Repeatability of anogenital distance in Holstein cattle and its association with embryo yield and quality

by

Iswarya Rajesh

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Reproductive phenotypes that are easy to measure, have high genetic variation and heritability along with good repeatability are being explored for genetic selection of cows for improved fertility. In this regard, anogenital distance (AGD; the distance from the center of the anus to the base of the clitoris) is a promising new reproductive phenotype. In Holstein cows, AGD was inversely associated with the measures of fertility, had high genetic variation and moderate heritability. The repeatability of AGD and the mechanism underlying its association with greater fertility in short-AGD cattle are not unknown. Therefore, we explored this novel reproductive phenotype for its repeatability through different physiological phases. Furthermore, to understand the mechanism behind the inverse association between AGD and fertility we hypothesized that embryo yield and quality will be greater in cattle with short AGD than in those with long AGD.

The first study was designed to test the repeatability of AGD at different ages (from 0 to 15 mo) and different physiological states (phases of the estrous cycle, gestation and lactation). We hypothesized that AGD at birth can be used to predict adult AGD and that AGD measurements will be highly correlated at different ages and physiological states. Contrary to our expectation, AGD at birth was not correlated with the adult AGD, but AGD measured at 6 mo of age was correlated with adult AGD. The AGD measurements at the different phases of the estrous cycle, gestation (except at 270 d of gestation), and lactation were highly repeatable within an individual.

The second study determined the differences in yield and quality of embryos in cows and heifers with short and long AGD using the superovulated model. We also posited that antral follicular count, anti-Müllerian hormone, and superovulation response will be positively associated. Although the mean number of preovulatory follicles and corpora lutea (indicators of superovulation response) or embryo yield did not differ, the proportion of preovulatory follicles

was greater, and transferable quality embryos tended to be greater in cows with short AGD than in cows with long AGD. Similarly, the proportions of fertilized eggs out of total structures recovered, and transferable quality embryos out of total structures recovered were greater in short-AGD cows than in long-AGD cows. In heifers, the proportions of total structures recovered (embryos/ova), fertilized eggs, transferable and Grade-1 (best quality) embryos were greater in cows with short AGD than in cows with long AGD. However, AGD was not associated with antral follicular count and anti-Müllerian hormone.

In conclusion, AGD measured before 6 mo of age did not reflect adult AGD, although AGD was highly repeatable at most physiological phases measured during adult life except during late gestation (270 d). No AGD-related differences in embryo yield and quality were found in heifers but the proportions of fertilized eggs and transferable embryos out of total recovered were greater in short-AGD cows than in long-AGD cows. Findings from Chapter 3 imply that AGD is measurable with high reliability at any physiological state. Findings from Chapter 4 imply that the improved fertility in short-AGD cows reported by others may be due to proportionately greater fertilization rate and embryo viability in short-AGD cows than in long-AGD cows. The latter findings must be validated in a larger population.

PREFACE

This thesis is an original work by Iswarya Rajesh. The research project titled "Anogenital distance – a promising new fertility trait in dairy cattle as a fertility trait", which, this thesis is a part of, received research ethics approval from the Animal Care and Use Committee (ACUC) for Livestock at the University of Alberta (AUP # 00002883).

Dr. Divakar J. Ambrose of the Department of Agricultural, Food and Nutritional Science, University of Alberta and the Livestock Research and Extension Branch, Alberta Agriculture and Forestry conceived the original ideas, developed the research proposal, and supervised the research. Dr. Masahito Oba co-supervised the research, administered the research budget and offered feedback on research progress.

For Chapter 3, I was responsible for performing data collection, data management, statistical analyses, results and interpretation and composing it as a chapter in the thesis. M. Gobikrushanth assisted in sample collection and statistical advice. J. Carrelli assisted in data collection. D.J. Ambrose supervised the study, conceived the study objectives and experimental design, assisted with sample collection and analyses and contributed to the writing and editing of this chapter.

For Chapter 4, I was responsible for animal monitoring, performing sample collection, implementing superovulation protocols, assist with embryo collection, data collection and management, statistical analyses, results interpretation, and composing it as a chapter in the thesis. M.G. Colazo was a co-investigator who assisted with the methodology of embryo collection. M. Gobikrushanth assisted in sample collection and statistical advice. J. Carrelli assisted in sample collection. D.J. Ambrose supervised the study, conceived the study objectives, assisted with

sample collection, interpretation of results, and contributed to the writing and editing of this chapter.

Dedications

To my mother, Mrs. Vijayalakshmi for all her trust, love, sacrifice and encouragement, my aunt, Mrs. Sathya and my friend Miss. Sangavi for their support and motivation

ACKNOWLEDGMENTS

I thank the Almighty for enlightening my soul and allowing me to enter the field of my desire with his blessings.

It is my pride and privilege to thank my professor, Dr. Divakar Ambrose, for accepting me as his student to pursue my post-graduate studies at the University of Alberta. I appreciate his continuous support and valuable guidance as it inculcated the spirit of self-reliance to work independently. His scholarly advice, meticulous scrutiny with patience helped me to run on the track throughout my Master's program. I greatly admire him for his passion, knowledge, patience, enthusiasm and dedication. His care and encouragement were worth more than I express in the paper. I could not have imagined a better advisor for enlightening me with my first research experience. I am glad to be his student and get to work under supervision and for which am highly indebted to him.

I greatly thank my co-supervisor and committee member Dr. Masahito Oba for his timely suggestions whenever in need throughout my research. His valuable comments, inquisitive questions, made me widen my knowledge of research. I owe a special thanks to Dr. Changxi Li for his time and for accepting to be my arm's length examiner. I greatly acknowledge the support of Dr. Marcos Colazo for taking the time to help and for his valuable suggestions in the embryo component of my thesis. His enthusiasm during embryo flushing was truly inspiring.

I am especially thankful to Dr. Mohanathas Gobikrushanth, who deserves a great appreciation for all his support in completing the research work and for his statistical advice. I thank my fellow grad student, Jennifer Carrelli, farm manager, Courtney Felton and other staff at the barn for their assistance in research whenever in need. A very big thanks to Lauren Engelking,

Satoko Hisadomi, and Matthew Burdick for their support, cooperation, invaluable assistance, last-minute favors and experiences which were essential to carry out my thesis especially in field works. I particularly appreciate them for their friendly nature, emotional support and pleasurable moments that made me feel home away from home.

I acknowledge WestGen Endowment Fund, Alberta Agriculture and Forestry, and Alberta Milk for their financial support. To my well-wishers and mentors, Dr. Shanthipoosan Sivaraman, Dr. R. C. Raja Sundaram, and Dr. C. Veerapandian, I am greatly indebted and a single word of thanks will not satisfy their contribution in various capacities in my life and to successfully start my research career. Their guidance and moral support will be cherished forever. Their constant encouragement both professionally and personally made me stand where I am today.

I owe a special thanks to my best friend, Sangavi Chokkalingam, without whom it would have been a hard journey to come this far. I appreciate her for her never-ending support in every aspect and I am glad for her being in my life from the start of my career in Veterinary Medicine till now. Words fall short to explain her love, support and sacrifice which constantly motivated me through my hard times.

This journey would not have been possible without the sacrifice, moral, emotional and financial support of my family members, my mother, Mrs. S. Vijayalakshmi, and her sister, Mrs. S. Sathiyavathi, who took care of me and supported me from childhood as her own. The sacrifices my mother made for me cannot be expressed in words. I am grateful for the opportunities and unselfish love she rendered to date to reach this stage in my life.

Table of Contents

List of tables

List of figures

List of abbreviations

ACUC	Animal Care and Use Committee
AFC	Antral Follicular Count
AGD	Anogenital Distance
AI	Artificial Insemination
AMH	Anti Müllerian Hormone
ANOVA	Analyses of Variance
BCS	Body Condition Scoring
bST	bovine Somatotropin
BTA	*Bos taurus* Autosome
CIDR	Controlled Intravaginal Drug Release
CL	Corpus Luteum
CV	Coefficients of Variation
DF	Daughter Fertility
DIM	Days in Milk
d	Day/Days
DO	Days Open
DP	Days Pregnant
DPR	Daughter Pregnancy Rate
DRTC	Dairy Research and Technology Center
FSH	Follicle-Stimulating Hormone
GDF	Growth Differentiation Factor

GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
GWAS	Genome-Wide Association Studies
IGF-1	Insulin-like Growth Factor
Kitlg	Kit-ligand
LH	Luteinizing Hormone
mo	Month/months
NEB	Negative Energy Balance
PGF2α	Prostaglandin F_2 alpha
PTA	Predicted Transmitting Abilities
QTL	Quantitative Trait Loci
r	Pearson Correlation of Coefficient
R^2	Coefficient of Determination
RTS	Reproductive Tract Scoring System
SD	Standard Deviation
SE	Standard Error
SNP	Single Nucleotide Polymorphism
SPS	Size and Position of the Reproductive Tract
TMR	Total Mixed Ration

Chapter 1. General Introduction

Demands for dairy and other food products from livestock will increase over the next three decades to meet the demands of an increasing world population; therefore, sustainable husbandry practices are essential to maintain animal health, fertility, and profitability of farms (Britt et al., 2018). Global milk production has increased in the last three decades by more than 59%, from 530 million tonnes in 1988 to 852 million tonnes in 2019 (Food and Agricultural Organization of the United Nations, 2020a; Food and Agricultural Organization of the United Nations, 2020b). This shows that there is a steady increase in milk production. Fertility in Holstein dairy cows declined between the early 1960s to early 2000s (VanRaden et al., 2004) and became a major concern among dairy farmers. In the United States, the decline was mitigated by the use of on-farm records to select animals with superior reproductive performance, widespread adoption of timed AI programs and improvement in reproductive management (Norman et al., 2009; USDA, 2019), and introduction of daughter pregnancy rate (DPR) in the mid-2000s into the breeding program (VanRaden et al., 2004). Though there has been a steady increase in genetic gain for milk production, an increase in reproductive performance has been minimal. The declining trend in reproductive performance has halted or reversed only after the introduction of DPR (USDA, 2019; Lima et al., 2020).

In Canada, the percentage of cows culled for reasons of reproductive failure decreased from 17.4 in 2014 to 15.9 % in 2019; still, it is the top reason for culling dairy cows in Canada (Canadian Dairy Information Centre, 2020). Fertility failure still is an ongoing challenge to the dairy industry which affects herd profitability.

Reproduction is the major factor that determines the longevity of cows, thereby the profitability of dairy farms. Maintaining cows with low fertility is expensive due to additional

input costs such as expenditures associated with semen, insemination, and increased days open (Britt, 1985). The fertility of a cow depends on various aspects such as animal-related factors, nutrition, environmental and managerial practices. Hence genetic selection of cows with efficient traits for reproduction and milk production is much needed for a profitable dairy operation. The physiology of dairy cows has been greatly altered by aggressive selection for milk production and altered feed and management practices (Lucy, 2001). So, novel reproductive phenotypes such as the ones that are closely related to the physiology of cows and embryo survival could improve the accuracy of estimated breeding value for fertility (Miglior et al., 2017). Anogenital distance (AGD; the distance from the center of the anus to the base of the clitoris in cows) falls under these criteria and it is reported to be an indicator of prenatal androgen exposure. In utero androgenization results in increased AGD in lambs (Gill and Hosking, 1995) and affected the postnatal fertility in gilts, lab animals (rats), sheep and humans (Drickamer et al., 1997; Zehr et al., 2001; Steckler et al., 2005; Wu et al., 2017). Recent studies in dairy cows found an inverse association between AGD and fertility i.e., pregnancy per artificial insemination in both Holstein cows (Gobikrushanth et al., 2017b, Akbarinejad et al., 2019) and heifers (Carrelli et al., 2020) where the cattle categorized as long AGD (>127 mm in cows; >110 mm in heifers) had inferior fertility compared to cattle with short AGD (≤127 mm in cows; <110 in heifers). The AGD was also found to be moderately heritable with a heritability estimate of 0.37 (Gobikrushanth et al., (2019), which is higher than that of other traditional fertility measures which range from 0.02 to 0.03 for binary (e.g., pregnant vs. not pregnant) and quantitative traits (e.g., number of services per conception) as reviewed by Berry et al, (2014). The characteristics mentioned above make AGD a promising candidate to be subjected to further exploration to determine if it qualifies as a reproductive phenotype in genetic selection of cows for fertility. In addition to heritability and association of AGD with fertility

measures, repeatability is one of the important criteria for a reproductive phenotype which helps to determine if a particular phenotype is reliable to be used at all time points. Further, for a thorough understanding of the association of cow AGD with fertility, differences in embryo yield and quality in association with AGD must be studied.

This thesis, therefore, will initially discuss the major factors affecting fertility and strategies to improve fertility with emphases on various genetic and genomic approaches and emerging reproductive phenotypes to improve the fertility of dairy cows (Chapter 2). Chapter 3 presents the results of studies conducted to determine if AGD measurements were repeatable across different ages and phases of productive and reproductive life. Chapter 4 presents the results of studies that determined the possible association of fertility differences between the AGD categories (short vs. long AGD) with the antral follicular count, anti Müllerian hormone, embryo yield and quality in cows and heifers. Finally, Chapter 5 presents a general discussion of findings, limitations of the study, and recommendations for future work.

