL) increase in serum IGF-1 concentration, the odds of estimated probability of P/AI increased by 1.1% in primiparous cows (P<0.01) and 0.9% in multiparous cows (P = 0.05), respectively.

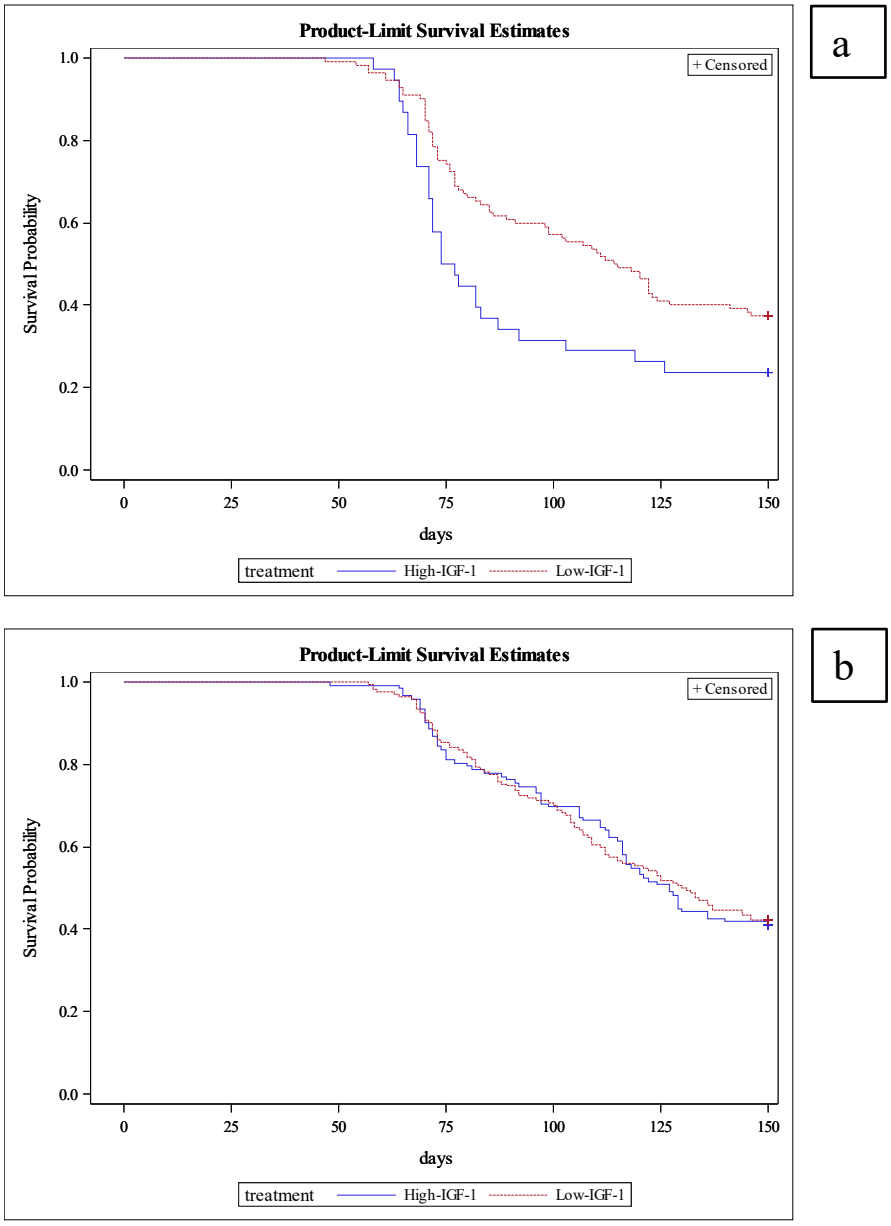

Figure 6. 5. Kaplan-Meier survival curve that illustrates the proportion of primiparous (a) and multiparous (b) cows (by IGF-1 category) pregnant by 150 DPP. In primiparous, the hazard of pregnancy by 150 DPP was greater (hazard ratio: 1.69 [confidence interval; 1.10-2.61; P = 0.02]) for high IGF-1 category cows (>85.0 ng/mL; n = 38; solid line) than those in low IGF-1 category (≤85.0 ng/mL; n = 114; broken line). In multiparous, however, the hazard of pregnancy by 150 DPP did not differ (hazard ratio: 1.02 [confidence interval; 0.75-1.39; P = 0.88]) between cows in the high IGF-1 (>31.0 ng/mL; n = 52; solid line) and low IGF-1 categories (≤31.0 ng/mL; n = 54; broken line).

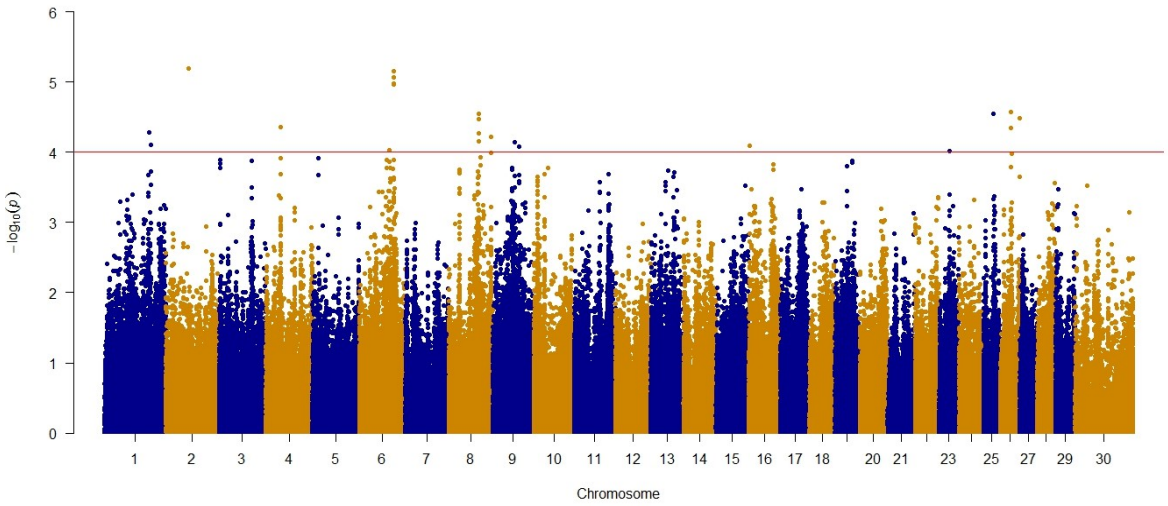

Figure 6. 6. Manhattan plot of the genome-wide association study for serum IGF-1 concentration in Holstein cows. The red horizontal line represents all single nucleotide polymorphisms with a P-value <0.0001 identified across ten *Bos taurus* autosomes BTA: 1, 2, 4, 6, 8, 9, 16, 23, 25 and 26).

Chapter 7. Characterization of ano-genital distance and its relationship to fertility in Canadian Holsteins

7.1. Abstract

Ano-genital distance (AGD) serves as a marker for genital development and fertility in humans and rodents. The primary objectives of this observational study in lactating dairy cows were to (1) characterize the distribution and variability of AGD, (2) determine the relationship among AGD and potential postnatal AGD determinants of age and height, and (3) evaluate the associations between AGD and pregnancy to first artificial insemination (P/AI) and cumulative pregnancy by 250 d in milk (DIM) within parity groups (1st, 2nd, and 3rd + parities). The secondary objective was to evaluate the association between AGD and testosterone concentrations. The AGD (mm), age (yr) and height at hip (cm) at the time of AGD determination, and aforesaid reproductive outcomes were determined in 921 Holstein cows (1st, 2nd and 3rd + parity; n = 360, 256 and 305, respectively). Plasma concentrations of testosterone were determined in a subset of 93 cows.

Overall, AGD had a normal distribution and high variability (mean [± SD]; 131.0 ± 12.2 mm), was weakly associated with cow age and height (R^2 = 0.09 and 0.04, respectively), and had an inverse relationship with P/AI in 1st and 2nd parity cows, but not in 3rd + parity cows. For every one mm increase in AGD, the odds of P/AI decreased by 3.4 and 2.4 % for 1st and 2nd parity cows, respectively. The optimal AGD threshold to predict probability of P/AI was 127.1 mm for both 1st (Sensitivity: 66.4; Specificity: 56.6 %) and 2nd parity cows (Sensitivity: 46.0; Specificity: 70.4 %). Accordingly, 1st and 2nd parity cows were categorized into either SHORT- or LONG-AGD

(≤ or > 127.1 mm), and associations with reproductive outcomes evaluated. First parity cows with LONG-AGD had lower P/AI (30.9 vs. 53.6 %) and decreased likelihood (hazard ratio: 0.68) of pregnancy by 250 DIM than those with SHORT-AGD. Similarly, 2nd parity cows with LONG-AGD had reduced P/AI (28.3 vs. 44.4 %) and a tendency for decreased likelihood (hazard ratio: 0.76) of pregnancy by 250 DIM than in cows with SHORT-AGD. The association between AGD and testosterone was weak and non-significant. In summary, AGD in Holstein cows was normally distributed, highly variable, and weakly associated with age and height. Besides, AGD had a strong inverse relationship with P/AI and cumulative pregnancy by 250 DIM in 1st and 2nd parity cows; however, such a relationship was not evident in older (3rd +parity) cows.

Key words: ano-genital distance, age, height, fertility

7.2. Background

Ano-genital distance **(AGD)** is the distance from the center of the anus to the anterior fourchette or to the base of the clitoris in female mammals (Salazar-Martinez et al., 2004; Sathyanarayana et al., 2010). The in-utero development of the perineum and caudal migration of genital tubercle, relative to the anus, are androgen-dependent in humans and rodents (Langman, 1975; Bowman et al., 2003). Therefore, the variation in AGD is a reflection of fetal androgen exposure during its reproductive programming window in those species (Macleod et al., 2010; Dean at al., 2012). In this regard, Mendiola et al. (2012) reported that AGD was normally distributed in a population of young women with high variability. Several other studies demonstrated that the AGD was approximately twice as long in males as in females (Salazar-Martinez et al., 2004; Swan, 2008; Thankamony et al., 2009; Macleod et al., 2010; Sathyanarayana et al., 2010). Hence, AGD is not only a biological indicator of prenatal

androgenization, but also a sexually dimorphic trait that may be used to determine fetal gender during early pregnancy.

Prenatal exposure to excess androgen in female fetuses leads to poor reproductive system development in-utero, subsequently resulting in long AGD and poor postnatal fertility outcomes in rodents, rabbits and humans (Zehr et al., 2001; Banszegi et al., 2012; Mendiola et al., 2012; Mira-Escolano et al., 2014a; Wu et al., 2017, respectively). The onset of puberty was delayed in female mice with long AGD (Zehr et al., 2001). Rabbit does with long AGD delivered fewer and lighter offspring, and had male-biased litters (Banszegi et al., 2012). Women with long AGD, presumably exposed prenatally to high androgen concentrations in-utero, had increased numbers of ovarian follicles (Mendiola et al., 2012) and greater testosterone concentrations during the early follicular phase (Mira-Escolano et al., 2014a) compared to women with short AGD. Recently, Wu et al., (2017) reported that women with longer AGD were approximately 18 times more likely to develop polycystic ovarian syndrome, which is characterized by hyperandrogenism and anovulation, than those with shorter AGD. The placenta is the primary source of androgens in dams bearing female fetuses in dairy cows (Mongkonpunya et al., 1975). Maternal concentrations of testosterone (110 to 166 pg/mL) and androstenedione (936 to 1400 pg/mL) during gestation were highly variable among individual cows bearing female fetuses (Gaiani et al., 1984). Thus, it is plausible that the high variability in in-utero exposure of female bovine fetuses to androgens affect AGD and postnatal reproductive functions as reported in humans and rodents. In women, AGD was not associated with the postnatal determinants of age, height and weight, but it was associated with body mass index (Mira-Escolano et al., 2014b; Wu et al., 2017). Moreover, in one study (Mira-Escolano et al., 2014a) for each mm increase in AGD in women, testosterone concentration increased by 0.006 ng/mL. Similar studies characterizing AGD and its

associations with age, height, reproductive outcomes and testosterone concentrations have not been conducted in dairy cows.

If an association exists between the simple morphologic measure of AGD and reproductive performance in dairy cows, AGD could become a new reproductive phenotype with potential for use in future genetic selection to augment fertility. Therefore, the primary objectives of this observational study were to (1) characterize the distribution and variability of AGD, (2) determine the relationship among AGD and potential postnatal AGD determinants of age and height, and (3) evaluate the associations between AGD and pregnancy to first AI **(P/AI)** and cumulative pregnancy by 250 d in milk **(DIM)** within parity groups (1^{st}, 2^{nd}, and $3^{rd\ +}$). The secondary objective was to evaluate the association between AGD and testosterone concentrations.

7.3. Materials and methods

7.3.1. Animals and management

This study was conducted at the Dairy Research and Technology Centre of the University of Alberta and three commercial dairy herds located in Alberta, Canada. Animals were housed and cared for in accordance with the requirements of Canadian Council on Animal Care (2009). Cows were fed a total mixed ration (primary ingredients were barley or corn silage, alfalfa silage, alfalfa hay, and concentrates) formulated according to NRC (2001) to meet the requirements of a 650 kg lactating cow producing 45.0 kg of milk/d, and had *ad libitum* access to water. While cows were subjected to presynchronization followed by Ovsynch (1^{st} AI) and Ovsynch only ($2^{nd\ +}$ AI) in the university research herd and one of the commercial herds (timed-AI; **TAI**; herds A and B, respectively), cows were predominantly inseminated based on estrus detection in two of

the commercial herds (insemination on detected estrus; **IDE**; herds C and D, respectively).

7.3.2. Determination of AGD, age, height, milk yield and reproductive measures

Ano-genital distance was defined as the distance from the center of the anus to the base of the clitoris (Figure 1a), and was measured using a stainless steel digital calipers (Procise, The Innovak Group, Montreal, QC, Canada). The age of the cow (yr) at the time of AGD measurement was calculated by subtracting the date of birth from the date of AGD determination. The height at hip (hereafter referred to as "height") was determined using a livestock measuring stick (Jeffers®, Dothan, AL) from the ground to the top of the cow's back (above hook bones). The AGD and height were measured by two individuals, with one person always measuring AGD and the other person measuring height. Data on AGD were collected during a single visit to each herd. Ano-genital distance and height measurements were obtained from 921 cows (mean ± SD: 171 ± 93 DIM) that had no apparent perineal abnormalities such as inflamed or lacerated vulva as indicators of trauma at parturition, and that were later than 14 DIM at the time of AGD determination. Data on 305-d mature-equivalent **(ME)** milk yield and reproductive measures (P/AI and pregnancy by 250 DIM) were retrieved for all cows using DairyComp 305 herd management software (CanWest DHI, Guelph, ON, Canada).

7.3.3. Blood sampling and determination of plasma concentrations of testosterone

Blood samples were collected in a subset of 93 cows (research herd only) from coccygeal blood vessel using evacuated Vacutainer® tubes (Becton Dickinson and Company, New Jersey, USA) containing sodium heparin immediately before the second GnRH of Ovsynch during a presynchronization/Ovsynch timed-AI protocol described elsewhere (Gobikrushanth et al., 2017). Samples were placed on ice upon collection, centrifuged at 1500 x *g* for 20 min at 4°C, plasma harvested and frozen at -20 °C until assayed. Plasma concentrations of testosterone were

determined at Endocrine Lab Services, University of Saskatchewan, Saskatoon, SK, Canada using a commercial solid-phase radioimmunoassay kit (ImmuChem^{TM}; MP Biomedicals, LLC, Orangeburg, NY). Procedures were carried out according to manufacturer's instructions except that standards were prepared by adding known quantities of testosterone to charcoal-stripped bovine serum, as the standards supplied with the kit were optimized for human samples. All samples were analyzed in a single assay; the intra-assay coefficient of variation was 1.4 % for low reference samples (mean, 4.2 pg/mL) and 5.1 % for high reference samples (mean, 8.8 pg/mL).

7.3.4. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Initially, AGD was determined by the same individual three times in 93 dairy cows from the university research herd, allowing sufficient time interval between measurements so that the subsequent readings were not influenced by the reader's memory (Salar-Martinez et al., 2004). Later, the reliability and variability of AGD measurements were determined by evaluating the Pearson correlation of coefficient (r) using CORR procedure and by evaluating coefficient of variation **(CV)** using Excel 2016 (Microsoft Corporation, Redmond, WA) an approach similar to that of Salar-Martinez et al. (2004). The correlation between the three AGD measurements was high (r = 0.98) and the CV was low 1.1 %, allowing AGD to be determined only once by the same examiner in the rest of the animals. In addition, in the same subset of 93 cows from the university research herd, the association between AGD and plasma concentrations of testosterone was evaluated by linear regression analysis using REG procedure of SAS.

The descriptive statistics such as minimum, maximum, mean, and standard deviation (amount of dispersion indicative of variability) for AGD were determined using MEANS

procedure of SAS for all cows (n =921) and separately for cows from 1st, 2nd, and 3rd + parity groups (n = 360, 256 and 305, respectively). The differences in mean AGD between 1st, 2nd and 3rd + parity cows were tested using GLIMMIX procedure of SAS, where AGD was modeled against parity and the effect of herd was treated as random. The associations among age, height and AGD was assessed by linear regression analysis using REG procedure of SAS.

The relationship between P/AI (binomial outcome) and AGD (predictor continuous variable) was first evaluated for each parity by logistic regression analysis using LOGISTIC procedure of SAS, and the estimated probabilities of P/AI were plotted against AGD using Excel 2016. As the logistic regression model was non-significant for 3rd + parity, the rest of the analyses only focused on 1st and 2nd parity cows.

The optimum threshold AGD that predicted the probability of P/AI, including specificity and sensitivity, was determined using receiver operating characteristic **(ROC)** curve analysis separately for 1st and 2nd parity cows. The ROC curves analyze sensitivity and 1 − specificity. Sensitivity is the proportion of cows above the threshold that was diagnosed as pregnant to 1stAI, and specificity is the proportion of cows below the threshold and diagnosed as not pregnant to 1stAI. The threshold AGD was chosen based on the highest Youden's J statistic index. The significance of the threshold AGD was determined based on the area under the curve **(AUC)**, where the AUC ranged from 0.50 to 1.00, with AUC of 0.50 considered non-informative and the AUC of 1.00 considered perfect as previously described (Swets, 1988).

Thereafter, cows were categorized as either SHORT- or LONG-AGD (≤ or > threshold) based on the threshold AGD. The associations among categories of AGD (SHORT- and LONG-AGD), herds (A, B, C and D), type of AI (IDE and TAI), 305-d ME milk yield (Low; ≤ average and High; > average) and P/AI were analyzed using the GLIMMIX procedure of SAS separately

for 1st and 2nd parity cows, while the model specifications included a binomial distribution and logit function, and an option to retrieve odds ratios. The P/AI was initially modelled against all of the aforementioned categorical variables and their interactions. As none of the interactions was significant, the final model only had the categorical variables modelled against P/AI.

The differences in intervals from calving to pregnancy between categories of AGD up to 250 DIM were evaluated using the Kaplan-Meier Survival analysis (LIFETEST procedure), separately for 1st and 2nd parity cows, and the results from Kaplan-Meier survival analysis were confirmed by a Cox proportional hazard model (PHREG procedure). Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

7.4. Results and discussion

Ano-genital distance has been identified as a marker of prenatal androgenization (Macleod et al., 2010; Dean at al., 2012) and associated with postnatal reproductive outcomes in female rats (Zehr et al., 2001), rabbits (Banszegi et al., 2012) and humans (Mendiola et al., 2012; Mira-Escolano et al., 2014a; Wu et al., 2017). To our knowledge, this is the first report to characterize the distribution and variation in AGD and evaluate its association with reproductive outcomes in lactating dairy cows. In the current study, AGD was normally distributed (Figure 1b) and highly variable (mean [± SD]; 131.0 ± 12.2 mm). The patterns of distributions and ranges reported (Table 1) remained approximately the same within parities even though the mean AGD differed ($P < 0.01$) between 1st, 2nd and 3rd + parity cows (126.9, 132.5 and 134.5 mm). The distribution and variability of AGD found in dairy cows was comparable to the pattern of distribution and variability reported for AGD in women (Mendiola et al., 2012). In this regard, Mendiola et al. (2012) showed that AGD was normally distributed in a population of young

women with a mean (± SD) of 80.4 ± 10.5 mm. The normal distribution and the degree of dispersion for AGD in dairy cows and in women indicate that large phenotypic variation in AGD exists in different species.

The current study determined the phenotypic variation in AGD that was attributable to postnatal factors such as cow age and height. The overall correlations between age and AGD (R^2 = 0.09; P < 0.01; Figure 2a) and between height and AGD (R^2 = 0.04; P < 0.01; Figure 2b) were very weak when evaluated across all cows, and remained weak when height and AGD were evaluated separately for 1st, 2nd and 3rd + parity cows (mean R^2 = 0.02; P < 0.10). In this regard, either weak or non-significant associations were reported between AGD and other anthropometric measures such as length and body weight in female infants (Thankamony et al., 2009) and age, height and weight in young women (Mendiola et al., 2012; Mira-Escolano et al., 2014b; Wu et al., 2017). While Thankamony et al. (2009) reported weak associations between AGD and length (R^2= 0.09), and between AGD and body weight (R^2= 0.03) in female infants at 24 months of age, others (Mendiola et al., 2012; Mira-Escolano et al., 2014b; Wu et al., 2017) have shown that age, height and weight were non-significant factors associated with AGD in women. However, Mira-Escolano et al. (2014b) and Wu et al. (2017) reported a positive association between body mass index and AGD. Collectively, the findings in human studies and the current results in dairy cows suggest that AGD measures are largely independent of postnatal factors, and perhaps primarily influenced by the prenatal in-utero concentrations of androgens as shown in rodents (Wolf et al., 2002; Hotchkiss et al., 2007; Dean et al., 2012).

Ano-genital distance and P/AI had a significant negative relationship in 1st and 2nd parity cows (Figure 3a and b, respectively); however, AGD was not associated with P/AI in older cows (P = 0.30). In confinement dairy management systems such as those in Canada, the average

longevity of a dairy cow is 2.5 lactations (about 5 years of age), and only cows that excel in both fertility and milk production are likely to remain in the herd longer, which would at least partially explain the absence of association reported between AGD and fertility in 3rd + parity cows. Interestingly, the variation in AGD observed in 3rd + parity cows was quite comparable to the variations in AGD for 1st and 2nd parity cows. Hence, the lack of association between AGD and P/AI in 3rd + parity cows was not attributable to a more homogenous AGD, but could be due to other potential factors that affect pregnancy establishment to first AI such as incidence of early postpartum diseases, negative energy balance, and higher milk production that are more common in older cows (Lee and Kim., 2006). Since the optimal AGD threshold to predict the probability of P/AI was 127.1 mm for both 1st and 2nd parity cows, only 1st and 2nd parity cows were categorized into SHORT- or LONG-AGD (≤ or > 127.1 mm) groups and the associations with reproductive outcomes evaluated. The sensitivity and specificity of the optimal AGD threshold that predicted P/AI were moderate in both 1st and 2nd parity cows (Figure 4a and b, respectively). We compared AGD groups separately for 1st and 2nd parity cows because, in general, fertility declines as parity increases (Norman et al., 2009). Therefore, analyzing parity groups separately would provide an insight into the true association between AGD and fertility within each parity.