Chapter 2. Literature Review

2.1. An overview of the dairy industry

Global demand for food due to population growth is increasing, creating a need to develop a sustainable food system and the profitability of dairy farms is the key to sustainability. Dairy consumption also increases due to the presence of an abundance of essential nutrients in them (Britt et al., 2018).

The Canadian dairy industry ranks second in the Canadian Agriculture Sector (Canadian Dairy Information Centre, 2020c), playing a crucial role in the food supply chain in Canada. The dairy industry in Canada operates under a supply management system, where the production is kept to meet the domestic consumption needs, with an average net income of dairy farms amounted to approximately 163,973 Canadian dollars in 2019 (Statista, 2021). It also contributes 19.9 billion to Canada's gross domestic product in 2015 (Dairy Farmers of Canada, 2018).

Canadian milk production increased (20.2%) in the 10-year period from 2010 to 2019 (Canadian Dairy Information Centre, 2020a), for an annual increase of approximately 2%. Aggressive selection of cows for milk production by rapid genetic advancement, artificial insemination (AI), using semen from proven sires for high milk production along with the development of advanced analytical techniques for traits and incorporation into selection programs has drastically improved the milk yield (Miglior et al., 2017)

While milk production has increased substantially, the fertility of dairy cows has slowly declined over the years (Walsh et al., 2011). A dairy herd with high fertility is important as it will

assist cows in entering production earlier in life i.e., within an optimum period after calving thus improving the economic benefits of a farm.

Conventionally, the genetic selection was largely based on production traits as they are directly linked to the profitability of a dairy farm. Selecting cows for fertility traits was not considered until recently (Miglior et al., 2017). Reproductive failure is considered as the top reason for a cow being removed from the herd (Canadian Dairy Information Centre, 2020b), thus indirectly affecting the longevity of cows. Though the decline in fertility was addressed by selecting for measures of fertility such as number of services per conception, non-returns to the first service, calving to conception interval and calving interval, they were influenced by management decisions like farmers decision on a voluntary waiting period, failure to detect estrus, and culling decision after the first insemination. Thus, recently, much more emphasis has been placed on traits such as fertility and health within Holstein cows (Miglior et al., 2017).

Breeding program encompassing traits for fertility would improve the fertility; as an example, the breeding program of Norwegian Red has included traits for fertility, production and health for five decades (since the 1970s) and it was reported to have high fertility with a better 60-day non-return rate after first AI (76.9%, 67.1%, 69.9% in heifers, first lactation and second lactation cows, respectively; Garmo et al., 2008). Besides, Ferris et al. (2014) reported that the conception rate to the first service was higher in Norwegian Red than in Holsteins. Fertility traits for Norwegian dairy cows have been included in the total merit index since 1972 by considering 56 – day nonreturn rate in virgin heifers, and 56 – day non-return rates in first lactation cows have also been considered since 2002 (Andersen-Ranberg et al., 2005).

2.2. Causes of fertility failure and strategies to improve fertility

The causes of poor fertility are multifactorial (Walsh et al., 2011). Though researchers reported that there is a negative relationship between milk production and reproduction (Hansen, 2000), the effects are only negligible compared to other effective factors like disease and nutrition (Lucy, 2001). High-producing cows might be suffering from negative energy balance that can have a significant negative effect on fertility (Buckley et al., 2003). The negative energy balance associated with high milk yield (Fetrow and Eicker, 2003) leads to a delayed resumption of ovarian activity (Butler and Smith, 1989; de Vries and Veerkamp, 2000). Lopez et al. (2004) reported that estrus expression (duration, standing events, and standing time of estrus) was shorter as well as the estradiol concentrations were less for high producing cows, despite the large preovulatory follicle. Environmental factors such as greater heat stress and endocrine disturbing chemicals also have negative effects on fertility. High lactating cows are associated with heat stress which causes reduced fertility in dairy cows (Wolfenson et al., 2000) due to impaired oocyte competence, embryo growth, gonadotropin secretion, ovarian follicular growth steroidogenesis, development of corpus luteum and uterine endometrial responses (Wolfenson and Roth, 2019). Endocrine-disrupting chemicals are available in the environment either from pollution or from plants / fungal origin like phytoestrogens (sweet clover toxicosis) and fungus fusarium and zearalenone (Kordić et al., 1992) in feed causes hyperestrogenism, thus affecting the reproductive performance and causes early embryonic loss. Inappropriate management of high producing cows will lead to poor fertility (LeBlanc, 2010); therefore, managemental strategies that were followed to effectively control the decline in fertility are proper nutrition (optimum pre-and post-partum nutrition), efficient estrus detection aids, estrus synchronization programs and timed AI. Efficient estrus detection is important to successfully breed a cow as it is linked to conception rate and pregnancy

rate of the herd (pregnancy rate = conception rate x estrus detection rate; review by Ambrose, 1999). Here, the conception rate is the number of cows pregnant per AI whereas the pregnancy rate is percent cows pregnant of all the eligible open cows in the herd. Estrus detection tools like tail chalk, Kamar heat mount detector, chin ball marker helps in the identification of the mounting behavior during estrus. Furthermore, a pedometer and body temperature measurements were used to detect estrus by measuring activity as the activity of cow increases to 2.8 folds during estrus (Redden et al.,1993) but a pedometer is not effective in detecting estrus in the tie-stall barn (Felton et al., 2012). Synchronization programs are particularly helpful in the herd with low estrus detection rate and various synchronization programs have been extensively reviewed in the literature (Colazo and Mapleloft, 2014; Nowicki et al., 2017).

2.3. Genetic/genomic approaches to improve fertility

The heritability of the traditional fertility traits is low but due to significant genetic variation for fertility within breeds (Spencer et al., 2014), genetic selection of cows can favor improving fertility (Veerkamp and Beerda, 2007). Conventionally, selection for reproductive traits was overlooked as the emphasis for selection was placed on production traits. It is as well important to study the heritability of a phenotype before using it in the genetic selection protocol as it determines the amount of difference in a particular trait affected by genetics, which is passed from generation to generation. A low heritable trait means that it does not respond well to selection as they are under less genetic control with the most part being controlled by the environment. Despite its low heritability, a trait that is of high economic value can be used in selection programs. Further, even with low heritability for reproductive traits, the coefficient of variation is high (Philipsson et al., 1981). So, incorporating fertility traits into genetic selection would mitigate the reproductive failures in the long run. Genomic selection is selection based on genomic breeding

values including gene assisted selection using individual genes or SNP (Single nucleotide polymorphism) in quantitative trait loci (QTL), which will also help in the genetic improvement of fertility (Veerkamp and Beerda, 2007). Marker-assisted genomic selection has been increasingly employed for the genetic selection of cows by identification of specific single nucleotide polymorphisms in the gene (Hayes et al., 2009). Genomic selection via, genome-wide association studies (GWAS) aids to identify the candidate genes contributing to the variation in fertility like various other traits (Pryce et al., 2010). The recessive lethal gene that affects fertility can also be identified using GWAS and helps to remove them from the dairy herd (VanRaden et al., 2011; Cooper et al., 2014) but SNP/QTL of fertility traits are hard to detect due to their polygenic nature and only one major QTL on *Bos taurus* autosome 18 (BTA18), has been identified so far and is associated with various fertility traits such as conception-to-calving-interval and body conformation traits, although the candidate gene or mutation has not been identified (Ma et al., 2019). Nevertheless, with the inclusions of daughter fertility in the lifetime production index, though the negative trend for fertility has reversed, the genetic gain has not increased greatly but the inclusion of genomics has increased the relative breeding value (RBV) to 1.78 RBV from 2011 to 2016 (Canadian Dairy Network, 2017).

2.4. Conventional selection indices for fertility

Efficient reproductive management involves selecting animals based on their reproductive performance or performance measures. The traditional measures of reproduction helped to reverse the trend for negative reproductive performance implies the importance of selecting cows for fertility traits. This is how farmers initially selected for high milk production based on the history and records of the milk yield. Conventional measures of reproduction detect the ability of a cow to be pregnant at an optimal time eliminating the disease and management problems that cause

poor reproductive efficiency (Leblanc, 2013). The reproductive performance was initially evaluated by measuring the days open (DO), calving interval, conception rate, number of services by measuring on-farm data and history stored in devices. The daughter fertility (DF) index included in the National Breeding Program in 2004 led to the initiation of genetic evaluation for female fertility (Miglior, 2007). It initially had four interval traits with the addition of two more traits lately. Currently, there are six traits measured in both cows and heifers in the daughter fertility index to determine indirectly the genetic potential of the sires and is measured by the following equation:

$$DF = 11\%\ AFS + 16\%\ NRR_H + 8\%\ FSTC_H + 15\%\ CTFS + 34\%\ NRR_C + 16\%\ FSTC_{c,}$$

Where AFS is the age at first service in the heifer, NRR_H and NRR_C are the 56- day non-return rate for heifers and cows, respectively, $FSTC_H$ and $FSTC_c$ are the interval from first service to conception in heifers and lactating cows, respectively and CTFS is the interval between calving to first service (Fleming et al., 2019). Canada has adopted DF into the lifetime production index since 2004 but the emphasis for the fertility trait is low from 2 % in Milking Short Horns to 6.7% in Holsteins (Canadian Dairy Network, 2020).

2.4.1 Daughter pregnancy rate

The percentage of cows that are become pregnant during the 21-day period after a voluntary waiting period of 60 d is called as daughter pregnancy rate (DPR), which was adopted in the United States in 2003, calculated from the records of days open (Van Raden et al., 2004) obtained from dairy herd information. The daughter pregnancy rate is incorporated in genetic selection as this trait is positively correlated with milk yield (Norman et al., 2009). The traits pregnancy rate and days open are highly correlated ($r = 0.99$) and with 1 percentage unit increase

in the pregnancy rate (PR) contributing to 4 lesser days open and calculated by a linear formula: PR = 0.25 (233 – DO) during genetic evaluation (Van Raden et al., 2004). Genetic selection for fertility is made possible by the use of predicted transmitting abilities for the daughter pregnancy rate and expressed in percentage, that is, a bull with predicted transmitting abilities (PTA) for the daughter pregnancy rate of 1 denotes that the bull's daughters are 1% more likely to become pregnant during that estrous cycle than the daughter of a bull with PTA DPR of 0 (Norman et al., 2007). Though the heritability of DPR is 4%, the reliability of the genomic prediction of daughter pregnancy rate increased to 17% (Wiggans et al., 2011). Genomic prediction of daughter pregnancy rate is also found to be positively associated with pregnant cows by the end of lactation, fewer services to pregnancy and fewer days to first service (Lima et al., 2020).

2.4.2 Binary, Interval, and other quantitative fertility traits

A binary trait (e.g. pregnancy) is one that has only two possible outcomes either non-pregnant) or pregnant and 21-day pregnancy rate (or pregnancy risk) indicates the effectiveness of reproductive management in a herd. The conception rate for heifer and calves were introduced into the Net Merit selection index in 2014 (Cole and VanRaden, 2018). The overall conception rate to the first service was 38.4% in Canada in 23 dairy herds (Ambrose and Colazo, 2007) and the conception rate at the first three services ranged from 40.5 to 43.4 % in 64 herds in the United States (Ferguson and Skidmore, 2013) and heritability of conception rate was higher for the first service than it is for all services combined in Holstein heifers (Kuhn et al., 2006). If a cow was not reported to have returned to estrus 70 d after breeding, she would be presumed pregnant and considered in the calculation of the 70 - d non-return rate. It has been also reported that conception rate (based on confirmed pregnancy) is a more useful variable than a non-return rate (based on presumed pregnancy) of any length. The first-breeding-non-return rate at 70 d in 2007 was lower

than that of 1996; furthermore, the all-breeding-non-return rate at 70 d was 1 to 3% lower than the first-breeding-non-return rate at 70 d (Norman et al., 2009). The heritability of these traits is very low ranging from 0.02 to 0.03 (Berry et al., 2014).

Examples of interval traits are age at first calving (interval from birth to first calving), calving interval (interval between two successive calvings), the interval from calving to the first service, the interval from calving to conception (days open), The shorter these intervals, the better the economic gain of the farm. The optimal age at first calving is 22 mo for Holstein cattle (Canadian Dairy Network, 2015). The heritability of interval traits is better (range from 0.03 to 0.10) compared to that of binary traits (Berry et al., 2014). The calving interval, commonly used in breeding programs along with calving to first service (Berry et al., 2014), shows the ability to regain normal physiology in the shortest time possible in cows post-calving. The coefficient of variation for the calving to first service and calving interval is 7% and 2%, respectively, suggesting a considerable genetic variation for these traits (Berry et al., 2014).

2.5. Emerging reproductive phenotypes

The traditional binary, interval and count traits are from the farm record, maybe biased by the decision to breed cows and incorrect farm records (Norman et al., 2009). As well, the low heritability of these traits makes reproductive failure a primary problem in dairy farms affecting profitability (Ribeiro et al., 2012). So, to improve genetic gain for fertility, discovering novel reproductive phenotypes having high genetic variation, that have moderate heritability, easy to measure, economical, and reliable to use, has been of recent interest among the scientific community. New reproductive phenotypes, which are less affected by the environmental factors and that more closely reflect the physiology of reproductive outcome would maximize lifetime

reproductive success (Miglior et al., 2017), leading dairy production to a sustainable path. Moreover, with the increasing demand for food due to the rising global population, it is important for infinitely sustainable dairy production.

2.5.1. Endocrine and estrus related traits

A trait based on hormones is called an endocrine-related trait. Due to the advancements of the automatic milking system, hormone concentrations, like progesterone in milk (endocrine trait) have been evaluated to indirectly predict the reproductive status of the cow, such as the commencement of the luteal activity, interval from luteal commencement to the first service, first luteal phase length, length of the inter-luteal interval and length of inter-ovulation interval for the possibility of genetic selection of the cows. Progesterone level thresholds such as 5 ng/ml (Tenghe et al., 2015) or 3 ng/ml (Garmo et al., 2009) have been established as indicators of luteal activity with above and below the established threshold indicating luteal and no luteal activity, respectively. Historically, progesterone and estrogen in the plasma are known to be highly variable during pregnancy, cyclicity and luteal activity (Robertson, 1972; Smith et al., 1975) and the milk progesterone concentration was not affected by the level of milk yield (Rabiee et al., 2002). Whereas, others have shown that progesterone concentration is affected by milk production with high producing cows having lower progesterone concentration in peripheral plasma - possibly due to increased liver metabolism (Sangsritavong et al., 2002; Wiltbank et al., 2005). Häggman et al. (2019) compared the traditional fertility traits and endocrine-related traits like calving to first estrus and commencement of luteal activity and found that heritability estimates were high for the endocrine-related traits than traditional fertility traits and the genetic correlation between calving to first heat and commencement of luteal activity was high ($r = 0.97$). So the cows need to regain

normal physiological status soon after calving (within a voluntary waiting period of at least 60 d) and establish a successful pregnancy.

Luteinizing hormone (LH) surge and its timing are important for ovulation to occur as delayed ovulation reduces the quality of follicle, affecting pregnancy. Here, Gobikrushanth et al. (2017a), characterized the variability and repeatability of LH and its association with fertility. They reported that GnRH-induced LH response in dairy cows had high variability but repeatability was low and it was not associated with measures of reproduction like pregnancy per artificial insemination.

Another endocrine trait of interest is the anti-Müllerian hormone, as it was reported to be an endocrine marker for gonadotropin responsive antral follicle in cows and it varies little through out estrous cycle allowing it to be used at random stages of the estrous cycle (Rico et al., 2009). The antral follicular count has been correlated with a total number of preovulatory follicles at all stages of development during folliculogenesis and AMH, suggesting AMH could as well be a predictor of ovarian reserve (Ireland et al., 2008; Ireland et al., 2011) The heritability estimate of AMH was moderate (0.46), but a consistent association of AMH with fertility was not found in dairy cows (Gobikrushanth et al., 2018).

Insulin-like growth factor–1 (IGF-1) is reported to be associated with fertility i.e., postpartum cows with high circulating IGF-1 were reported to have greater pregnancy to first AI than those with low circulating IGF-1 (Taylor et al., 2004). The IGF-1 has been positively associated with interval to first ovulation, conception rate to the first service, interval to commencement of luteal activity and calving to conception interval in cows and is a highly variable and moderately heritable trait (reviewed by Velazquez et al., 2008). Recently Gobikrushanth et al.

(2018) found 37 single nucleotide polymorphisms associated with variation in serum IGF-1 by a genome-wide association study. However, the IGF-1 has interaction with other factors like nutrition and hormones (e.g., gonadotropins) which might constrain its use as a sole predictor of reproductive events.

Estrous cyclicity is an indirect indicator of a cow's ability to return to cyclic estrus and time from calving to first ovulation (Petersson et al., 2007). Early-onset of estrus activity with pronounced estrus behavior post-calving increases the probability of successful insemination and conception rate (Darwash et al., 1997). Hence, estrus-related traits like calving to first high activity (increase in physical activity measured by activity tags), estrus duration, estrus strength were analyzed to determine if they can be considered into genetic selection as they are physiological and behavioral traits (Ismael et al., 2015). In this, the phenotypic and genetic correlation between calving to first high activity and calving to the first insemination was strong (0.38 and 0.96; $P = 0.05$); the heritability estimate was 0.16 for calving to first high activity (Ismael et al., 2015), which was moderate compared to the heritability estimate of estrus duration, estrus strength (0.05 and 0.02, respectively). Explicit behavioral signs during estrus (duration and strength) are associated with the blood and follicular estradiol concentration, yet the heritability and variability of the estradiol concentrations have not been characterized.

2.5.2. Anatomic Phenotypes

Reproductive tract health and normality are important for better fertility (Bell et al., 2007; Bonneville-Hébert et al., 2011). Pathology of the reproductive tract starting from the cervix, uterus, ovaries either physically or physiologically will lead to a decline in the fertility of the cows (Opsomer et al., 2000).

A reproductive tract scoring system (RTS; Score 1 to 5) was developed and has been reported to be positively associated with pregnancy rate to 50 d AI season (Holm et al., 2009). Recently, Young et al. (2017), characterized the size and position of the reproductive tract score (SPS) based on its location pertaining to the pelvic cavity as small (SPS1), intermediate (SPS2), and large (SPS3). The same group has also reported that cows with SPS1 had higher pregnancy / AI than cows with SPS2 and SPS3. However, though the heritability of RTS was better (0.32; Anderson et al., 1991), the repeatability was low (Holm et al., 2009; Gutierrez et al., 2014).

Antral follicular count (AFC) is another anatomic trait that counts the number of antral follicles present in the ovary by transrectal ultrasound scan, and it reflects the ovarian follicular reserve, ovary function and fertility (Cushman et al., 2009, Mossa et al., 2012). In addition, high AFC is associated with a high heifer pregnancy rate and AFC decreases after 5 years of age relating to reduced fertility as the parity increases (Mossa et al., 2012). In other studies, a greater AFC number (> 50) was negatively correlated with pregnancy per AI (Burns et al., 2005; Maculan et al., 2018). The follicular number among animals is highly variable with high repeatability (0.85 – 0.95) within each cow (Burns et al., 2005; Ireland et al., 2007). The estimated heritability of antral follicles is 0.31 ± 0.14 in dairy cows and 0.25 ± 0.13 in the heifer, suggesting that it is a moderately heritable trait (Walsh et al., 2014). However, it has an inconsistent association with fertility and the optimum follicular number that reflects the good fertility status is not known.

Anogenital distance (AGD) is relatively new of all the anatomic phenotypes associated with fertility. Anogenital distance is a sexually dimorphic trait (Salazar-Martinez et al., 2004; Thankamony et al., 2009) that reflects the degree of androgen exposure and is defined as the distance from the center of the anus to the posterior convergence of the fourchette (Salazar-

Martinez et al., 2004), clitoral hood (Swan, 2008) in female mammals and the distance from the center of the anus to the base of the scrotum in male mammals (Salazar-Martinez et al., 2004). Numerous studies on AGD have been reported in humans (Wu et al., 2017), laboratory animals such as rat and rabbit (Zehr et al., 2001; Banszegi et al., 2012), and farm animals like sheep (Steckler et al., 2005) and gilt; (Drickamer et al., 1997) on its association with fertility.

Measuring AGD by ultrasonography during the first trimester of gestation in human fetuses has been recently used to predict gender (Najdi et al., 2019). The genital tubercle (predecessor of the clitoris) was used to determine the fetal sex at 56 to 80 d in gestation in cattle and goat fetuses as it moves towards the umbilical cord in the male and towards the tail in female fetuses (Curran et al., 1989; Kamimura et al., 1994; Santos et al., 2007).

Recently, Gobikrushanth et al. (2017b) characterized AGD as the distance from the center of the anus to the base of the clitoris in dairy cows. In litter bearing species, positioning of female fetuses between two male fetuses increases the androgen exposure in female rodents (vom Saal, 1981) and gilts (Drickamer et al., 1997), increasing the distance from anus to genitals and masculinization of the external genitalia, which indicates that AGD is androgen dependant (Jainudeen and Hafez, 1965; Vom Saal and Bronson, 1978; Drickamer et al., 1997). Domenici et al. (2018) measured AGD in a different group of women before menopause and after menopause. They reported a significantly short-AGD in postmenopausal women compared to premenopausal women and inferred that reduction in estrogen after menopause would have reduced the AGD.

Women with polycystic ovarian syndrome have more testosterone concentration during gestation (Caanen et al., 2016). The exact source of androgen to the fetus is unknown and it is believed that placental barriers filter the excess testosterone during gestation unless it exceeds the

threshold level to be metabolized by the placenta (Kanova and Bicicova, 2011). In female sheep, exposure to testosterone propionate increases the anogenital distance (Gill and Hosking, 1995), decreases primordial follicle (Steckler et al., 2005), and increases LH pulse frequency (Savabieasfahani et al., 2005) altering the feedback of the hypothalamus-pituitary-ovarian axis (Sharma et al., 2002). Prenatal exposure to androgens in sheep reduces the sensitivity to the estradiol negative feedback, causing an increase in mean LH, LH pulse frequency and amplitude (Veiga-Lopez et al., 2009) and it also affects the estradiol positive feedback by delay in the timing of LH surge (Padmanabhan et al., 2015), causing neuroendocrine defects.

2.6. Developmental biology of anogenital distance

Endocrine-disrupting chemicals prenatally have been shown to increase the AGD postnatally in rats (Hotchkiss et al., 2007). Endocrine-disrupting chemical like testosterone concentration in the male fetus was two-fold higher than in female fetus, causing sex differentiation and its excess exposure to the dam during 30 to 80 d of gestation with female fetus leads to masculinization of sheep's reproductive tract (Veiga-Lopez et al., 2013) and impaired fertility in sheep (Steckler et al., 2007).

Androgens like testosterone and androstenedione were reported to be higher in the serum of pregnant cows (Gaiani et al., 1984). Progressively increasing testosterone levels were found from day 18 of gestation which decreased at the term in the placenta of the rat under in-vitro conditions (Matt and Macdonald, 1984). Barry et al. (2011) reviewed the results from studies on the testosterone concentration in umbilical cord blood between human male and female fetuses and reported that the testosterone concentration was high in male fetuses compared to female fetuses and further suggested that cord blood in female fetuses can be used to identify the prenatal

androgen exposure. Exposure of testosterone at 30 to 80 d of gestation in female sheep fetuses resulted in complete masculinization of the external genitalia, and exposure at 50 to 100 d of gestation showed pronounced elongation and fusion of the labia with enlarged clitoris (Clarke et al., 1976). This change might be because of the androgen hitting the sexually dimorphic nucleus in the preoptic area of the hypothalamus, as this dimorphic nucleus is smaller in females with short AGD than in females with long AGD when the rats were exposed to transplacental testosterone exposure (Faber and Hughes, 1992). Before sexual differentiation, the embryo has both primitive ducts (Müllerian and Wolffian) which then retains one, the Müllerian duct in case of female corresponding to the hormonal environment (absence of male hormones such as Müllerian inhibiting substance, testosterone and insulin-like growth factor 3) in which the fetus is exposed (Kobayashi and Behringer, 2003). This Müllerian duct, in turn, develops into female genital organs the vagina, cervix, uterus, and ovaries. In the absence of androgens, the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) suppresses growth factor and finally causes degeneration of the Wolffian duct (Zhao and Yao, 2019), which allows the development Müllerian duct. In contrast, androgen receptors are activated by androgens binding to them, leading to gene transcription in androgen dependant cell proliferation (Lim et al., 2017).

Bowman et al. (2003) reported that male rats exposed to anti-androgens had hypospadias and testicular retention, which proves the fact that the growth of the perineal region by the migration of genital tubercle caudally is androgen dependant in male, making the AGD two-fold greater in male. As well, the androgen receptor present in the genital tubercle called R-1881, stimulated by the increasing dihydroxy testosterone from day 19 of gestation (Veyssiere et al., 1985) suggests that the genital tubercle is androgen dependant. Thus, when female sheep are exposed to excess androgens during a critical reproductive programming window of reproductive

development (30 to 80 d of gestation), abnormal growth of perineal development occurs leading to greater AGD in sheep (Clarke et al., 1976).

This is evident by the formation of male leaning phenotype in female fetuses positioned between male fetuses in polytocous species like rodents and swine (vom Saal., 1981; Drickamer et al., 1997). It also applies to monotocous species bearing twins where the androgen from the male fetus is exposed to the female fetus through the anastomosis of chorioallantois, resulting in freemartin heifers (Lillie, 1917).