Overall reproductive performance (P/AI and pregnancy by 250 DIM) was poorer in cows with LONG-AGD than those with SHORT-AGD (Table 2 and Figure 5). The results of the present study were broadly comparable to the outcomes reported for different AGD categories and fecundity/fertility in other species (Zehr et al., 2001; Banszegi et al., 2012; Mendiola et al., 2012; Mira-Escolano et al., 2014a; Wu et al., 2017). Specifically, the onset of puberty was delayed in female mice with long AGD (Zehr et al., 2001). Rabbit does with long AGD had smaller, lighter and male-biased litters (Banszegi et al., 2012). Women with longer AGD had

increased follicular recruitment and higher testosterone concentrations (Mendiola et al.,2012; Mira-Escolano et al., 2014a) during the early follicular phase, and were 18 times more prone for polycystic ovarian syndrome (Wu et al., 2017) than those with shorter AGD. Likewise, Steckler et al. (2005) showed that pregnant ewes treated twice weekly with testosterone propionate from 30 to 90 days of pregnancy had increased ovarian follicular recruitment in fetal ovaries. The authors (Steckler et al., 2005) suggested that this observation was due to activation of large number of primordial follicles into primary follicles by increasing the androgen receptor expression in primordial follicles as reported previously (Vendola et al., 1999).

We evaluated the association between AGD and testosterone in a subset of 93 cows, using an approach similar to that used by Mira-Escolano et al. (2014a) in women to determine whether cows with long AGD, presumably exposed to high androgens during fetal life, also had higher testosterone concentrations during postnatal life. In the current study, the association between AGD and testosterone was weak and non-significant ($R^2 = 0.02$; $P = 0.19$), perhaps due to the relatively small sample size. In this regard, Mira-Escolano et al. (2014a) reported that in women, testosterone concentration increased by 0.006 ng/mL for each mm increase in AGD. Interestingly, a recent study (Mossa et al., 2010) that evaluated antral follicle counts (AFC) in dairy cattle have shown that cows with high AFC (≥ 25) had almost double the concentrations of circulating testosterone (~ 60 vs. 30 pg/mL) throughout the estrous cycle than cows with low AFC (≤ 15). In addition, dairy heifers with high AFC had poorer fertility and reduced longevity (Jimenez-Krassel et al., 2017). It is plausible that exposure of the female fetus to high levels of maternal androgens results in alterations in the development of female reproductive tissues including the external manifestation of long AGD, and these animals subsequently have increased follicular recruitment and androgen concentrations during estrous cycles that consequently lead to poor fertility

outcomes and reduced longevity in dairy cattle. The proposed associations between maternal concentrations of androgens during the first trimester of pregnancy (under natural [endogenous] and experimental [exogenous] scenarios) and AGD at birth, puberty and adulthood, and subsequent associations with fertility outcomes in dairy cows warrant further in depth investigations. The heritability of AGD and whether AGD could be used as a reproductive phenotype in genetic selection of dairy cattle to improve fertility also remain to be determined.

In conclusion, the present study has demonstrated for the first time that AGD is normally distributed and has high variability in lactating dairy cows. The variation identified for AGD was weakly associated with postnatal factors such as cow age and height. Furthermore, our results indicate an inverse relationship between AGD and pregnancy to the first AI and cumulative pregnancy by 250 DIM in 1st and 2nd parity cows.

7.5. Acknowledgments

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Table 7. 1. Descriptive statistics for ano-genital distance (AGD) in Canadian Holsteins

	Minimum (mm)	Maximum (mm)	Mean [± [1]SD] (mm)
Overall [2]AGD, all parities (n = 921)	96.0	170.0	131.0 ± 12.2
AGD in 1st parity cows (n = 360)	96.0	169.0	126.9 ± 11.9
AGD in 2nd parity cows (n = 256)	100.0	170.0	132.5 ± 11.7
AGD in 3rd+ parity cows (n = 305)	103.0	164.0	134.5 ± 11.4

[1] SD: standard deviation

[2]AGD: ano-genital distance: is the distance from the center of the anus to the base of the clitoris

Table 7. 2. Associations among ano-genital distance (AGD) categories, herd, type of AI, 305-d mature-equivalent (ME) milk yield and pregnancy to first AI in first and second parity Canadian Holsteins

Variable	Pregnancy to 1st AI, %	Odds ratio estimates	95 % confidence interval	*P*-value
First parity cows				
[1]AGD category (mean ± SEM)				
[2]LONG-AGD (135.6 ± 1.5 mm)	30.9	0.41	(0.22 - 0.74)	<0.01
[2]SHORT-AGD(117.5 ± 1.5 mm)	53.6	Reference		
Herd				
A	47.0	.	.	0.81
B	51.0	.	.	
C	45.9	.	.	
D	35.4	Reference		
[3]Type of AI				
IDE	45.9	0.87	(0.50 - 1.51)	0.61
TAI	50.0	Reference		
[4]305-d ME milk yield				
High (> 10,693 kg)	35.3	0.37	(0.14 - 0.96)	0.04
Low (≤ 10,693 kg)	49.3	Reference		
Second parity cows				
[1]AGD category (mean ± SEM)				
[2]LONG-AGD (138.3 ± 1.3 mm)	28.3	0.33	(0.15 - 0.72)	<0.01
[2]SHORT-AGD(119.8 ± 1.4 mm)	44.4	Reference		
Herd				
A	31.0	.	.	0.24
B	38.0	.	.	
C	38.3	.	.	
D	30.8	Reference		
[3]Type of AI				
IDE	38.3	1.08	(0.53 - 2.20)	0.82
TAI	34.4	Reference		
[4]305-d ME milk yield				
High (> 11,827 kg)	28.1	0.21	(0.27 - 1.45)	0.27
Low (≤ 11,827 kg)	38.8	Reference		

[1]Ano-genital distance: the distance from the center of the anus to the base of the clitoris

[2]AGD categories: cows that had their AGD ≤ or > the threshold AGD (127.1 mm) that predicted probability of pregnancy for the first insemination in 1st and 2nd parity cows were designated into either SHORT- or LONG-AGD categories

[3]Type of AI; Cows were inseminated at detected estrus (IDE) or timed-AI (TAI) following synchronization of ovulation

[4]305-d ME milk yield categories were based on the average 305-d ME milk yield determined for 1st (10,693 kg) and 2nd (11,827 kg) parity cows

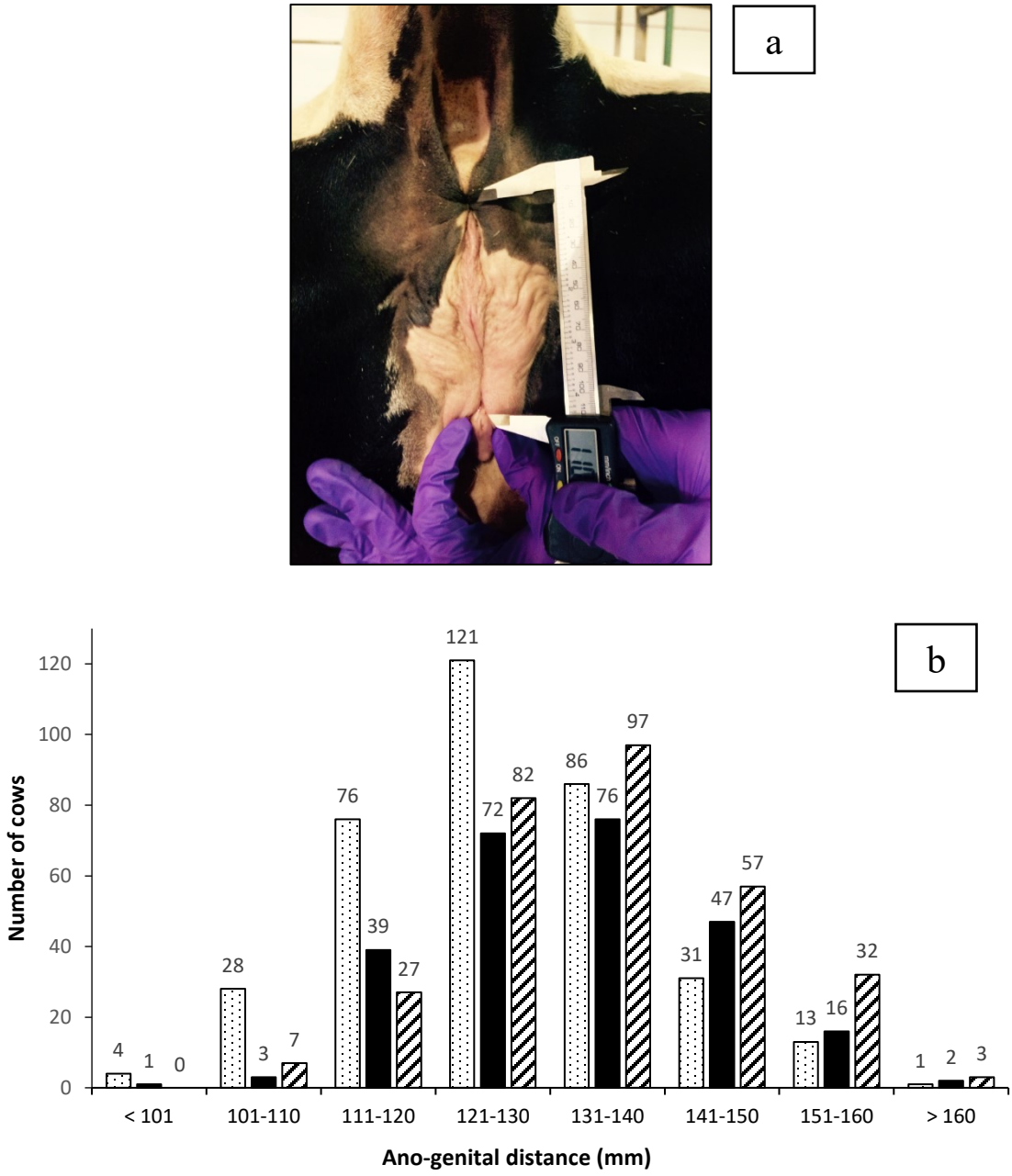

Figure 7. 1. Positioning of digital calipers to measure ano-genital distance (a): the distance from the center of the anus to the base of the clitoris. The distribution of ano-genital distance (b) in 1st (dotted bars; n = 360), 2nd (filled bars; n = 256) and 3rd +(hatched bars; n = 305) parity cows.

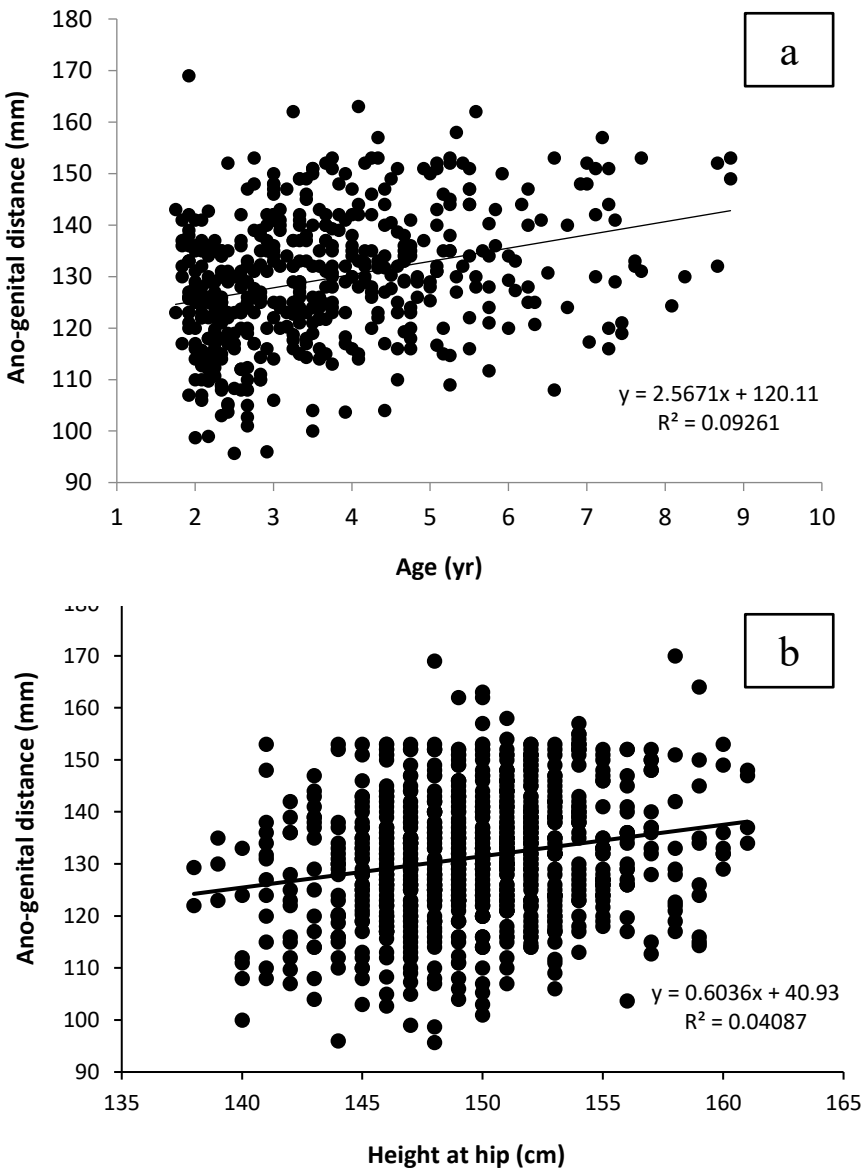

Figure 7. 2. Association between age and ano-genital distance (a; R2 = 0.09; P < 0.01) and height at hip and ano-genital distance (b; R2 = 0.04; P < 0.01) in lactating dairy cows (n = 921).