2.6.1. AGD and its association with fertility in mammals

Anogenital distance reflects the degree of prenatal androgen exposure. Excess prenatal exposure to androgens has been related to poor reproductive outcomes postnatally in animals' like rodents (vom Saal, 1981; Zehr et al., 2001), rabbit (Banszegi et al., 2012), gilt (Drickamer et al., 1997), sheep (Padmanabhan et al., 2006), humans (Mendiola et al., 2012; Mira-Escolano et al., 2014; Wu et al., 2017) and recently dairy cows (Gobikrushanth et al., 2017b).

Females with short AGD had an early onset of puberty in rats (Zehr et al., 2001). Similarly, does with long-AGD reported having a smaller, lighter litter with more males to female ratio (Banszegi et al., 2012).

Wu et al. (2017) reported that prenatal testosterone is positively associated with AGD and polycystic ovarian syndrome, thus affecting the fertility of females. It is also plausible to affect the next generation, that, the daughter of the polycystic ovarian syndrome women also had long-AGD (Barrett et al., 2018). The pathophysiology behind the negative effect of androgen in antral follicular development was reported by Lim et al. (2017), where the polycystic ovarian syndrome

condition in rats exposed to androgen may be due to the inhibition of Growth Differentiation Factor 9 (GDF 9) and Kit-ligand (Kitlg) derived from the oocyte and granulosa cells, which are essential for the follicular growth.

Recently, AGD has been shown to be highly variable in dairy cows. The cows with short AGD (<127 mm) were associated with better fertility, measured as pregnancy per artificial insemination, than in cows with long-AGD (≥127 mm) (Gobikrushanth et al., 2017b), and has a moderate heritability (h^2) of 0.37 (Gobikrushanth et al., 2019).

In another study (Akbarinejad et al., 2019) cows with long-AGD were less fertile than cows with short AGD, more specifically the cows with long AGD had significantly prolonged calving to conception interval (as a measure of fertility). Carrelli et al. (2020) reported that AGD was associated with fertility i.e., nulliparous heifers with short AGD achieved greater pregnancy to AI (62.2 ± 5.7 *vs.* 52.5 ± 5.8%; $P = 0.01$), earlier pregnancy with a low number of services compared with long AGD. This makes AGD a promising new reproductive phenotype to be considered when selecting cows for fertility.

2.7. Knowledge gaps

In dairy cows and heifers, AGD was reported to be inversely associated (Gobikrushanth et al., 2017b; Akbarinejad et al., 2019; Carrelli et al., 2019) with measures of reproduction like pregnancy to artificial insemination (greater), calving to conception interval (shorter), number of services (less). In addition it is found to have a high genetic variation and moderate heritability in cows. Therefore, if AGD is to be used as a fertility trait, it is important to know if AGD at birth reflects adult AGD and whether it changes during the estrous cycle or under the influence of lactation and gestation. In other words, for a reproductive phenotype to be a potential fertility trait,

other criteria such as consistency or reliability in measurements are very important as they would play a role in its heritability. A phenotype that is very reliable at all phases of physiological states is not influenced by environmental variation or postnatal determinants.

The extent of consistency in AGD measurements at different phases of life in dairy cows is not known. Hence, one of the objectives of this thesis is to determine if AGD can be consistently measured at different physiological states of life or influenced by postnatal factors like changes in the physiology of cows associated with the estrous cycle, pregnancy and milk production. In addition, it would be useful to determine the early age at which the AGD is correlated closely with that of breeding age heifers, as this could allow for an early life selection of potentially "more fertile" animals preventing the cost associated with maintaining a sub fertile population.

Gobikrushanth et al. (2017b) reported a 22.7% and 16.1% greater fertility in first- and second- parity cows with short AGD. From a more fundamental perspective of improving knowledge or understanding the mechanism underlying the association of high fertility in cows with short AGD, we hypothesized that cows with short AGD will produce more embryos and of better quality (more transferable quality) than cows with long AGD. Therefore, to test the above hypothesis we have used superovulated cows as a model in this study. In addition, as AMH was previously identified as an indirect indicator of ovarian reserve, we also hypothesized that AMH in cows with short AGD would be positively associated with antral follicles and preovulatory follicles.

CHAPTER 3

Repeatability of anogenital distance measurements from birth to maturity, and at different physiological states in female Holstein cattle

3.1. Abstract

The inverse association of anogenital distance (AGD) with fertility and its moderate heritability reported in dairy cows make AGD an appealing candidate for further exploration as a reproductive phenotype. In addition to heritability, repeatability (that is, consistency in measurements taken at different time points) is important for a reproductive phenotype to be considered useful in genetic selection. Our objectives were, therefore to, determine the 1) association and repeatability of AGD from birth to breeding age in heifer calves (0, 2, 6, 9, 12 and 15 mo of age) 2) at phases of the estrous cycle, 3) gestation (30, 90, 180, 270 d of pregnancy), and 4) lactation (monthly, 30 to 300 d in milk) in cows. The anogenital distance was measured using digital calipers. Calf birth weight (kg) was collected from farm records and calf height (cm) at the hip was measured within 7 d of birth. In the cows used to determine AGD changes through an estrous cycle, phases (proestrus, estrus, metestrus, and diestrus) were established based on plasma progesterone concentrations and ovarian ultrasonography performed every other day. The association and repeatability of AGD at different ages, and phases of the estrous cycle, gestation, and lactation were determined by PROC CORR. The differences in AGD measurements at different ages, phases of the estrous cycle, gestation and lactation were determined using PROC MIXED.

The anogenital distance was normally distributed with a high phenotypic variation (mean ± SD); 133.0 ± 9.4, 132.9 ± 12.4, 128.8 ± 8.7 mm in cows enrolled in the estrous cycle, gestation and lactation studies, respectively. The associations of AGD with height and birth weight were

weak, yet positive. The AGD differed by age, with a linear increase from birth to breeding age. There was no association between the AGD at birth and breeding age in heifers. Although any two consecutive AGD measurements were correlated, the earliest age at which AGD correlated with that of breeding age heifers, was 6 mo, with moderate repeatability (r = 0.41). The AGD was not influenced by the different phases of the estrous cycle and highly correlated (r ≥0.98). Likewise, AGD was not influenced by the different phases of lactation and highly correlated (r ≥0.95). The AGD measurements at 30, 90 and 180 d of pregnancy were strongly associated (r ≥0.97), whereas, the associations among AGD at 30, 90, 180 and 270 d of pregnancy were moderate (r ≥0.62), with AGD at late gestation (270 d) differing significantly from AGD at all earlier phases of gestation.

In summary, AGD measured at birth did not reflect adult AGD, and AGD measurements had high repeatability at all phases of the estrous cycle, lactation, and gestation, except for late (270 d) gestation. Results indicate that although the association and repeatability of AGD measurements from birth to breeding age in nulliparous heifers was poor, AGD could be reliably measured at any physiological state in dairy cows except during late gestation. In conclusion, the high repeatability of AGD indicates that this novel reproductive phenotype could be reliably measured at random physiological phases of life in cattle.

Keywords: reproductive phenotype, genetic selection, fertility

3.2. Introduction

The use of novel reproductive phenotypes with greater genetic variation within the population that have moderate to high heritability, and are easily obtainable, could augment the genetic gain for fertility (Berry et al., 2014, Miglior et al., 2017). For a potential trait to be

considered in genetic selection program, it should have reasonably large genetic variation, heritability, be clearly defined, consistently recorded, measurable at a low cost, have a direct economic value or high genetic correlation with the economically important trait(s), and be measurable earlier in life than the economically important trait (Shook, 1989).

Conventional fertility traits such as interval traits (days open), binary traits (pregnant vs. non-pregnant) and count traits (services per conception), all have a low heritability estimate range of 0.02 to 0.03 and affected by management decision like voluntary waiting period or error in recording and poor nutritional management (Berry et al., 2014) and poor nutritional management, resulting in slow genetic progress. Research on novel reproductive phenotypes with greater heritability, high repeatability, and that are closely related to physiology and easy to measure is of current interest among the scientific community and considered important for improving fertility. Emerging fertility traits, for example, endocrine traits such as commencement of luteal activity were found to have greater (0.16 – 0.30; Ismael et al., 2015) heritability than conventional traits. Anatomically manifested physiologic traits such as antral follicular count (AFC) had moderate heritability in heifers (0.31; Walsh et al., 2014) and repeatability (Gobikrushanth et al., 2017a; Koyama et al., 2018) in dairy cows, but difficult to obtain. Other traits, such as hormone concentrations, not only require frequent monitoring but are also time-consuming, invasive in nature, and expensive to quantify, making it hard to implement in commercial dairy operations. The heritability estimate of 0.32 for the reproductive tract scoring system (Anderson et al., 1991) was positively associated with pregnancy per AI. This trait, however, is subjective, labor-intensive, and has low repeatability (Holm et al., 2009; Gutierrez et al., 2014).

Anogenital distance (AGD) is a relatively new reproductive phenotype that has been of research and clinical interest in some species. In mammals, AGD is defined as the distance from the center of the anus to the posterior convergence of either the fourchette (Salazar-Martinez et al., 2004) or the clitoral hood (Swan, 2008) in female infants. In male infants, it is defined as the distance from the center of the anus to the junction of the smooth perineal skin with the rugated skin of the scrotum in male infants (Salazar-Martinez et al., 2004). Disturbance in androgen signaling occurs when androgen receptor present in the genital tubercle (R-1881; Veyssiere et al., 1985) gets exposed to excess androgens during the critical window (30 – 80 d in pregnancy) of reproductive development cause masculinization of the reproductive tract in sheep (Veiga-Lopez et al., 2013). Excess androgen exposure (Zehr et al., 2001; Banszegi et al., 2012; Mendiola et al., 2012; Mira-Escolano et al., 2014; Wu et al., 2017) prenatally, results in long-AGD and poor postnatal fertility outcomes in rodents, rabbits, and humans. Similarly, female piglets from the male-dominated litter were less fertile (Drickamer et al., 1997) due to exposure of androgens (Meisel and Ward, 1981) from male littermates to females and larger anogenital distance.

Recently, Gobikrushanth et al. (2017b) characterized AGD in Canadian Holstein cows, measured as the distance from the center of the anus to the base of the clitoris, and found an inverse relation between AGD and fertility. That is, pregnancy per AI was greater, and calving-to-conception interval was shorter in first and second parity cows with short AGD than in those with long AGD, and a heritability estimate (h^2) of 0.37 was reported in Irish Holstein cows (Gobikrushanth et al., 2019). In addition to heritability, repeatability (consistency in measurements taken at different time points) is important for a reproductive phenotype to be considered more useful in genetic selection. Repeatability is defined as the proportion of variation attributed to variation among individuals and is most frequently obtained as the interclass correlation

coefficient or Pearson correlation coefficient (Wolak et al., 2012). Repeatability helps to derive within individual consistency of measurements and only traits that display consistency within individual and variation between individual can respond well to selection. Repeatability is said to lay the upper limit of heritability as it measures both genetic and environmental sources of variation whereas heritability only measures the influence of genetics over a particular trait (Boake, 1989). If AGD is highly repeatable, i.e., low variation within an individual, then it has greater potential to be included in genetic selection programs with other complex reproductive traits and could be used in the selection index for fertility.

We hypothesized that 1) anogenital distance of a newborn calf is proportionately associated with that of breeding-age heifers; thus, AGD at birth can be used to predict the AGD of mature heifers; 2) anogenital distance is not affected by physiological states of life, that is, phases of the estrous cycle, gestation, and lactation. The main objectives of the present study, therefore, were to, determine if anogenital distance:

1. Measured at a young age, could predict AGD at breeding age (13 to 16 mo).
2. Is affected by phases of the estrous cycle (i.e., proestrus, estrus, metestrus, diestrus)
3. Is affected by different phases of lactation
4. Is affected by different phases of gestation

A secondary objective was to determine the associations among AGD, height and birthweight in the newborn calf.

3.3. Materials and Methods

Animals and Experimental design

The studies were conducted from January 2019 to July 2020 at the University of Alberta's dairy unit (Dairy Research and Technology Center), a 146-cow tie-stall barn located in Edmonton, Alberta. After weaning, heifers were housed at the Roy Berg Research Ranch in Kinsella, Alberta.

Animals were cared for as per the guidelines of the Canadian Council on Animal Care (2009) and all animal use protocols were approved by the Animal Care and Use Committee for Livestock, University of Alberta (Animal Use Protocol number 00002883). Cows were housed individually in tie-stalls provided with rubber mattresses and wood shavings. The cows went out for up to 3 h, on weekdays, for exercise. Cows were fed *ad libitum,* a total mixed ration (TMR) of alfalfa silage, barley silage, chopped hay and concentrates once daily (0800 h) and had free access to drinking water. Feed was formulated according to the National Research Council guidelines (2001) to meet the requirement of a 650 kg lactating cow producing 45 kg of milk per day. After weaning, calves and heifers were kept in outdoor dry-lot pens with homogenous age groups housed together. Heifer calves were raised on milk with ad libitum access to concentrates and chopped hay till weaning. After weaning, they were fed with free choice alfalfa hay, mash supplement of grain. At approximately 14 to 15 mo, heifers are moved into the breeding pen, they are offered with barley/oat silage and free choice of alfalfa hay. Heifer calves at all stages were provided with ad-libitum access to water.

All the studies were prospective longitudinal studies with repeated measures over time (repeated measures design). Within each objective, we measured the same set of animals at different time periods and the change in response (AGD) overtime was the variable of interest.

AGD from birth to breeding age (Objective 1): Forty-eight female calves were enrolled; Anogenital distance measurements were obtained in the first week (0 mo) of birth (3.3 ± 0.3, 0 to 8 d), then at 2 mo (62.5 ± 0.6, 55 to 72 d), 6 mo (184.6 ± 1.8, 161 to 209 d), 9 mo (270.9 ± 1.6,

255 to 285 d), 12 mo (359.9 ± 2.2, 341 to 376 d), and breeding age (489.0 ± 6.5, 423 to 533 d). Breeding age is the target age of first breeding around 13 to 15 mo in order to achieve the optimal age at first calving of around 24 mo.

Anogenital distance (Figure 3.1; AGD) was measured with a stainless-steel digital caliper (Procise, The Innovak Group, Montreal, QC, Canada) as described by Gobikrushanth et al. (2017b). The anogenital distance was measured three consecutive times in the same session, in each animal, and the mean of the three measurements was used; AGD measurements collected at different phases (d), given as mean ± standard error (SE), with minimum and maximum days. For heifers, birth dates and birth weights, and for cows, fresh (calving) dates and conception dates were obtained from herd records using Dairy Comp 305 software (CanWest DHI, Guelph, ON, Canada).

The height at the hip (height; length from the ground to the back of the hook bone) of calves was measured along with the AGD measurements using a livestock measuring stick (Jeffers®, Dothan, AL). The height and AGD were measured by the same individual in all animals with a second individual assisting in restraining calves.

AGD at different phases of the estrous cycle (Objective 2):

Anogenital distance was measured every other day through one complete estrous cycle from one ovulation to the subsequent ovulation (Figure. 3.2).

Synchronization protocol, AGD measurement and blood sampling

Twenty cows in their early lactation were enrolled (33.3 ± 2.3, 24 to 55 days in milk) and were subjected to transrectal ultrasonography (Aloka 500, Aloka Co Ltd., Tokyo, Japan) by using a 7.5 MHz linear array transducer to determine the cyclicity status of the cow by the presence of corpus luteum (CL) and preovulatory follicles (>10 mm). Upon confirming cyclicity, cows were subjected to estrous synchronization (Figure. 3.2) by using controlled intravaginal drug release

(CIDR; EAZI-breed™ CIDR®, containing 1.9-g progesterone; Zoetis, United States) and administration of Gonadotropin-releasing hormone injection (GnRH; Fertiline®, 100 µg gonadorelin acetate, i.m; Vetoquinol N. A. Inc. Lavaltrie QC, Canada). Seven days later, cows were again examined for the presence of CL. If CL was present, prostaglandin F2 alpha (PGF2α: Estrumate®; 500 µg, i.m; Merck Intervet Corp. Kirkland, QC, Canada) were administered followed by second PGF2α 24 hr later. In cows with no CL, ovaries were re-examined the next day for the presence of CL and PGF2α injected followed by a second dose. The ovaries, thereafter, scanned every other day, through one ovulation to consecutive ovulation for one complete estrous cycle. Ovarian maps were drawn at each ultrasound session to record major ovarian structures.

Blood samples were collected from the coccygeal vein into 10 mL heparinized evacuated tubes (Vacutainer®, BD life sciences, New Jersey, USA). The blood samples were collected every other day throughout the cycle from one ovulation to the subsequent ovulation to determine the plasma concentrations of progesterone (ng/mL). Samples were centrifuged at 1500 x *g* for 20 min within 30 min of collection, plasma harvested and frozen at -20 °C for progesterone estimation

Quantifying progesterone in plasma

Plasma concentrations of progesterone were analyzed at Endocrine Lab (Vet Biomed Sciences, Saskatoon, Saskatchewan, Canada) using a commercial radioimmunoassay kit (ImmuChem^{TM} Progesterone^{125} kit; ICN Pharmaceuticals, INC. Diagnostic Division, Costa Mesa, CA). The samples were analyzed in a single assay with the intra-assay coefficients of variations being 7.1 % for low- (mean, 0.9 ng/mL), 12.6 % for medium (mean, 5.2 ng/mL) and 6.7% for high reference samples (mean, 12.5 ng/mL), respectively.

Determining the phases of the estrous cycle

The phases of the estrous cycle such as proestrus, estrus, metestrus and diestrus were determined retrospectively based on corpus luteum (CL) size and plasma concentrations of progesterone. As the start of estrus could not be precisely determined, the first day when the ovulation was confirmed was considered as metestrus, followed by diestrus, proestrus and estrus. The progesterone value with a range 0 to 0.4ng/ mL was considered as metestrus, 2.3 to 4.3 ng/ mL as diestrus, 0 to 1.9 ng/ mL as proestrus and 0 to 0.04 ng/ mL as estrus. Similarly, the day of ovulation confirmation was marked as diestrus to a 50% reduction in CL size. Other phases were determined by assessing the progesterone value and CL size in individual cows.

AGD at different phases of lactation (Objective 3):

Thirty lactating dairy cows were enrolled; AGD measurements were collected at 30 (33.8 ± 0.4, 29 to 38 d), 60 (64.9 ± 0.5, 60 to 74 d), 90 (94.2 ± 0.5, 87 to 99 d), 120 (124.7 ± 0.5, 120 to 133 d), 150 (154.9 ± 0.7, 147 to 162 d), 180 (184.1 ± 0.6, 177 to 193 d), 210 (212.8 ± 0.6, 205 to 219 d), 240 (242.4 ± 0.6, 236 to 251 d), 270 (272.9 ± 1.0, 262 to 286 d), and 300 (304.2 ± 1.2, 294 to 319 d) days in milk (DIM).

AGD at different phases of gestation **(Objective 4):**

Seventy-eight dairy cows confirmed pregnant at 30 to 32 d post insemination were enrolled for this study**.** The AGD measurements were collected at 30 (36.6 ± 0.3, 32 to 48 d), 90 (98.6 ± 0.5, 90 to 109 d), 180 (187.9 ± 0.5, 177 to 199 d), and 270 (270.5 ± 0.4, 264 to 286 d) days of pregnancy (DP).

3.4. Statistical analysis

Data were analyzed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC). The descriptive statistics such as mean, standard error, standard deviation (extent of deviation in AGD;

indicative of variability), minimum, maximum for the days of AGD measurements, AGD, height, weight was determined using the MEANS procedure and the normality of the data was tested by UNIVARIATE procedure of SAS for all four studies. Significant differences were reported if $P \leq 0.05$ and considered to be a tendency if $0.051 < P < 0.10$.

Pearson correlation coefficient (PROC CORR) was used to determine the associations of AGD,

a. *Among birth weight, height, and AGD at birth (0M) (Objective 1)*
b. *Among four phases of the estrous cycle (Objective 2)*
c. *Among different phases of gestation (Objective 3)*
d. *Among different phases of lactation (Objective 4)*

The repeatability estimate of AGD measurements at different phases of the estrous cycle, gestation and lactation was estimated from the Pearson correlation coefficient (r; -1 to +1)

The linear regression model was used to determine the relationship among AGD, birth weight and height (PROC REG).

The MIXED procedure of SAS was used to determine the differences in individual least squares means (LSM) of AGD among different ages, phases estrous cycle, gestation, and lactation. Anogenital distance was paired against fixed effects such as parity and age, phases of the estrous cycle, phases of gestation and lactation and their interaction were included in the model. The interaction term was excluded from the model if it was not statistically significant ($P > 0.05$). The cow was the experimental unit (random effect) for this model and included as a repeated term for AGD measurements.

3.5. Results and Discussion

Anogenital distance measurements were influenced by age from birth until breeding age. ($P < 0.01$) and normally distributed ($P > 0.05$) in heifers at birth, 2 mo, 9 mo, 12 mo and at 15 mo of age. However, AGD was not normally distributed at 6 mo of age ($P = 0.01$) which might be because of the small sample size and one heifer being an outlier i.e., the AGD measurement fell outside the normal range. However, in studies with a large number of samples, the distribution of AGD has been normal (Gobikrushanth et al., 2017b; Akbarinejad et al., 2019; Carrelli et al., 2019). Thus, it was deemed not necessary to transform the data in this study. The AGD was normally distributed ($P > 0.05$) within the phases of the estrous cycle, gestation and lactation in the current study.

The association between AGD at birth and calf birth weight (r = 0.28, $P = 0.04$) was moderate and significant; however, only 6 % of the variation in AGD was associated with birth weight (Figure 3.3a). The AGD tended to be influenced by height measured at the hip within a week of birth and 5% of the variation in AGD was explainable by height (Figure 3.3b). The calves that were heavier and taller had longer AGD than that of the calves that were lighter and shorter. Thus, AGD increased as the calf grew and reached breeding age, following an isometric growth pattern. In other words, the increase in AGD was in proportion to the size and shape of the body and the relative growth was at a constant rate. A weak association was previously reported between AGD and height ($R^2 = 0.04$; $P < 0.01$) and age ($R^2 = 0.09$; $P < 0.01$) in lactating dairy cows (Gobikrushanth et al., 2017b) and no association between AGD and height in adult women (Wu et al., 2017).

Measurements at 0 and 2 mo (r = 0.57, $P <0.01$), 2 and 6 mo (r = 0.33, $P < 0.02$), 6 and 9 mo (r = 0.54; $P < 0.01$) and 9 and 12 mo (r = 0.62, $P = 0.02$) were moderately correlated. The

associations were strong (P <0.05) between adjacent age intervals. i.e., 0 and 2 mo, 2 and 6 mo, 6 and 9 mo, 9 and 12 mo, and 12 and 15 mo (Table 3.1). In a human study (Priskorn et al., 2018) a significant correlation (r = 0.19, P = 0.02) was found between AGD at 3 and 18 mo of age in female infants.

Anogenital distance at birth did not reflect the adult AGD in the present study. The younger age at which the AGD reflected the AGD at breeding age was 6 mo (r = 0.41, P = 0.05). Based on these findings, it is possible that AGD at 6 mo could have some predictive value for adult AGD. However, because of the small sample size in this study, these findings need to be corroborated in the future with a larger number of animals. Though the number of heifer calves was determined by priori power analyses, the limitation in this study was that animals completed the study is only less than half (n = 22) of the animals enrolled (n = 48) due to the pandemic situation caused by COVID – 19. Furthermore, there was no association between AGD at 0 and 6 mo (r = 0.09, P = 0.95). Puberty could occur from as early as 6 mo to 24 mo and varies greatly between heifers (Kinder et al., 1995). Rapid growth, pubertal growth of reproductive organs, occurs from around 6 mo of age in Holstein heifers (Desjardins and Hafs, 1969). This difference might have contributed to the huge variation and so AGD measured at 6 mo was not associated with AGD at 0 mo of age and thereafter would have grown at a constant rate till breeding age.

Rapid growth of AGD occurs around the onset of puberty and the time to reach this stage varies as puberty is multifactorial such as body weight gain, nutrition and complex hormonal pathway. The variation in the onset of puberty in calves controlled for feed and environment might be due to genetic factors and changes in hormonal action. Chelikani et al., (2003) reported age at puberty depends on constant body weight and composition, independent of diet. The same research group has underscored the association of early onset of puberty in heifers given a diet of high

energy and protein with the high-frequency low amplitude luteinizing hormone (LH) pulse, which has a strong correlation with the onset of puberty (Day et al., 1987). Prenatal androgen exposure can cause a carryover effect on reproductive organs leading to neuroendocrine irregularities by dampening LH surge amplitude in sheep (Padmanabhan et al., 2013; Padmanabhan et al., 2015).

The placenta is the primary source of androgens for a bovine fetus is highly variable (Mongkonpunya et al., 1975). In cows, plasma levels of androstenedione (100 to 200pg/ml to 1400pg/ml) and testosterone (20 to 50 pg/ml to 220pg/ml) increased. Androstenedione increased from 90 to 200 days of pregnancy and testosterone increased from 90 to 270 days of pregnancy and testosterone is highly variable within the same species (Gaiani et al., 1984). In litter-bearing species, androgen exposure to female fetuses is through the androgen secretion by the testes of a male fetus of the same litter by transplacental and transamniotic diffusion (Meisel and Ward, 1981; Clemens et al., 1978). This mechanism might be in play in monotocous species like the bovine, in the case of twin pregnancies, wherein mixing of the male and female fetal blood will lead to freemartin female (masculinized female with non-functional ovaries) due to exposure of Müllerian inhibiting substance and testosterone through an anastomosis between the two fetus' chorioallantoic vessels (Lillie, 1917). It is important to note that cholesterol and progesterone are precursors of estradiol with androgens like androstenedione and testosterone act as an intermediary in steroid biosynthesis (Lacroix et al., 1974). Therefore, it is possible that androgens produced in excess by feeding high-fat content could have aromatized to estradiol in some prepubertal calves altering the neuroendocrine signals associated with puberty and the excess androgens by itself leading to abnormal growth of genital organs. Eisenberg et al. (2012) reported that every 1 cm increase in AGD in human males is associated with an increase in testosterone concentration by 20 ng/dl.