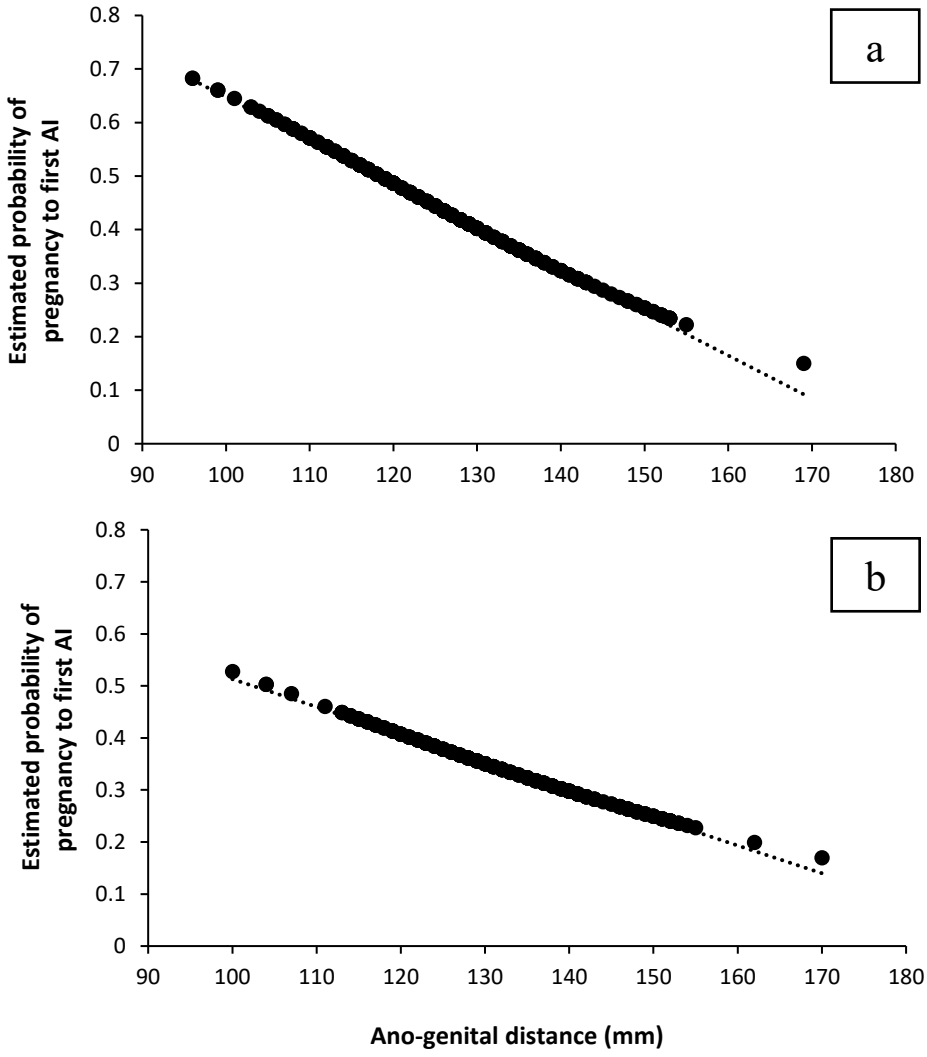

Figure 7. 3. The estimated probability of pregnancy to first AI (P/AI) plotted against ano-genital distance (AGD) in 1st and 2nd parity cows (a; n =360 and b; n = 256). For every one-unit (mm) increase in AGD, the odds of conceiving to first AI (P/AI) decreased by 3.4 and 2.4 % for 1st and 2nd parity cows, respectively ($P < 0.05$).

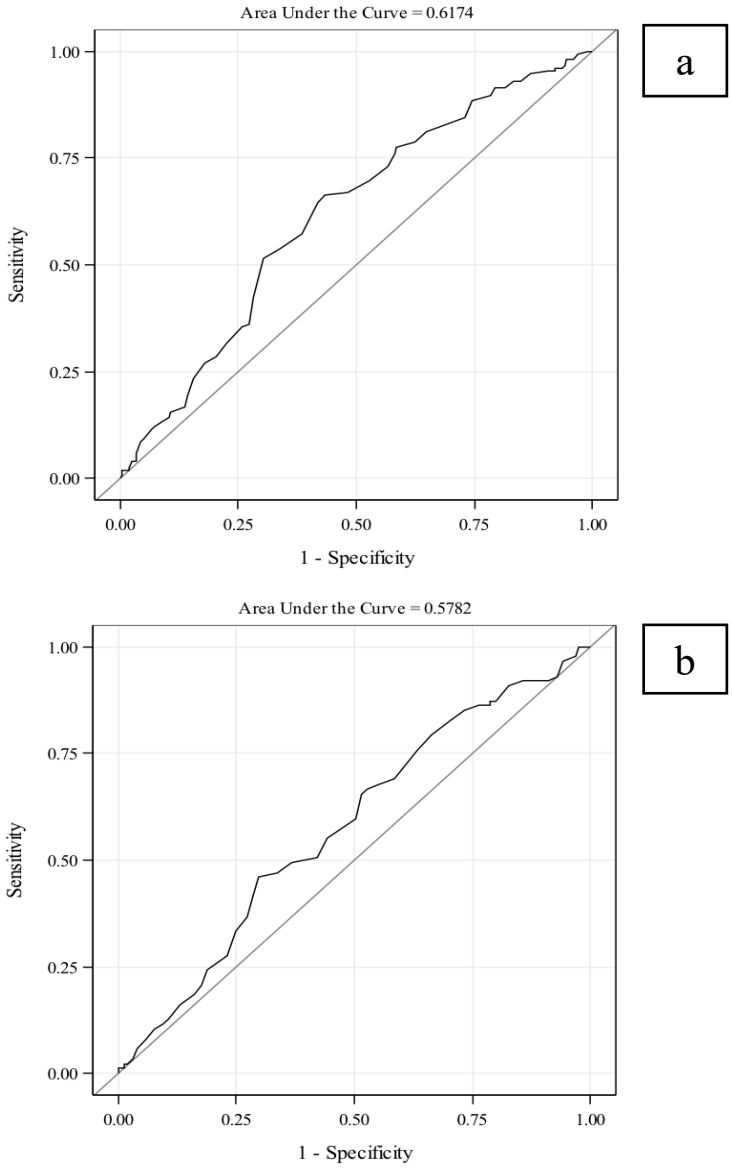

Figure 7. 4. The receiver operating characteristic (ROC) curve analysis for ano-genital distance (AGD) that predicted probability of pregnancy to the first AI (P/AI) in 1st (a; n = 360; area under the curve: 0.62; Sensitivity: 66.4 and Specificity: 56.6 %; P < 0.01) and 2nd parity cows (b; n = 256; area under the curve: 0.58; Sensitivity: 46.0 and Specificity: 70.4 %; P = 0.04), respectively.

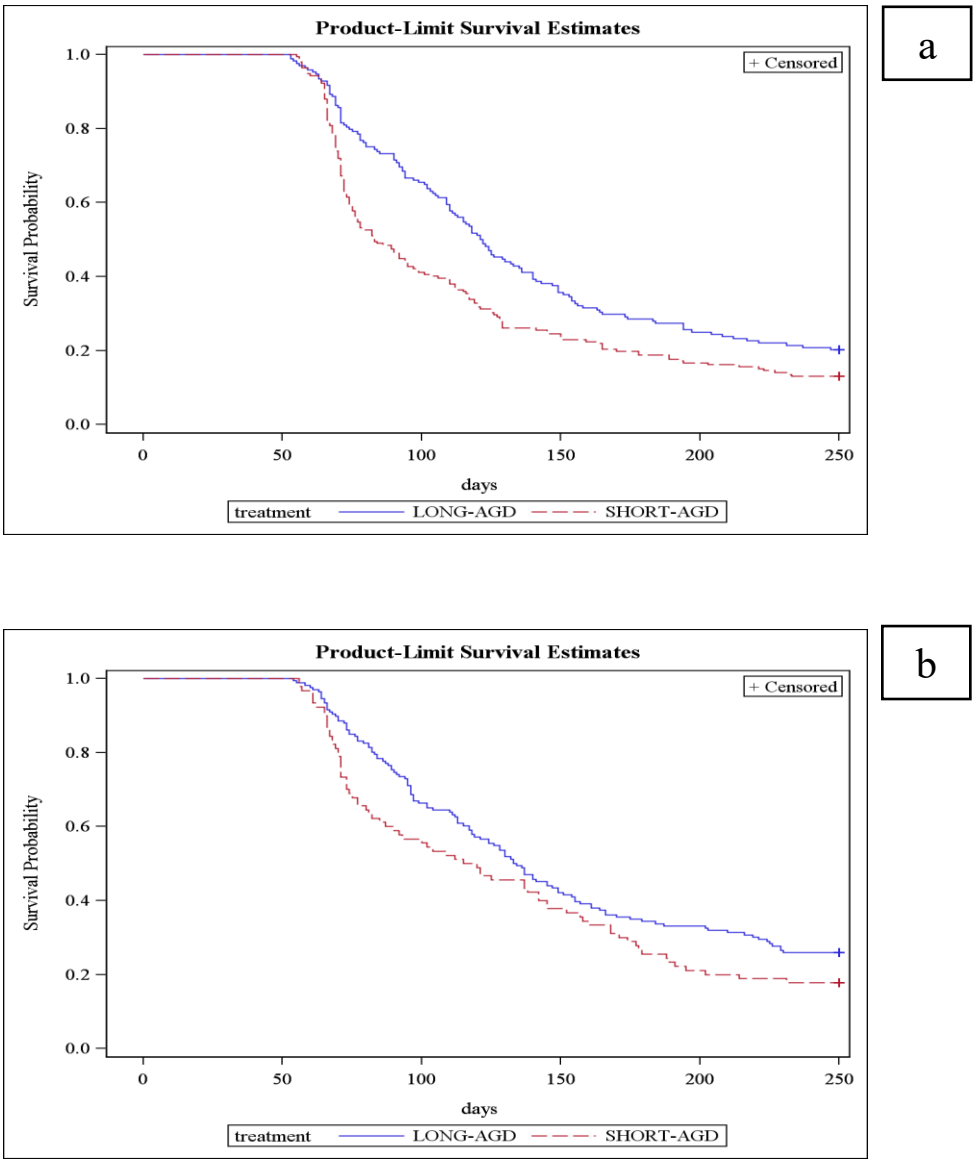

Figure 7. 5. Kaplan-Meier survival curve that illustrates the proportion of cows (by AGD category) that remained non-pregnant by 250 DIM in 1st (panel a) and 2nd parity cows (panel b). The 1st parity cows of LONG-AGD (135.6 ± 1.5 mm; n =168) had significantly lower significantly lower likelihood of pregnancy by 250 DIM (hazard ratio: 0.68; $P < 0.01$) than cows of SHORT-AGD (117.5 ± 1.5 mm; n = 192). Similarly, the likelihood of pregnancy by 250 DIM tended to be lower (hazard ratio: 0.67; $P = 0.06$) for LONG-AGD cows (138.3 ± 1.3 mm; n = 166) than those of SHORT-AGD (119.8 ± 1.4 mm; n = 90) in the 2nd parity.