Prenatal exposure to androgens reduces the sensitivity to the estradiol negative feedback causing a rise in mean LH, LH pulse frequency and amplitude (Veiga-Lopez et al., 2009), which might again increase androgens in calves through theca cells from the preovulatory follicle which produces androgens under the influence of LH. The secretion of androgens in theca cells is further accelerated by the simultaneous supply of pregnenolone or progesterone by granulosa cells (Fortune, 1986). Thus, the carryover effect of prenatal androgen exposure on the endocrine milieu could affect the ovarian function and could lead to polycystic ovaries as documented in a human study by Wu et al. (2017) culminating in early reproductive failure (Birch et al., 2003).

Mean (± SE) AGD (mm) was greater in multiparous cows compared to that of primiparous cows enrolled in these studies, Objective 2 (134.8 ± 1.1 *vs.* 127.4 ± 2.01, $P < 0.02$), Objective 3 (135.8 ± 0.6 *vs.* 126.2 ± 1.0, $P < 0.01$) and Objective 4 (133.1 ± 0.5 vs. 123.4 ± 0.6, $P < 0.01$). These results indicate that AGD increases as parity advances and is in agreement with previous findings of Gobikrushanth et al. (2017b) who reported differences in mean AGD between multiparous (132.5 mm) and primiparous cows (126.9 mm). In contrast, parity did not influence AGD in a study with a smaller number (n = 86) of cows (Akbarinejad et al., 2019). In a human study, AGD increased rapidly from birth to 12 mo of age in both male and female infants with only a minor increase after the first year (Papadopoulou et al., 2013). Similarly, although AGD growth is linear and relatively faster in heifers, the change in AGD with increasing parity is much less, albeit significant in the present study.

The AGD was not influenced by the different phases of the estrous cycle ($P = 0.99$) and were highly correlated (repeatability; $r > 0.98$) among the different phases within the same animal (Figure 3.5, Table 3.2). Estrogen is the major hormone found at a greater level during the estrus phase of the estrous cycle which gradually increases from 3 to 10 pg/ml during proestrus and >

10pg/ml at the onset of estrus with a non - detectable level of progesterone (Henricks et al., 1971). Mild swelling and reddening of the vulva (Diskin and Sreenan, 2000) commonly found to be associated with estrus, though not measured in this study, did not influence AGD. In contrast, Dusˇek and Bartos, (2012) observed for developmental instability in the anogenital distance in female mice during estrous cyclicity and found that AGD was significantly influenced by the phases of the estrous cycle ($P < 0.05$) but the repeatability of AGD measurements was good (> 0.66). This study suggests that in addition to prenatal androgenization, estrous cyclicity influences the anogenital distance.

The AGD was not influenced by the phases of gestation at 30, 90, 180 DP but it was influenced by the late gestation stage, i.e., 270 DP (Figure 3.6, Table 3.3). The tissue around the perineal region becomes edematous with the progression of mild swelling of the vulva several days before calving to pronounced swelling of the vulva at impeding parturition, which aids in ease of parturition (Berglund et al., 1987). Relaxin and estrogen in the placenta of cows have a major role in the relaxation of pubic ligaments and preparation of the maternal birth canal for parturition (Schuler et al., 2018). The correlations of AGD at 30, 90 and 180 DP were high (r >0.97) and was moderate at 270 DP (r >0.63). Although the vulva enlargement during impending parturition might alter the AGD at 270 DP, it remains moderately correlated with the AGD measured at previous phases of gestation.

The phases of lactation at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300 DIM did not affect AGD (Figure 3.7). The correlation among AGD measured at different phases of lactation was very high (Table 3.4; r >0.95). As hypothesized, we did not find changes in AGD during the different stages of lactation.

The cows that were pregnant were also lactating, however, lactational stages have not influenced AGD. Though 270 DP influenced AGD, the average gestational stage when the cows were at 300 DIM was 172 days pregnant. In this regard, lactation and gestation interaction were not considered in this study.

In summary, this study, for the first time, determined the repeatability of AGD at various ages and phases of productive and reproductive life in dairy cows. The key findings were that 1) AGD measured at birth did not reflect the AGD of breeding age in heifers; 2) AGD was not affected by any phase of the estrous cycle, 3) AGD was not influenced by most phases of gestation except during late gestation (270 DP), and 4) AGD remained unaffected by phases of lactation.

Thus, we infer that AGD is highly repeatable and not affected by postnatal physiological states in cows. Thus, the implication is that one measurement is taken during any of these phases i.e., estrous cycle, gestation (except 270 DP) and lactation would be sufficient to reliably obtain AGD in lactating dairy cows.

Table 3. 1. Pearson correlation Coefficients denotes the association of AGD between and among the different phases of heifer development (i.e., 0, 2, 6, 12 and 15 mo [M] of age).

Pearson Correlation Coefficients (Prob > |r| under H0: Rho=0)						
	AGD0M	AGD2M	AGD6M	AGD9M	AGD12M	AGD15M
AGD0M	1.00000 48					
AGD2M	0.57 <.001* 48	1.00000 48				
AGD6M	-0.009 0.95 46	0.33 0.02* 46	1.00000 46			
AGD9M	0.32 0.07¥ 32	0.31 0.08¥ 32	0.54 0.001* 32	1.00000 32		
AGD12M	0.24 0.28 22	0.35 0.09¥ 22	0.36 0.09¥ 22	0.62 0.002* 21	1.00000 22	
AGD15M	0.27 0.21 22	0.32 0.14 22	0.41 0.05* 22	0.31 0.16 21	0.62 0.003* 20	1.00000 22

* Correlation value is significant

¥ tendency of the correlation value

Table 3. 2. Pearson correlation Coefficients denote the correlation of AGD among the different phases of the estrous cycle (Proestrus, Estrus, Metestrus, Diestrus).

Pearson Correlation Coefficients, N = 20 (Prob > |r| under H0: Rho=0)				
	Metestrus	Diestrus	Proestrus	Estrus
Metestrus	1.00000			
Diestrus	0.99 <.0001*	1.00000		
Proestrus	0.98 <.0001*	0.98 <.0001*	1.00000	
Estrus	0.98 <.0001*	0.98 <.0001*	0.98 <.0001*	1.00000

* Correlation value is significant

Table 3. 3. Pearson correlation Coefficients denote the correlation of AGD among the different phases of gestation (i.e., 30, 90, 180 and 270 days pregnant [DP])

	Pearson Correlation Coefficients (Prob > |r| under H0: Rho=0)			
	AGD30DP	AGD90DP	AGD180DP	AGD270DP
AGD30DP	1.00000 79			
AGD90DP	0.97 <.0001* 79	1.00000 79		
AGD180DP	0.96 <.0001* 78	0.97 <.0001* 78	1.00000 78	
AGD270DP	0.62 <.0001* 69	0.62 <.0001* 69	0.63 <.0001* 68	1.00000 69

* Correlation value is significant

Table 3. 4. Pearson correlation Coefficients denotes the correlation of AGD measurements among the different phases (30 to 300 d) of lactation.

	Pearson Correlation Coefficients, N = 30 (Prob > |r| under H0: Rho=0)									
	DIM 30	DIM 60	DIM 90	DIM 120	DIM 150	DIM 180	DIM 210	DIM 240	DIM 270	DIM 300
DIM 30	1.00000									
DIM 60	0.95 <.0001*	1.00000								
DIM 90	0.94 <.0001*	0.96 <.0001*	1.00000							
DIM 120	0.94 <.0001*	0.95 <.0001*	0.96 <.0001*	1.00000						
DIM 150	0.94 <.0001*	0.97 <.0001*	0.96 <.0001*	0.97 <.0001*	1.00000					
DIM 180	0.90 <.0001*	0.94 <.0001*	0.96 <.0001*	0.94 <.0001*	0.96 <.0001*	1.00000				
DIM 210	0.90 <.0001*	0.94 <.0001*	0.96 <.0001*	0.95 <.0001*	0.96 <.0001*	0.97 <.0001*	1.00000			
DIM 240	0.90 <.0001*	0.93 <.0001*	0.95 <.0001*	0.95 <.0001*	0.95 <.0001*	0.96 <.0001*	0.98 <.0001*	1.00000		
DIM 270	0.90 <.0001*	0.94 <.0001*	0.96 <.0001*	0.97 <.0001*	0.95 <.0001*	0.94 <.0001*	0.98 <.0001*	0.97 <.0001*	1.00000	
DIM 300	0.90 <.0001*	0.92 <.0001*	0.94 <.0001*	0.95 <.0001*	0.93 <.0001*	0.94 <.0001*	0.96 <.0001*	0.96 <.0001*	0.97 <.0001*	1.00000

* Correlation value is significant

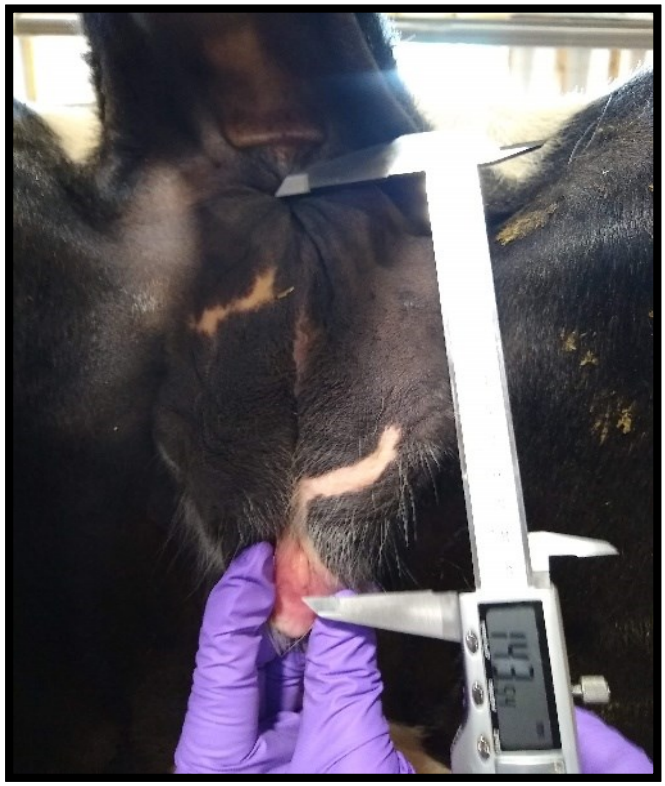

Figure 3. 1. Digital caliper positioned from the center of the anus to the base of the clitoris; anogenital distance

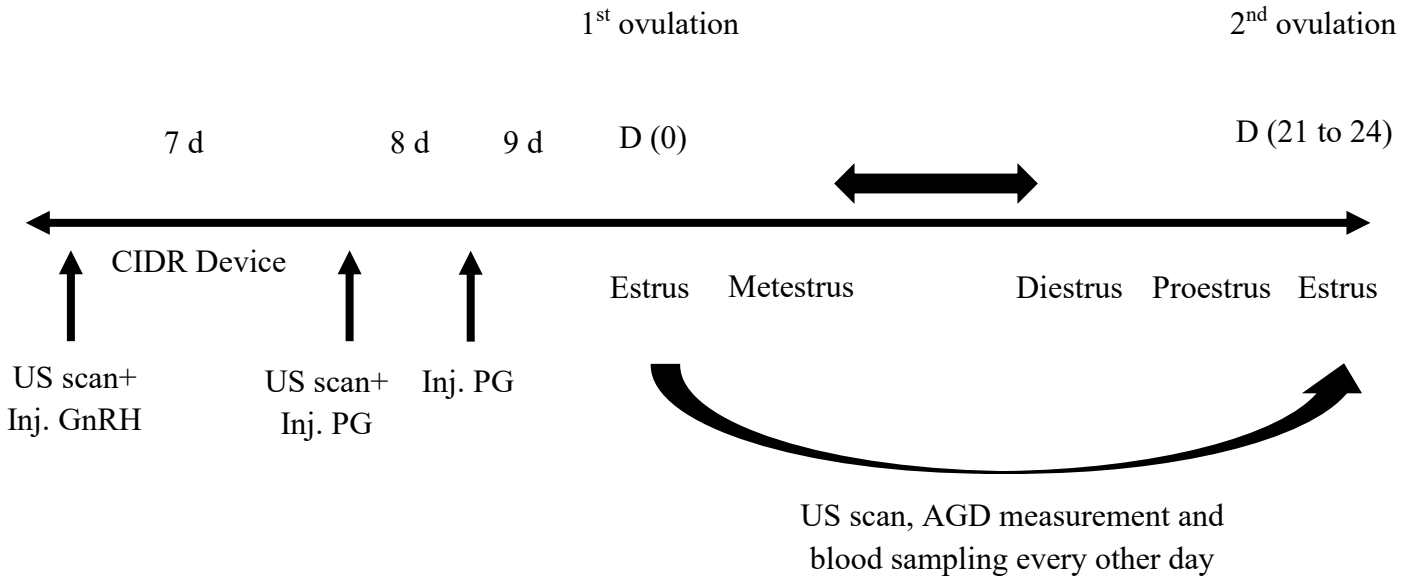

Figure 3. 2. Line diagram of the experimental design.

Ovarian status of 20 early lactating dairy cows were synchronized from d 0 to d 8, followed by ultrasound scanning of ovaries from one ovulation to the next, AGD measurement and blood sampling were performed every other day for a complete estrous cycle.

CIDR device – Control intra vaginal drug release
Inj. GnRH – Gonadotrophin releasing hormone
Inj. PG – Prostaglandin F2 alpha
US Scan – Ultrasound scan
AGD – Anogenital distance
d - days
D – day

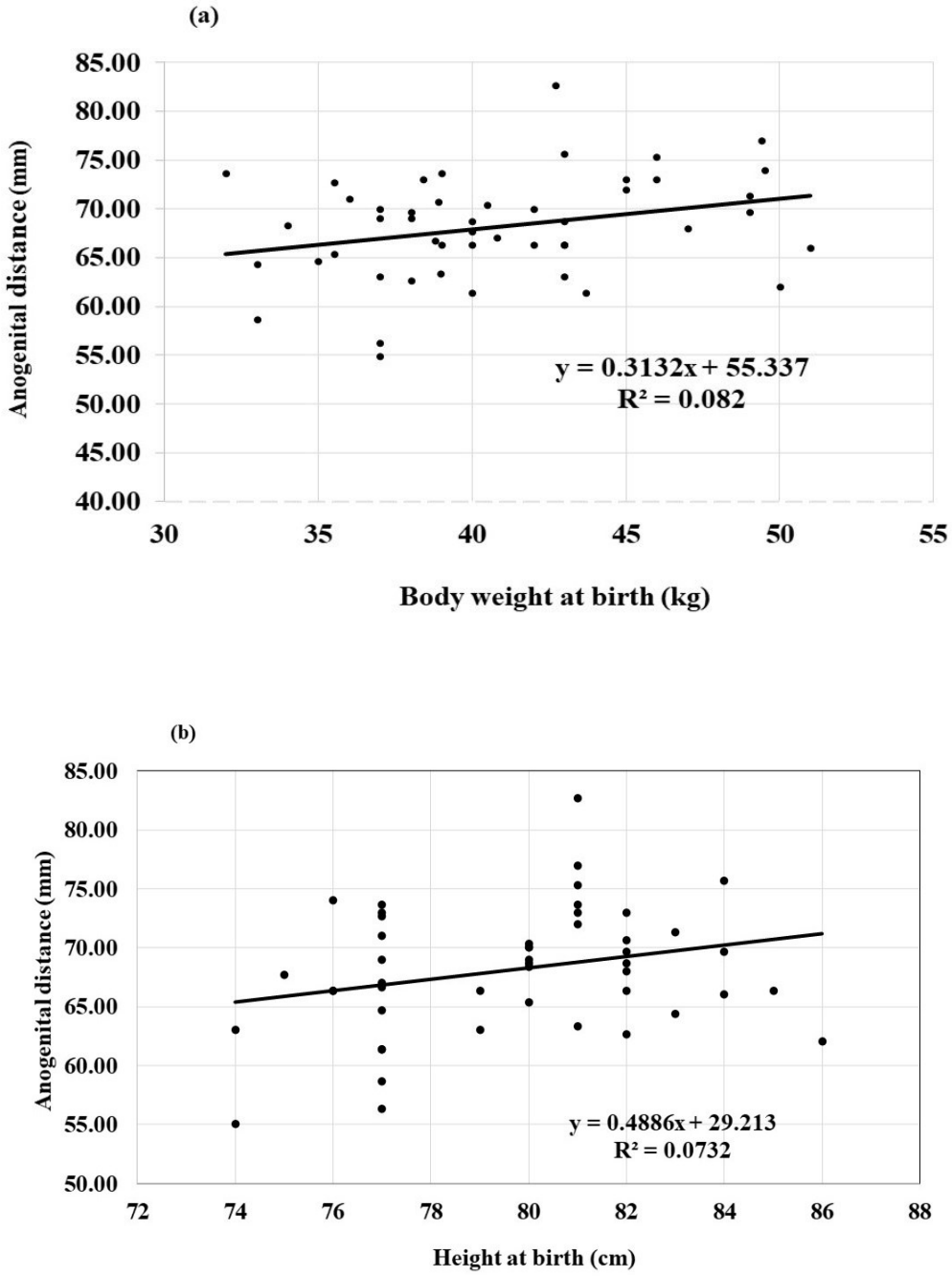

Figure 3. 3. Association between AGD and body weight at birth (a; $R^2 = 0.08$; P = 0.04), height at birth (b; $R^2 = 0.07$; P = 0.06) in 48 calves

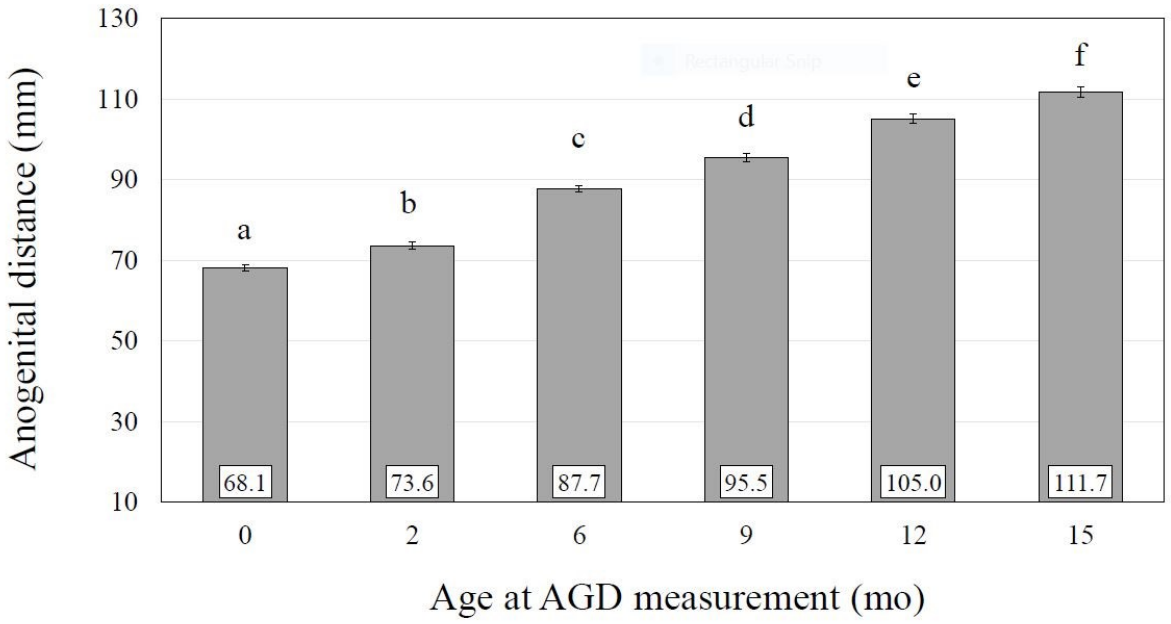

Figure 3. 4. Bar chart indicates differences (P = <0.0001) in the mean AGD (mm) measurements at different ages with error bars (SE). Number of animals used is 48.

[abcdef] Means with different superscript letters differ (P < 0.05). The numbers in the box at the base of bar chart indicates anogenital distance measured at 0, 2, 6, 9, 12, and 15 mo of age.

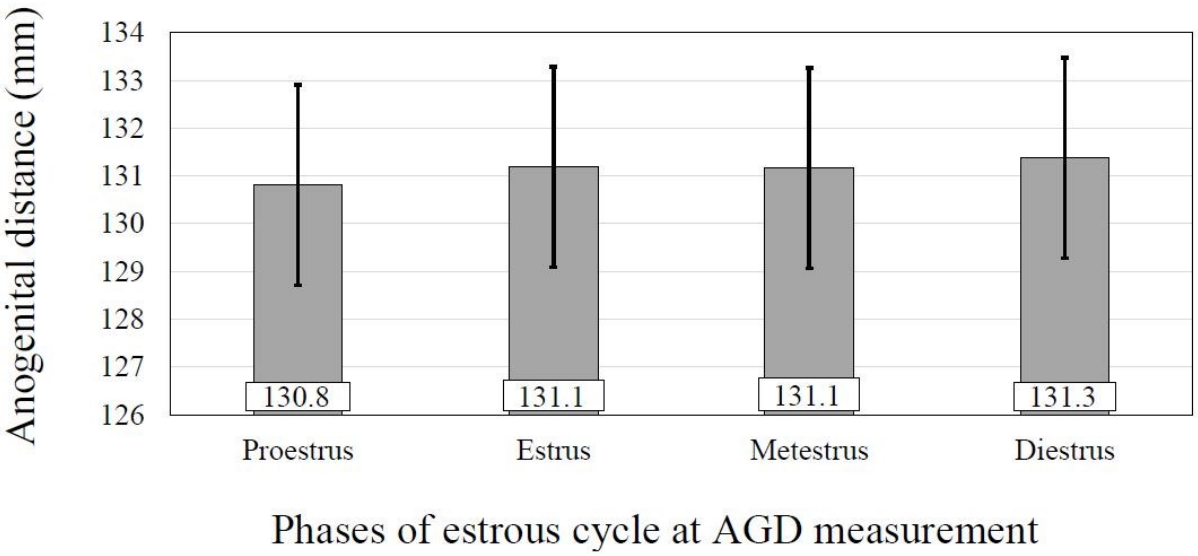

Figure 3. 5. Bar chart indicates mean AGD (mm) measurements at different phases of estrous cycle with error bar (SE). Means did not differ (P = 0.99). Number of animals used is 20.

The numbers in the box at the base of bar chart indicates anogenital distance measured at proestrus, estrus, metestrus and diestrus.

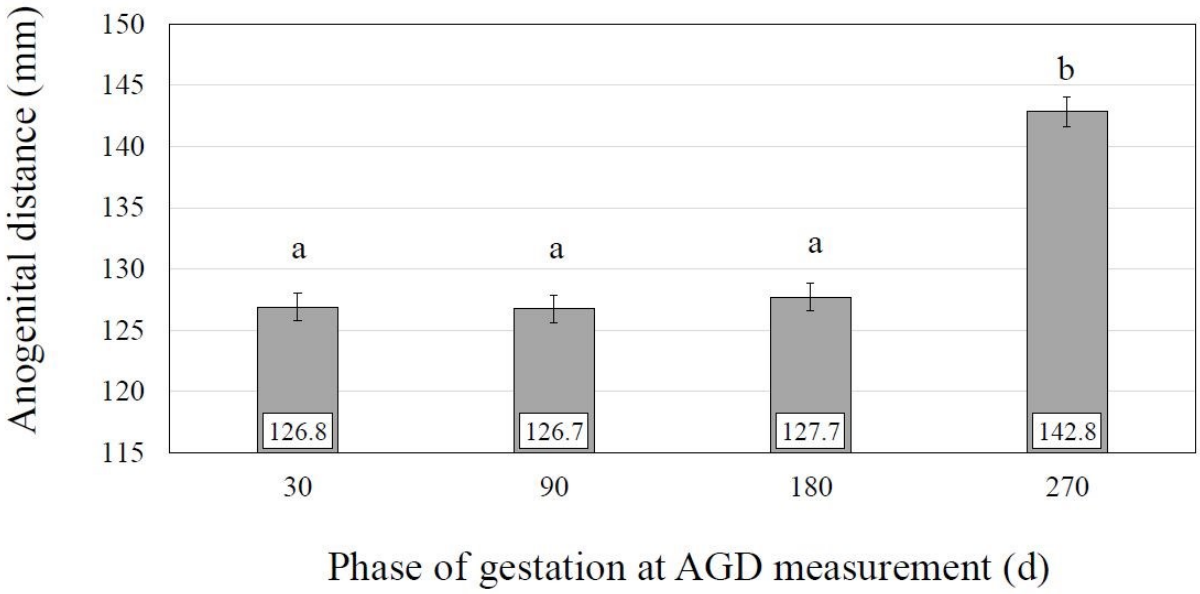

Figure 3. 6. Bar chart indicates mean AGD (mm) measurements at different phases of gestation with error bar (SE). Number of animals used is 78.