Chapter 8. The relationship between ano-genital distance (AGD) and fertility, and genome-wide associations for AGD in Irish Holstein-Friesian cows

8.1. Abstract

The evaluation of ano-genital distance (AGD; the distance from the centre of the anus to base of the clitoris) in dairy cows as a potential fertility trait for genetic selection is of recent interest. The objectives of this cross-sectional observational study were to (1) characterize the distribution and variability of AGD, (2) determine factors associated with AGD, (3) estimate heritability for AGD, (4) identify single nucleotide polymorphisms (SNP) associated with phenotypic variation of AGD, and (5) validate the relationship between categories of AGD and fertility in Irish Holstein-Friesian cows. Ano-genital distance was measured using digital calipers in 1180 Holstein cows (mean ± standard deviation: 171 ± 93 d in milk) from 10 dairy herds located in Munster, Ireland. In addition, age (yr), weight (kg), height at hip (cm) and body condition score (BCS) at the time of AGD measurement were determined in a subset of 281 cows. Genotype information available from 908 cows was subsequently imputed to the Illumina Bovine High Density Bead Chip for genome wide association analysis for phenotypic variation in AGD.

Overall, AGD had a normal distribution and high variability (mean [± standard deviation]; 119.2 ± 11.6 mm). Ano-genital distance was weakly but positively associated with cow age, height and body weight, and negatively associated with BCS; the phenotypic variation in AGD that was explainable by these variables was small (coefficient of determination; R^2 = 0.09, 0.06, 0.10 and 0.02, respectively). The estimated heritability for AGD was 0.37 (standard error of mean ± 0.08). Six SNP of suggestive significance ($P<1x10^{-5}$) were identified on *Bos taurus* autosomes 6, 15, 20 and 26; however, none of these SNP was related to previously identified candidate genes for

fertility. Cows were categorized into quartiles (Q1; 86 to 111 mm; n = 295, Q2; 111 to 120 mm; n = 295; Q3; 120 to 127 mm; n = 295, and Q4; 127 to 160 mm; n = 295) based on AGD and the association with reproductive outcomes examined (21 d submission rate, pregnancy to first AI, pregnancy rate within 21, 42 and 84 d after the farm mating start date, and number of times bred). None of the reproductive parameters evaluated significantly differed between AGD categories. In summary, despite identification of high variability and moderate heritability for AGD in Irish Holstein-Friesian cows, the reproductive outcomes did not differ between categories of AGD. This latter result differs from our previous finding of an inverse relationship between AGD and pregnancy outcomes in first and second parity Canadian Holstein cows, emphasizing the need to test and validate this new phenotype in diverse cow populations.

Key words: fertility traits, heritability, reproductive efficiency, genomic selection

8.2. Background

Anogenital distance **(AGD)** has been defined as the distance from the center of the anus to either the posterior fourchette (Salazar-Martinez et al., 2004) or the clitoris (Sathyanarayana et al., 2010) in women and base of the clitoris in dairy cows (Gobikrushanth et al., 2017c). Prenatal exposure of female fetuses to excess androgen leads to androgenization of the reproductive system in-utero (Langman, 1975; Bowman et al., 2003), which results in longer AGD and poor postnatal fertility outcomes in mice (Zehr et al., 2001), rabbits (Banszegi et al., 2012), gilts (Drickamer et al., 1997), and women (Mendiola et al., 2012; Mira-Escolano et al., 2014a; Wu et al., 2017).

A reproductive phenotype that has high variability, repeatability, heritability and strong associations with fertility would be an ideal candidate for genetic selection to improve reproductive performance in dairy cows. Recently, we reported for the first time that AGD is normally

distributed, highly variable, minimally influenced by postnatal factors such as age and height, and inversely related to pregnancy to first and subsequent insemination events in first and second parity Canadian Holsteins cows (Gobikrushanth et al., 2017c). In this regard, first and second parity Canadian Holstein cows with long AGD (>127.1 mm) had lower conception rate to first AI (first parity; 30.9 vs. 53.6% and second parity; 28.3 vs. 44.4%) and decreased likelihood of pregnancy by 250 d in milk (hazard ratio of 0.68 for first parity and 0.76 for second parity) compared to cows with short AGD (≤127.1 mm). These results are intriguing and if an association between the simple morphologic measure of AGD and reproductive performance can be validated in diverse populations of dairy cows, AGD could become a novel fertility trait for use in future genetic selection programs in dairy cows.

In Ireland, cows have been predominantly selected for improved fertility over the last two decades. For example, the Irish national breeding program first introduced a multi-trait selection index called the Economic Breeding Index **(EBI)** in 2001, placing 30% (currently at 35%) weightage for fertility, which includes calving interval and survival (Irish Cattle Breeding Federation, 2017). In contrast, in Canada, daughter fertility sub index was introduced to the Lifetime Profit Index **(LPI)** in 2007, placing only 12% weightage for fertility, which includes daughter fertility and herd life (currently at 21.4%; Canadian Dairy Network, 2018). This indicates that a relatively more fertile population of cows might be present in Ireland compared with Canada. Indeed, fertility in general, is greater for Holstein-Friesian cows managed in pasture-based seasonal calving system such as those in Ireland (Dillon et al., 2006) and New Zealand (Macdonald at al., 2008) than those managed under confinement system in the UK (Pryce et al., 2004) and the USA (Norman et al., 2009). In addition, heritability of AGD, and genome wide association studies

(**GWAS**) identifying single nucleotide polymorphisms (**SNP**) associated with phenotypic variation in AGD are novel aspects yet to be explored in dairy cows.

We hypothesized that seasonal-calving pasture-based cows in Ireland with short AGD have greater fertility outcomes than cows with long AGD, as observed in Canadian Holstein cows (Gobikrushanth et al., 2017c), and SNP are associated with phenotypic variation in AGD. Therefore, the objectives of this observational study were to (1) characterize the distribution and variability of AGD, (2) determine the factors associated with AGD, (3) estimate heritability for AGD, (4) identify SNP associated with phenotypic variation of AGD, and (5) validate the association between categories of AGD and fertility in Irish Holsteins.

8.3. Materials and methods

8.3.1. Animals and management

A cross-sectional observational study was conducted on a convenience sample of ten dairy herds located in the province of Munster in Ireland. Herds were operating pasture-based seasonal calving systems that ranged in size from 41 to 274 milking cows. The study population included a total of 1180 Holstein Friesian cows (308, 306 and 566 first, second, and third + parity cows, respectively). All cows calved during the spring calving season of 2016. All experimental procedures involving cows were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority, which is the competent authority in Ireland responsible for the implementation of European Union legislation (Directive 2010/63/EU) for the protection of animals used for scientific purposes.

8.3.2. Determination of AGD, age, height, body weight, and BCS

Ano-genital distance was defined as the distance from the center of the anus to the base of the clitoris, and was measured using a stainless steel digital caliper (Silverlinec, Group Silverline Limited, Yeovil, Somerset, United Kingdom). The age (yr), height (cm), body weight (kg) and body condition score **(BCS)** were determined on the day of AGD determination in a subset of 281 cows. The age of the cow at the time of AGD measurement was determined from birth records. The height at the hip (hereafter referred to as "height") was determined using a livestock measuring stick from the ground to the top of the cow's back (above the tuber coxae). Body weight was measured using an electronic farm scale. The BCS was determined by the same trained person on a 1 to 5 scale system measured in increments of 0.25 units (1 = thin, 5 = fat) as described (Edmonson et al., 1989). Ano-genital distance measurements were obtained from 1180 cows (mean ± SD: 171 ± 93 DIM) that had no apparent perineal abnormalities such as inflamed or lacerated vulva as indicators of trauma at parturition, and that were later than two wk post-calving at the time of AGD determination.

8.3.3. Definition for interval from calving to mating start date categories and reproductive parameters

All cows calved during the spring calving season of 2016 and mating by AI commenced on a fixed calendar date on each farm between 11th of April and 3rd of May 2016. Detection of estrus was conducted using the standard reproductive management protocols within each farm, which typically involves periods of cow observation aided by use of tail paint, and the total duration of the breeding season was 12 wk. Pregnancy diagnosis using transrectal ultrasound was conducted five to seven weeks after the end of the breeding period, and conception date for pregnant cows was identified using a combination of the ultrasound results and breeding records.

Cows were grouped into three categories based on the interval from calving to mating start date **(MSD)** as early-calving (≥88 d; n = 387), mid-calving (71 to 87 d; n = 402), and late-calving (≤70 d; n = 391), respectively.

The proportion of cows that received an AI within the first 21 d after the farm MSD was defined as 21-d submission rate. The proportion of cows pregnant to first AI was defined as pregnancy to first AI **(P/AI)**. The 21-, 42-, and 84-d pregnancy rate were defined as the proportion of cows pregnant within the first 21, 42 or 84 d of the MSD. Total number of inseminations received during the entire breeding season was defined as "times bred".

8.3.4. Genotypes, quality control and imputation

Of the 1180 animals with AGD information, 907 animals also had genotype information available. These animals had been genotyped on a variety of panels including the Illumina bovine 3K genotype panel (n=6; SNP = 2,900), Illumina Low Density panel (n=156; SNP = 6,909) or the custom genotyping panel International Dairy and Beef **(IDB)** version 1 (n=73; SNP = 17,137), version 2 (n=578; SNP = 18,004 or version 3 (n=94; SNP = 53,450). All animals had a call rate ≥ 90% and only autosomal SNP, SNP with a known chromosome and position and SNP with a call rate ≥ 90% were retained. To increase the density of the SNP panel for GWAS, imputation to the Illumina Bovine High Density **(HD)** beadchip was performed using FImpute2 (Sargolzaei et al., 2014). Imputation was completed using a two-step approach, whereby all animals were first imputed to the Illumina BovineSNP50 chip using a reference population of 3,532 Holstein-Friesian BovineSNP50 genotyped animals, and subsequently imputed to HD density using a multi-breed population of 5,504 HD genotyped as the reference population. After imputation, each individual had 648,572 autosomal SNP with a minor allele frequency >0.002 available for analysis.

8.3.5. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The descriptive statistics such as minimum, maximum, mean, and standard deviation (amount of dispersion indicative of variability) for AGD were determined using MEANS procedure of SAS for all cows (n =1180) and separately for cows from first, second, and third + parity groups (n = 308, 306 and 566, respectively).

The differences in mean AGD between first, second and third + parity cows were tested using GLIMMIX procedure of SAS, where AGD was modeled against parity and the effect of herd was treated as random. The associations among cow age, height, weight, BCS and AGD was assessed by coefficient of determination **(R^2)** using REG procedure of SAS.

Initially, the linear association between AGD (continuous independent variable) and P/AI (binomial dependent variable) was tested by the logistic regression analysis using LOGISTIC procedure of SAS. As no significant association was found using this approach, cows were categorized into quartiles based on AGD (Q1; 86 to 111 mm; n = 295, Q2; 111 to 120 mm; n = 295; Q3; 120 to 127 mm; n = 295, and Q4; 127 to 160 mm; n = 295) and the association with reproductive outcomes (21 d submission rate, P/AI, pregnancy rate within 21, 42, 84 d after MSD, and times bred) were analyzed using the GLIMMIX procedure of SAS. The interval from calving to MSD (early-calving; ≥88 d, mid-calving; 71 to 87 d, and late-calving; ≤70 d, respectively) and parity (first, second and third + parity) were considered as fixed effects along with categories of AGD, while herd was considered a random effect. The model specifications included a binary distribution and logit function, and an option to retrieve odds ratios. The reproductive outcomes were initially modelled against categories of AGD, interval from calving to MSD, parity and their interactions. As none of the interactions was significant, the final model only had the categorical

variables modelled against each reproductive outcome while the effect of herd was treated as random variable. Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $P > 0.05$ and ≤ 0.10.

8.3.6. Heritability and Genome-wide association study for AGD

Variance component of AGD was estimated by restricted maximum likelihood in ASREML (Gilmour et al., 2009) using a univariate animal model. Whole genome association analysis was performed in genome-wide complex trait analysis (Yang et al., 2011) using a mixed linear model based association based on the leave-one-chromosome-out method (Yang et al., 2014). The following model was used: $y = \mu + bx + g^{-} + e$, where y is the AGD measure, μ is the overall mean, b is a vector of fixed effects including parity coded as first, second and third+ parity, herd id, and the additive effect of the candidate SNP tested for association, x is the incidence matrix for the parameters b, g^{-} is the accumulated polygenic effect of all SNP except those on the chromosome where the candidate SNP is located and e is the residual. False discovery rate (FDR) control was performed using the Benjamini-Hochberg method using a FDR of 0.05. Gene search was completed using Ensembl (http://ensembl.org/) and NCBI map viewer (http://www.ncbi.nlm.nih.gov/mapview/) on the UMD 3.1 genome build. The relative roles of nearest candidate genes were searched using Bovine Genome Database (http://bovinegenome.org/).

8.4. Results and discussion

Overall, AGD had a normal distribution and high variability in Irish Holstein-Friesian cows managed under pasture-based seasonal calving systems (mean [± standard deviation]; 119.2 ± 11.6 mm; Table 1 and Figure 1). The pattern of distribution and variability reported herein was

comparable to that of our previous finding (Gobikrushanth et al., 2017c) in Canadian Holstein cows (mean [± SD] of 131.0 ± 12.0 mm) except that the overall mean AGD was 12 mm shorter in Irish Holstein-Friesian cows compared to Canadian Holsteins. A large phenotypic variation for AGD (mean [±SD] of 80.4 ± 10.5 mm) also has been reported in women (Mendiola et al., 2012). The normal distribution and the high variability reported for AGD in dairy cows and women indicate that large phenotypic variation exists for AGD among different species. There could be numerous factors that contribute to the variation of AGD in these species; therefore, we intended to identify potential postnatal factors associated with phenotypic variation of AGD in Irish Holstein-Friesian cows.

In the present study, AGD was positively associated with cow age, height and body weight, and negatively associated with BCS (Figure 2). However, the phenotypic variation in AGD explainable by these variables was small ($r^2 = 0.09$, 0.06, 0.10 and 0.02, respectively). In agreement with the current findings, our previous study (Gobikrushanth et al., 2017c) has shown that phenotypic variation in AGD was also weakly associated with age ($r^2 = 0.09$) and height ($r^2 = 0.04$) in Canadian Holstein cows. Similarly, either weak or non-significant associations were reported between AGD and length and body weight in female infants (Thankamony et al., 2009) and age, height and weight in young women (Mendiola et al., 2012; Mira-Escolano et al., 2014b; Wu et al., 2017). These observations indicate that the phenotypic variation in AGD is largely independent of postnatal factors, not only in women but also in dairy cows, and likely influenced by prenatal in-utero concentrations of androgens, as reported in rodents (Wolf et al., 2002; Hotchkiss et al., 2007; Dean et al., 2012). It is also possible that genotypic differences among individuals affect phenotypic AGD. Thus, we aimed to estimate heritability and identify genetic markers (SNP) associated with phenotypic variation in AGD for Irish Holstein-Friesian cows in the current study.

The heritability estimate of 0.37 (standard error of mean ± 0.08) reported for AGD in the current study is comparatively higher than what has been reported for most of the traditional female fertility traits (0.02 to 0.04; Berry et al., 2014) and closer to the heritability estimates recently reported for other reproductive phenotypes such as antral follicle count (0.31 by Walsh et al., 2014) and circulating anti-Müllerian hormone concentration (0.36 and 0.46 by Nawaz et al., 2017 and Gobikrushanth et al., 2018a, respectively) in dairy cows. Although, these novel reproductive phenotypes have relatively higher heritability estimates than traditional fertility traits used in the dairy industry, their positive relationship with fertility has been inconsistent. Identification of potential SNP associated with phenotypic variance of any trait may have the potential to increase the genomic prediction accuracy of the trait if eventually incorporated into SNP selection panels. However, in the current study, no SNP remained significantly associated with phenotypic variation in AGD after adjustment for multiple testing. Six SNP of suggestive significance ($P<0.0001$) were identified across four *Bos taurus* autosomes (BTA; 6, 15, 20 and 26; Figure 3). Genomic regions associated with phenotypic variation in AGD and putative candidate genes within 250 kb up and downstream of the strongest association are listed in Table 2. However, none of these candidate genes is known to be related to fertility in dairy cows.

In our previous study (Gobikrushanth et al., 2017c), P/AI and pregnancy up to 250 DIM were lower for cows with long AGD compared to those with short AGD in both first and second parity cows. Several studies have documented that females with long AGD had poorer fecundity/fertility outcomes than those with short AGD in gilts (Drickamer et al., 1997), rodents (Zehr et al., 2001), rabbits (Banszegi et al., 2012) and women (Mendiola et al., 2012; Mira-Escolano et al., 2014a; Wu et al., 2017). Drickmer et al. (1997) reported that the AGD of newborn gilts was significantly larger in litters that had a greater proportion of male piglets. In a second

experiment from the above study (Drickmer et al., 1997), using 13 yr of data on breeding and litter composition, the authors reported that the majority of gilts from male-biased litters failed to become pregnant at the first breeding, suggesting a negative relationship between AGD and fertility in gilts. Furthermore, the onset of puberty was delayed in female mice with long AGD (Zehr et al., 2001) and rabbit does with long AGD had smaller, lighter and more male-biased litters (Banszegi et al., 2012). Based on these results in litter bearing species, females fetuses exposed to a high proportion of male fetuses have longer AGD and subsequently impaired fertility in adulthood. Recent studies reported that women with longer AGD had increased follicular recruitment and greater testosterone concentrations (Mendiola et al.,2012; Mira-Escolano et al., 2014a) during the early follicular phase, and were at a much higher risk of developing polycystic ovarian syndrome (Wu et al., 2017) than those with shorter AGD. All these results lend support to our previous finding that longer AGD in Canadian Holstein cows is associated with poor reproductive performance (Gobikrushanth et al., 2017c). In the present study, however, none of the reproductive parameters evaluated significantly differed between AGD categories in Irish Holstein-Friesian cows (Table 3). Hence, the current results in Irish Holstein-Friesian cows do not support our previous finding of an inverse relationship between AGD and fertility in Canadian Holsteins.

The inverse relationship between AGD and fertility in Canadian Holsteins was only evident in first and second parity cows, not in third + parity cows, despite the latter having a phenotypic variation similar to that of first and second parity cows (Gobikrushanth et al., 2017c). An optimum AGD of 127 mm was identified as predictive of P/AI using the receiver operating characteristic curve analysis for both first and second parity cows with moderate sensitivity and specificity. Thus, we previously concluded that this finding was likely because only cows that excel in both fertility

and milk production would typically remain in the herd beyond two lactations, thereby leaving a relatively fertile pool of older (third + parity) cows, within which the association between AGD and fertility was less evident. Extending that notion to the present study, the absence of an inverse relationship between AGD and fertility in Irish Holstein-Friesian cows is probably attributable to the strong emphasis placed on selecting for fertility traits in Ireland during the last two decades, and aggressive culling of sub-fertile cows, resulting in a relatively more fertile population of cows in Ireland compared to Canada. For example, overall P/AI was 55% in Irish Holstein-Friesian cows in the current study compared with 37% reported in Canadian Holstein cows in our previous study (Gobikrushanth et al., 2017c). Of note, as mentioned previously, mean AGD was 12 mm shorter in Irish Holstein-Friesian cows compared to Canadian Holsteins (119 vs. 131 mm, respectively) despite similar pattern of distribution and variation for AGD between these two populations of cows, indicating a relatively larger population of cows with short AGD in Ireland compared to Canada. In addition, the differences in the overall reproductive management system between these two countries could also be a contributing factor. For example, in our previous study, Canadian Holstein cows were managed under confinement system with timed-AI as the predominant breeding method, whereas in the current study Irish Holstein-Friesian cows were managed under pasture based seasonal-calving system with AI at detected estrus as the predominant method of breeding, which may have further improved P/AI (Tenhagen et al., 2004; Thangavelu et al., 2015).

Despite no relationship observed between categories of AGD and fertility, in the current study, late-calving cows had poorer reproductive outcomes compared to mid- or early-calving cows, categorized based on the interval from calving to MSD (Table 4). Cows that have longer interval between calving and MSD (e.g. mid- or early-calving cows) would have adequate time for uterine involution, clearance of postpartum uterine infections, resumption of ovarian cyclicity and

have a greater number of estrous cycles prior to the first insemination compared to cows that have shorter interval between calving and MSD (e.g. late-calving cows), which predispose to greater fertility in dairy cows (Thatcher and Wilcox, 1973; Galvao et al., 2004; Sheldon and Dobson, 2004; Santos et al., 2009).

In conclusion, the present study failed to demonstrate an inverse relationship between AGD and fertility in Irish Holstein-Friesian cows managed under pasture-based seasonal calving systems despite high variability for AGD in that population. The moderate heritability reported for AGD is promising and will remain important if an association between AGD and fertility could be established in other dairy cattle populations. Thus, the association between AGD and fertility warrants further investigation, especially in the less-fertile North American dairy cattle, to corroborate our previous findings in Canadian Holstein cows.

8.5. Acknowledgments

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Table 8. 1. Descriptive statistics for ano-genital distance (AGD) in Irish Holstein-Friesian cows

	Minimum (mm)	Maximum (mm)	Mean [± [1]SD] (mm)
Overall [2]AGD, all parities (n = 1180)	86.0	160.0	119.2 ± 11.6
AGD in first parity cows (n = 308)	86.0	145.0	115.7 ± 10.8
AGD in second parity cows (n = 306)	89.0	154.0	117.6 ± 10.9
AGD in third + parity cows (n = 566)	86.0	160.0	122.0 ± 11.6

[1] SD: standard deviation

[2]AGD: ano-genital distance: is the distance from the center of the anus to the base of the clitoris

Table 8. 2. Genomic regions of suggestive association with ano-genital distance in Irish Holstein-Friesian cows. The strongest association within each region is identified as well as the nearest putative candidate genes

[1]BTA	Position ([2]Mb)	[3]Number of SNP/s	Lead SNP	Lead SNP ([4]bp)	[5]P	Minor allele frequency	Genes within 250kb of the strongest association
6	32.427-32.427	1	rs133726266	32427292	$9.79x10^{-6}$	0.48	ENSBTAG00000046413 ENSBTAG00000046473
15	40.947-41.007	3	rs137211667	41007334	$8.67x10^{-6}$	0.09	*PARVA,* ENSBTAG00000047691, ENSBTAG00000008193, MICAL2
20	25.028-25.028	1	rs132798046	25028988	$1.46x10^{-6}$	0.47	ARL15
26	2.383-2.383	1	rs133491966	2383663	$6.14X10^{-6}$	0.12	-

[1]BTA: *Bos taurus* autosome
[2]Mb: Mega base
[3]Number of SNP/s: Number of single nucleotide polymorphisms of suggestive significance ($p<1x10^{-5}$) within the region
[4]bp: Base pairs
[5]P: Unadjusted p-value of the most strongly associated SNP within the region

Table 8. 3. Associations among AGD categories and reproductive outcomes (least square means; LSM) in Irish Holstein-Friesian cows

	[1]Categories of AGD				*P*-Value
	Q1 (range, 86 to 111 mm; n = 295)	Q2 (range, 111 to 120 mm; n = 295)	Q3 (range, 120 to 127 mm; n = 295)	Q4 (range, 127 to 165 mm; n = 295)	
[2]21-d Submission rate (%)	92.3	92.3	92.3	92.0	0.99
[3]Pregnancy to first AI (%)	53.8	52.9	53.2	54.1	0.99
[4]21-d Pregnancy rate (%)	55.3	52.7	52.7	54.0	0.92
[5]42-d Pregnancy rate (%)	76.1	76.1	75.2	79.7	0.61
[6]84-d Pregnancy rate (%)	91.6	92.1	91.9	95.2	0.32
[7]Times bred	1.65	1.65	1.67	1.64	0.97

[1]Cows were categorized into quartiles based on AGD.
[2]21-d submission rate was constructed by coding cows with an insemination record within the first 21 d from mating start date as 1, and cows with no insemination record within the first 21 d coded as 0.
[3]Pregnancy to first AI was coded as 1 if a cow received only one insemination and was diagnosed as pregnant by the end of the breeding season.
[4,5,6]The 21-d**,** 42-d and 84-d pregnancy rate were coded as 1 if cows became pregnant within 21, 42 and 84 d from the mating start date and as 0 if the animal was diagnosed as non-pregnant.
[7]Times bred: total number of inseminations per cow during the 12 wk breeding season.

Table 8. 4. Associations among interval from calving to mating start date categories and reproductive outcomes (least square means; LSM) in Irish Holstein-Friesian cows

	[1]Interval from calving to mating start date categories			*P* - Value
	Early-calving (≥88 d; n = 387)	Mid-calving (71 to 87 d; n = 402)	Late-calving (≤70 d; n = 391)	
[2]21-d Submission rate (%)	88.4[a]	96.5[b]	88.9[a]	< 0.01
[3]Pregnancy to first AI (%)	59.4[a]	56.2[a]	44.7[b]	< 0.01
[4]21-d Pregnancy rate (%)	57.7[a]	60.2[a]	42.9[b]	< 0.01
[5]42-d Pregnancy rate (%)	78.6[a,x]	80.0[a]	71.2[b,y]	< 0.01
[6]84-d Pregnancy rate (%)	92.8	93.4	92.2	0.80
[7]Times bred	1.57[a]	1.60[a]	1.79[b]	< 0.01

Different superscripts within the same category differ (a,b; $P < 0.05$) or tended to differ (x,y; $P > 0.05$ and ≤ 0.10).

[1]Cows categorized into early-calving, mid-calving, and late-calving based on the interval from calving to mating start date.
[2]21-d submission rate was constructed by coding cows with an insemination record within the first 21 d from mating start date as 1, and cows with no insemination record within the first 21 d coded as 0.
[3]Pregnancy to first AI was coded as 1 if a cow received only one service and was diagnosed as pregnant by the end of the breeding season.
[4,5,6]The 21-d**,** 42-d and 84-d pregnancy rate were coded as 1 if cows became pregnant within 21, 42 and 84 d from the mating start date and as 0 if the animal was diagnosed as non-pregnant.
[7]Times bred: total number of inseminations per cow during the 12 wk breeding season.

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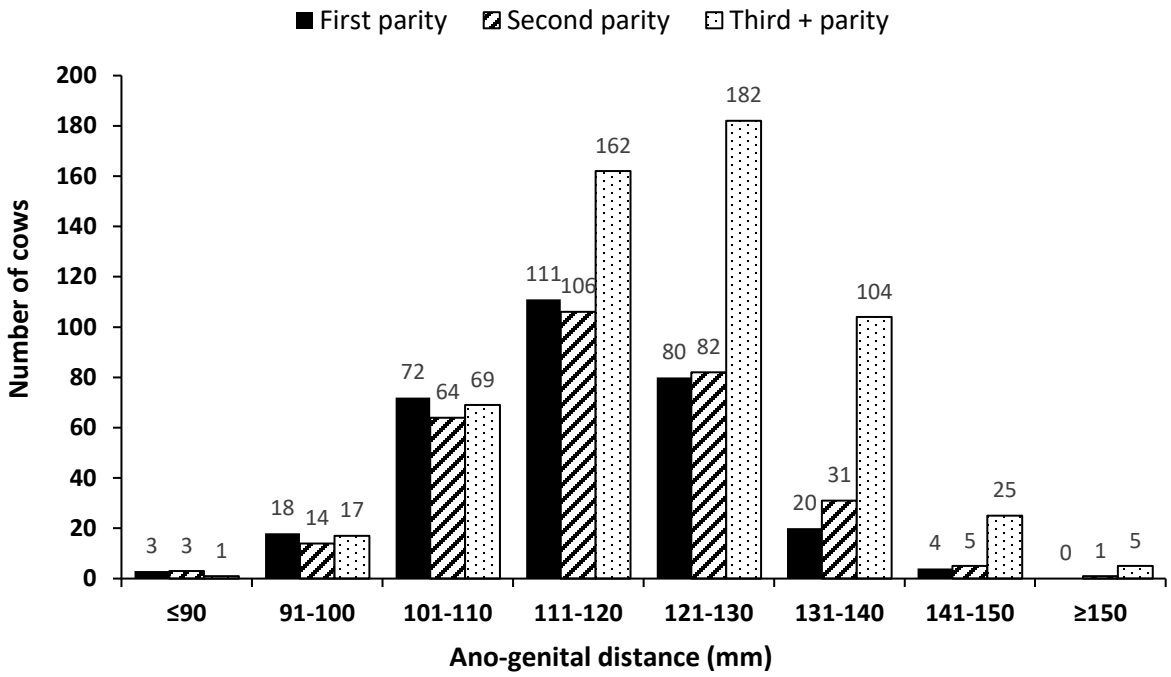

Figure 8. 1. The distribution of ano-genital distance in first (filled bars; n = 308), second (hatched bars; n = 306) and third + (dotted bars; n = 566) parity Irish Holstein-Friesian cows.

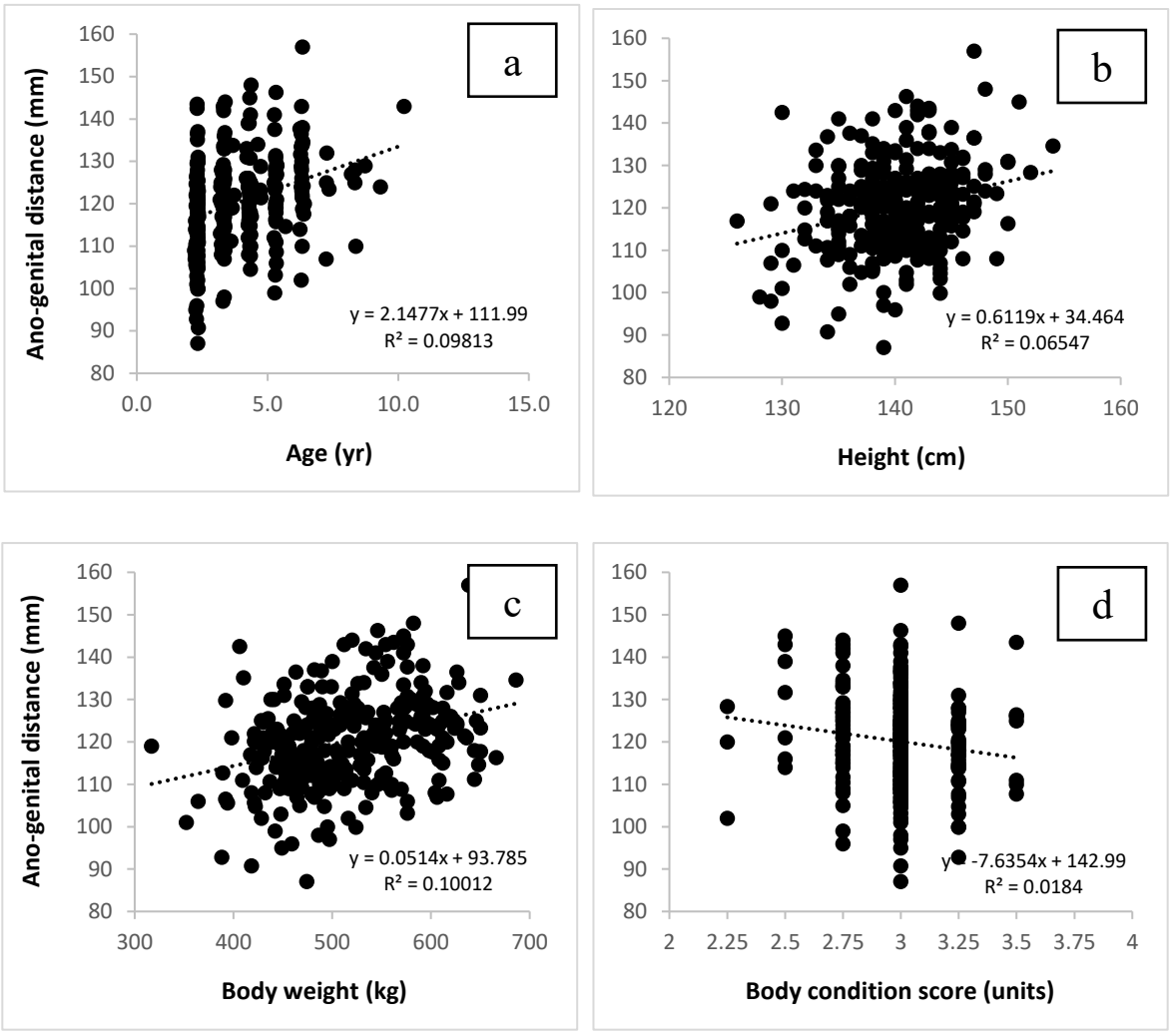

Figure 8. 2. Association between ano-genital distance (AGD), age, height at hip, body weight and body condition score (BCS) in a subset 281 Irish Holstein-Friesian cows (a; $r^2 = 0.09$; $P < 0.01$, b; $r^2 = 0.06$; $P < 0.01$, c; $r^2 = 0.10$; $P < 0.01$, d; $r^2 = 0.02$; $P < 0.01$, respectively).

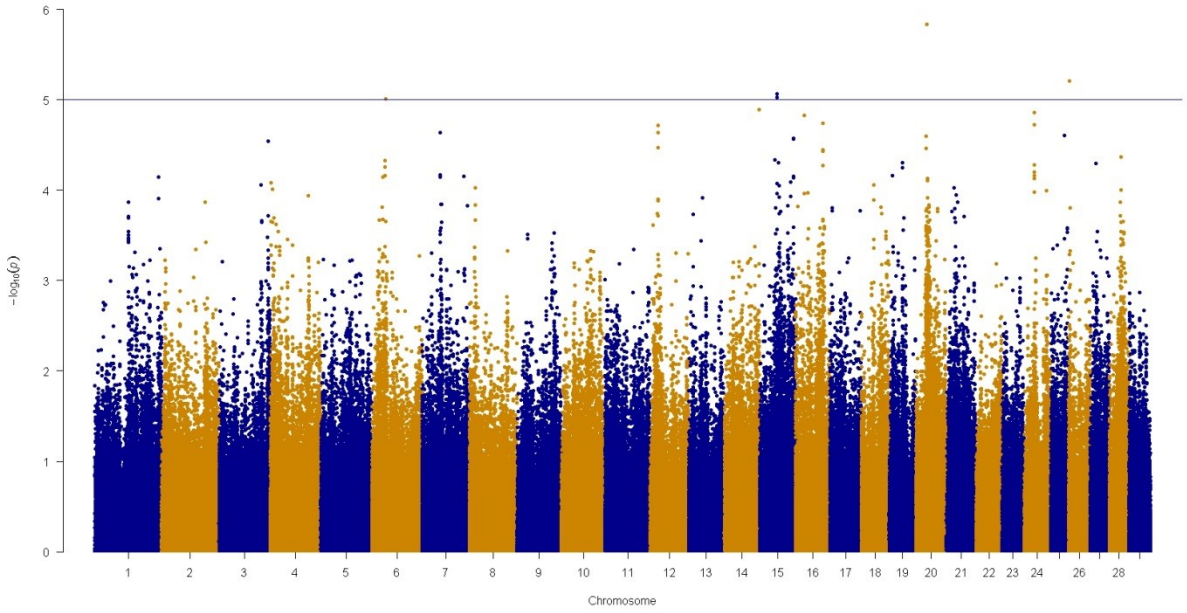

Figure 8. 3. Manhattan plot of the genome-wide association study for ano-genital distance in 908 Irish Holstein-Friesian cows. The blue horizontal line represents all single nucleotide polymorphisms with a suggestive significance (P-value <0.00001) across four Bos taurus autosomes (BTA: 6, 15, 20 and 26) before adjustment for false discovery rate of $P < 0.05$.

Chapter 9. General discussion and future directions

A reproductive phenotype that has high variability, repeatability, heritability, and association with fertility could become an excellent fertility trait for genetic selection to enhance fertility in dairy cows. Therefore, we intended to evaluate the aforementioned characteristics for five novel reproductive phenotypes in dairy cows, namely, gonadotropin releasing hormone (GnRH)-induced luteinizing hormone (LH) response, antral follicle count (AFC), anti-Müllerian hormone (AMH), insulin-like growth factor-1 (IGF-1), and ano-genital distance (AGD). In addition, we determined genetic markers (single nucleotide polymorphisms; SNP) associated with three of the aforementioned reproductive phenotypes (AMH, IGF-1, and AGD) using genome-wide association studies (GWAS).

9.1. Key findings

In the first study, we showed that GnRH-induced LH responses were normally distributed, highly variable and had positive relationship with other reproductive hormones like circulating FSH and E2 in dairy cows. However, the repeatability for this reproductive phenotype was weak and not significant. Although, in our second study, we found that both AFC and AMH were repeatable between an unknown stage of follicular growth (14 d postpartum) and the expected day of follicular wave emergence (approximately 10 h after induced ovulation), the repeatability was significantly greater for AMH than for AFC. In addition, AMH and AFC were moderately correlated. In the third study, serum AMH determined during the first week postpartum was positively skewed, highly variable, and moderately heritable, but it was not associated with fertility. However, for the first time, numerous (n=670) genetic markers associated with

phenotypic variation of AMH were identified in dairy cows. Interestingly, some of these genetic markers were linked to candidate genes of fertility as previously reported in rodents. In the fourth study, serum IGF-1 determined during the first week postpartum had positively skewed distribution, high variability and positive associations with postpartum ovarian cyclicity in multiparous cows and pregnancy to first AI in both primiparous and multiparous cows. However, the diagnostic values were moderate in predicting pregnancy outcomes to first AI. As in the case of AMH, we identified several (n=37) genetic makers that were associated with phenotypic variation of IGF-1 in dairy cows, some of which have been linked to candidate genes of fertility in previous reports. For the first time, we characterized AGD in dairy cows and found that it was normally distributed, highly variable, moderately heritable, and minimally influenced by postnatal factors.-While, AGD was inversely related with fertility in Canadian Holstein cows of first and second parity, this relationship was not evident in older (third + parity) Canadian Holsteins. This was likely because only cows that excel in both fertility and milk production would typically remain in the herd beyond two lactations, leaving a relatively fertile pool of older (third + parity) cows in Canadian Holstein herds. The inverse relationship between AGD and fertility found in Canadian Holsteins, however, was not found in Irish Holstein-Friesian cows of any parity. Irish Holstein- Friesian cows are known to be more fertile than North American Holstein cows due to the strong emphasis placed on selecting for fertility traits in Ireland in the past two decades combined with an aggressive strategy of culling sub-fertile cows, within which the inverse association between AGD and fertility was less evident.

9.2. General discussion, limitations, and future directions

The use of GnRH-induced LH response as a fertility trait for genetic selection remains questionable due to its poor repeatability despite having normal distribution, high variability and positive associations with other reproductive hormones such as FSH and E2. Circulating AMH would be a more reliable phenotype than AFC to test associations with reproductive outcomes due to its greater repeatability between unknown and known stages of follicular growth. The use of serum AMH as a fertility trait remains questionable despite having greater phenotypic variation, moderate heritability and numerous genetic markers linked to previously identified candidate genes of fertility. Nevertheless, circulating AMH has been suggested as a useful predictor of superovulation responses in dairy cows (Rico et al., 2012; Rozner and Verstegen., 2012; Souza et al., 2015). Therefore, genetic markers that were in linkage disequilibrium with *AMH* gene on chromosome 7 could be of value in identifying donors from within a pool of elite cows, that have higher potential for yielding a greater number of good quality transferable embryos. The early postpartum circulating IGF-1 was identified as an important factor necessary for postpartum cyclicity and establishment of pregnancy to first AI in dairy cows. However, due to its moderate sensitivity, circulating IGF-1 concentration cannot be used as a biomarker to predict pregnancy outcomes to first AI to strategically delay the breeding of cows with lower circulating IGF-1. Yet, genetic markers identified to be associated with phenotypic variation of IGF-1 could be incorporated into SNP panels to increase selection accuracy of cows having high IGF-1 if IGF-1 is to be considered as a fertility trait in future. Ano-genital distance had almost all of the preferred characteristics for a novel fertility trait including high variation, moderate heritability, and negative association with fertility, at least in first and second parity Canadian

Holsteins. The absence of association noted between AGD and fertility in older parity (third +) Canadian Holsteins and Irish Holstein-Friesian cows is interesting given that these are relatively more fertile animals. Nevertheless, the association between AGD and fertility warrants further investigation in the relatively less fertile North American cows.

Luteinizing hormone plays an important role in reproductive function. Therefore, selecting cows with greater capacity for LH secretion could be a strategy to improve fertility in dairy cows. The possible influence of negative energy balance (NEB) and high P4 concentration were controlled or avoided by evaluating GnRH-induced LH responses twice within a short interval (8 and 10 wk postpartum) and by eliminating cows that had circulating P4 concentration $\geq$ 0.5 ng/mL at GnRH of presynchronization and Ovsynch in our first study. However, it has been previously reported that dairy cows might take up to 20 wk postpartum to regain positive energy status (Taylor et al., 2003). Therefore, in order to determine the true potential of anterior pituitary gland in the production and release of LH, future experiments could be designed using pre-pubertal heifers or cows beyond 20 wk postpartum. Albeit not statistically significant, we have shown that cows with high LH responses had 12.5 percentage units greater ovulation rate compared to cows with low LH responses following GnRH treatment. Thus, the association between GnRH-induced LH responses and fertility warrants further investigation. If GnRH-induced LH responses are adequately repeatable and strongly associated with fertility, then the next steps are to determine (1) heritability, (2) genetic and phenotypic correlations with other traits of interest (i.e., production), and (3) genetic markers associated with its phenotypic variation through GWAS. Of note, a moderate heritability of 0.44 has been previously reported for GnRH-induced LH responses in ewes (Haley et al., 1989), and GWAS identifying genetic markers

associated with reproductive hormones in human subjects have been reported more recently (Chen et al., 2013; Ruth et al., 2016).

The use of AFC and circulating AMH as potential fertility traits is of recent interest to many researchers. Therefore, it is important to identify when these phenotypes should be measured relative to postpartum and/or ovarian cyclicity stages. Given this, in the second study, we determined AFC and AMH once during early postpartum where cows are at their unknown stage of follicular growth and again during timed-AI protocol which is 10 h after induced ovulation when cows are approximately at their follicular wave emergence. The first measurements for AFC and AMH were taken during the second week postpartum when cows are known to be in a state of negative energy balance (NEB). Therefore, this sampling time point could be considered inappropriate given potential negative influence of NEB on postpartum follicular growth and thereby production of AMH. The rise of FSH and emergence of first postpartum follicular wave occur within 3 to 5 d postpartum in dairy cows regardless of state of energy balance (Crowe, 2008). Indeed, earlier studies from Savio et al., (1990) and Beam and Butler (1997) reported that the first dominant follicle was selected between 10 and 12 d postpartum in all dairy cows irrespective of dietary deficiencies and periparturient diseases. In addition, Monniaux et al. (2012) have shown that circulating concentration of AMH were consistent through 8, 18, 28, 38, and 48 d postpartum despite dramatic changes in NEB. Therefore, determination of AFC and AMH at early postpartum period in our study should not have been affected by the state of NEB. The moderate-to-high correlation reported between AFC and AMH in the present study and in previous studies (Ireland et al., 2008; Rico et al., 2009) indicates that either of the two phenotypes (AFC or AMH) could be used interchangeably in future studies. Nevertheless, while the determination of AFC is inexpensive and rapid, it needs

the expertise of a trained individual to perform transrectal ultrasonography. Quantifying circulating AMH, on the other hand, is relatively expensive and takes more time, albeit easily measured from a single blood sample.

Due to the ease of determining AMH from a single blood sample and its greater repeatability, circulating AMH concentration was determined during first week postpartum and associated with fertility outcomes in the third study. Nevertheless, an optimum circulating AMH threshold predictive of pregnancy to first AI could not be established and categories of serum AMH had associations with none of the reproductive outcomes studied. The potential limitations of our study are the use of a relatively small sample size (n = 460) and the use of cows that were subjected to either timed AI or insemination at detected estrus. A posteriori power analysis based on an actual difference of 6% in pregnancy to first AI between low and high AMH category cows called for ~ 2000 cows in total to attain statistical significance. Though not significant, the difference of 6% in pregnancy to first AI increased to 8% when only cows inseminated at detected estrus were considered in the analysis. Therefore, future studies could focus on evaluating the association between circulating AMH and fertility in dairy cows subjected to insemination at detected estrus.

The fourth study determined circulating concentration of IGF-1during the first week postpartum in dairy cows, and we have shown that the variation in circulating IGF-1 was influenced by differences among herds, age, parity, body condition score and season of sampling. It is important to note that the differences among cows in their dry matter intake (DMI) and energy status (i.e. NEB) during the first week postpartum could have been some other factors influencing phenotypic variation of IGF-1, and a potential limitation of our study. Moreover, heritability for circulating IGF-1 was not estimated in this study due to extremely skewed

distribution for serum IGF-1 because nearly a third of the cows had IGF-1 concentration below the detection limit of the assay. Therefore, more sensitive assays should be used in future studies involving measurement of circulating IGF-1 in dairy cows.

The last two studies were designed to test the potential of using AGD as a novel fertility trait in dairy cows. Despite having almost all of the preferred characteristics of an ideal fertility trait, the association between AGD and fertility was inconsistent based on two studies conducted in Canada and Ireland. For example, the negative relationship observed between AGD and fertility in first and second parity Canadian Holsteins was not evident in older parity (third +) Canadian Holsteins as well as in Irish Holstein-Friesian cows. It is noteworthy that Irish Holstein-Friesians are inherently more fertile than Canadian Holsteins and this might have masked the actual relationship between AGD and fertility in Irish Holstein-Friesian cows. Nevertheless, the association between AGD and fertility warrants further investigation, especially using a large population of relatively less fertile North American Holsteins before ruling out the potential of using AGD as a fertility trait for genetic selection in future. If AGD is proven to be associated with fertility, then the next steps are to (1) unravel the underlying physiological mechanisms that lead to poor fertility in cows with longer AGD, and (2) determine the phenotypic and genetic correlations with production and health related traits that are currently used in our national selection indices.

9.3. Final thoughts

Vigilant and systematic implementation of genetic selection over the past decades has contributed greatly to the vast improvements seen in milk production potential of the modern dairy cow. While selection based on production-oriented traits has received much attention, little

emphasis has been placed on genetic selection for fertility traits, until recently. Even so, the current focus is on conventional fertility traits, which rely on interval data collected from the field. However, these conventional fertility traits are highly influenced by environmental factors and have low heritability, slowing genetic progress. On the other hand, novel reproductive phenotypes that are closely related to the reproductive physiology of dairy cows such as commencement of luteal activity, estrus-related phenotypes, and hormone concentrations may be less influenced by the environment and have higher heritability, contributing to more rapid genetic progress, but are often difficult and expensive to quantify in a larger population. Whilst, commencement of luteal activity (Berry et al., 2012) and duration and intensity of estrus (Ismael et al., 2015) have already been evaluated as potential fertility traits, the knowledge on the use of reproductive hormones as fertility traits for genomic selection is uncommon. In this regard, this dissertation is an attempt to characterize novel reproductive phenotypes of physiological relevance in dairy cows, resulting in the creation of new knowledge and several original contributions that provide a deeper insight into five important novel reproductive phenotypes related to reproductive physiology of the Holstein cow.

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