[ab] Means with different superscript letters differ ($P < 0.05$). The numbers in the box at the base of bar chart indicates anogenital distance measured at 30, 90, 180 and 270 days pregnant.

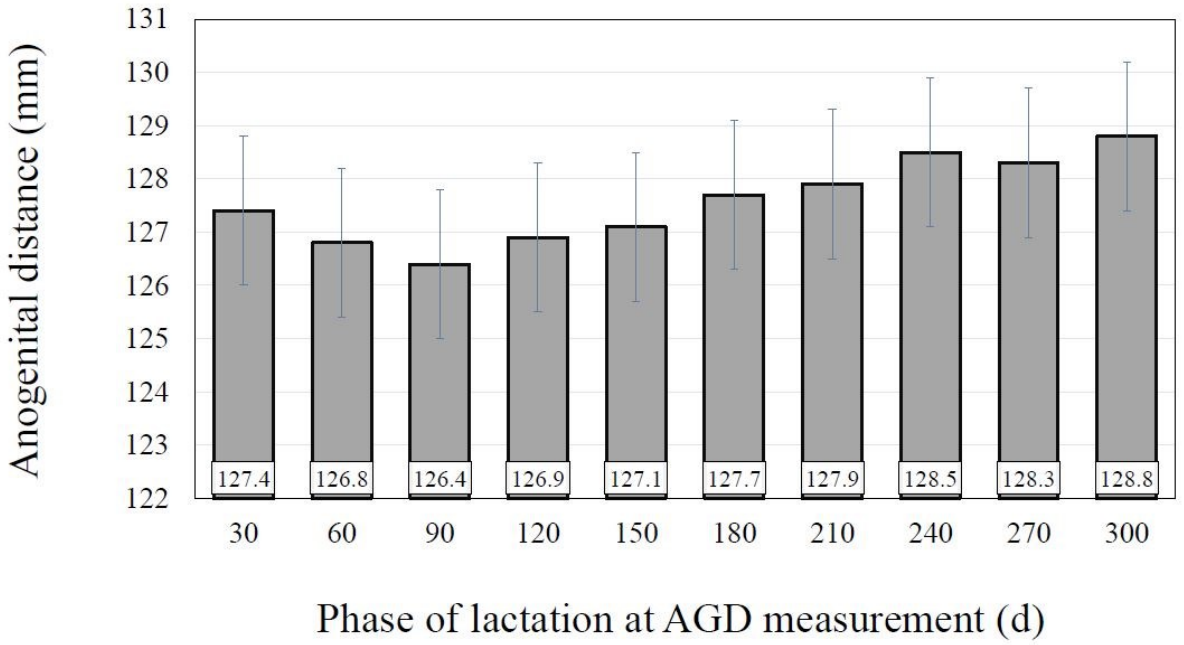

Figure 3. 7. Bar chart indicates mean AGD (mm) measurements at different phases of lactation with error bar (SE); Means did not differ (P = 0.96). Number of animals used is 30.

The numbers in the box at the base of bar chart indicates anogenital distance measured at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 days in milk.

CHAPTER 4

Yield and quality of embryos in Holstein cattle of short and long anogenital distance

4.1. Abstract

To better understand the mechanisms underlying previous reports of greater fertility in dairy cattle with short vs. long anogenital distance (AGD), we hypothesized that cattle with short AGD will have more antral follicles and high-quality preovulatory follicles and embryos than those with long AGD in response to FSH-induced superovulation. Two studies were carried out to test these hypotheses. In the first study, we determined a) associations between anti Müllerian hormone (AMH), antral follicular count (AFC) and superovulation response b) differences in yield and quality of embryos in cows of short and long AGD, c) in vitro survivability and development of embryos obtained from short- and long-AGD cows. At 43.7 (±1.6) d postpartum, AGD was measured using digital calipers, in lactating dairy cows, which were then classified as either short AGD (n=12) or long AGD (n=12) based on a previously established AGD threshold of 127 mm. Cows were subjected to FSH treatments, artificially inseminated (AI), and embryos collected 7 d after AI. Antral follicular count, and superovulation response based on the numbers of preovulatory follicles at AI and corpus luteum (CL) on the day of embryo collection, were evaluated by ovarian ultrasonography. A blood sample was taken to quantify serum AMH at the beginning of superovulation treatment. Data were analyzed using SAS using FREQ, GENMOD and REG procedures.

The associations among AFC and superovulation response were moderate (r = 0.52 to 0.61, P <0.05). Likewise, preovulatory follicles, total structures recovered (embryos/ova), fertilized eggs and transferable quality embryos were moderately associated (r = 0.43 to 0.47, P <0.05). Mean (±SE) AMH (pg/mL:132.5 ± 23.4 *vs.* 169.4 ± 23.4), AFC (22.2 ± 3.4 *vs.* 22.5 ± 3.4), preovulatory

follicles (11.9 ± 1.7 *vs.* 9.4 ± 1.7), CL (7.1 ± 1.2 *vs.* 7.1 ± 1.2), structures recovered (2.4 ± 0.9 *vs.* 2.2 ± 0.9), fertilized (1.9 ± 0.7 *vs.* 1.6 ± 0.7), and transferable embryos (1.0 ± 0.5 *vs.* 1.1 ± 0.5) did not differ ($P > 0.10$) between cows with short and long AGD, respectively. Likewise, the proportions of total structures recovered out of CL (31.4 *vs.* 25.5), fertilized eggs (77.7 *vs.* 72.7) and transferable embryos of the total recovered (44.4 *vs.* 50.0), and transferable embryos of total fertilized (57.1 *vs.* 68.7) did not differ ($P > 0.10$) between cows with short and long AGD.

In the second study, data relating to embryo yield and quality in cows and heifers from two commercial dairy herds were collected and analyzed retrospectively, for AGD-related differences. Cows and heifers were grouped in each of short and long AGD category based on the median AGD (mm) cut off of 134 and 113, respectively. There were no differences in the mean numbers of total structures recovered (7.3 ± 1.2 *vs.* 9.0 ± 1.5), fertilized (5.5 ± 1.0 *vs.* 6.2 ± 1.2) and transferable embryos (4.3 ± 0.8 *vs.* 4.7 ± 1.0) between heifers with short and long AGD categories. Similarly, the means of total structures recovered (8.7 ± 3.1 *vs.* 14.0 ± 3.1), fertilized (6.5 ± 2.0 *vs.* 5.5 ± 2.0) and transferable (5.7 ± 1.6 *vs.* 4.3 ± 1.6) did not differ ($P > 0.10$) between cows of short and long AGD categories. The proportions of fertilized eggs (75.5 *vs.* 69.2) and transferable of total recovered (59.3 *vs.* 52.9) and transferable embryos of total fertilized (78.6 *vs.* 76.4) were not different in heifers, but fertilized eggs (74.6 *vs.* 39.6, $P < 0.01$) and transferable embryos (65.8 *vs.* 30.9, $P < 0.01$) of total recovered were significantly higher in cows with short AGD than in cows with long AGD. Transferable embryos out of total fertilized (88.1 *vs.* 78.0) did not differ ($P = 0.16$) between cows of short and long AGD.

In conclusion, AFC was associated with superovulation response in cows. The number of preovulatory follicles was associated with total structures recovered, fertilized and embryos of transferable quality. There were no AGD-related differences in superovulation response in heifers.

But, the fertilized eggs and transferable embryos out of total recovered were greater in cows with short AGD than in cows with long AGD. This study has to be further investigated with a greater population of cattle.

4.2. Introduction

Genetic selection using fertility traits in addition to traits for milk production has reportedly decreased the decline in fertility in Holstein cows (Garcia-Ruiz et al., 2016). As the heritability for fertility traits is low genetic gain is slow. Hence rapid genetic gain for fertility may be achieved by using novel reproductive phenotypes with high heritability. Recent research on anogenital distance (AGD), a novel reproductive phenotype, indicates an inverse relationship between AGD and fertility in dairy cattle (Gobikrushanth et al., 2017b; Akbarinejad et al, 2019; Carrelli et al., 2020). The degree of androgen exposure during the critical period of reproductive development led to caudal migration of genital tubercle in rats (Bowman et al., 2003). Prenatal androgenization has been found to masculinize (i.e., increase) AGD (Lamm et al., 2012) affect fertility in sheep (Steckler et al., 2007). Ultrasonographic measurement of AGD was recently reported to predict gender in the fetus in a human study (Najdi et al., 2019). It is reported to reflect the degree of androgen exposure during the prenatal period and widely used as a marker for prenatal exposure to endocrine-disrupting chemicals in humans as they have shown to increase the AGD (Hotchkiss et al., 2007). Recently, Gobikrushanth et al, (2017a) characterized AGD in cows and reported that cows with short AGD (≤127 mm) had greater pregnancy to artificial insemination (P/AI; 53.6 vs. 30.9% in the first parity and 44.4 vs. 28.3% in the second parity) compared to cows with a long AGD (> 127 mm). Similarly, Akbarinejad et al, (2019) reported that the days to the first service was 17 d lesser and the first service conception rate was 18.6% greater in cows with short AGD compared to those with long AGD. Furthermore, repeat breeders (cows that failed to conceive after

three inseminations) were 16.3% higher in cows with long AGD compared with short AGD. Carrelli et al. (2020) characterized AGD in heifers and reported that heifers with short AGD (<114 mm) conceived earlier and had greater pregnancy to AI compared to heifers with a long AGD. The mechanism behind the inverse association between AGD and fertility is not known. It is plausible that an inverse association between prenatal androgen exposure and poor fertility might be because of the negative effect of androgen on ovarian follicular reserve (Steckler et al., 2005; Forsdike et al., 2007). Moreover, a high variation in ovarian size, ovarian reserve between-animals, potentially caused by maternal environment such as prenatal hyper-androgenization, have a negative impact on ovarian function (Ireland et al., 2011; Evans et al., 2012). Given this knowledge, prenatal hyper-androgenization could negatively affect ovarian function, the endocrine milieu of a dominant follicle, oocyte competence and its ability to undergo fertilization and develop into an embryo. Studies in the sheep model found that prenatal androgen exposure causes neuroendocrine defects thereby affecting the estradiol positive feedback and delaying LH surge (Padmanabhan et al., 2015). Antral follicular count (AFC), is highly repeatable (Burns et al., 2005; Ireland et al., 2007) within individual cows and positively associated with healthy oocytes and follicles in the ovaries and AMH concentration in blood (Ireland et al., 2008). Thus, AMH has been suggested as an indirect predictor of the number of antral follicles (Ireland et al., 2011) that are 3 to 7 mm in diameter, and also identified as a marker for ovarian response to superovulation treatment (Rico et al., 2009; Souza et al., 2015). Therefore, AMH and AFC are positively associated with the quality of the follicles. Normally, healthy follicles are expected to produce good quality embryos, provided other conditions are conducive; but in contrast to this, Ireland et al. (2007) reported an inverse association of the follicular number (> 3 mm in diameter) before super stimulation with follicle-stimulating hormone (FSH) and proportion of good quality embryos in Holstein cows.

Akbarinejad et al, (2019) reported a tendency for high serum AMH concentration in long-AGD cows when compared to short-AGD cows. It is unknown if the AGD is associated with the AMH, AFC, superovulation response, embryo yield and quality in dairy cattle. We hypothesized that cattle with short AGD will have more antral follicles, good yield and high-quality embryos than those with long AGD in response to FSH-induced superovulation.

Two studies were conducted to identify the association between AGD and AMH, ovarian reserves (AFC, follicles), oocyte competence and possible differences in yield and quality of embryos. The objectives of the first study conducted in a research herd were to determine (a) the differences in the superovulation response, yield and quality of embryos in cows with short and long AGD, (b) survivability and hatchability of embryos between short- and long-AGD cows, and (c) the associations among AFC – AMH –superovulation response – embryo yield/quality. The objectives of the second study were to determine if there are AGD-related differences in embryo yield and quality in cows and heifers, based on the retrospective analysis of embryo collection records from commercial dairy herds.

We expect that the findings of these studies will provide insights into the mechanism behind the inverse association between AGD and fertility in dairy cattle. If an association exists between short-AGD cows and higher follicle numbers, embryo quality, this finding could have a significant implication in (1) selection of donor cows for assisted animal reproduction and (2) supporting AGD as a potential reproductive phenotype in the genetic selection of cows for fertility.

4.3. Materials and Methods

Animals, housing and diet

The studies were conducted at the University of Alberta Dairy Research and Technology Center (DRTC) located at the Edmonton Research Station (Study 1) and two commercial dairy farms (Study 2) from January 2019 to February 2020.

All experimental procedures were approved by the Animal Care and Use Committee (ACUC) for Livestock at the University of Alberta (Animal use protocol number 00002883) and animals were cared for and maintained as per the guidelines of the Canadian Council on Animal Care (2009). Cows were individually housed in tie-stalls and fed a total mixed ration of alfalfa silage, barley silage, chopped hay and concentrates once daily (0600 to 0800 h) with ad libitum access to water. The cows were let out for exercise for approximately 3 h daily during weekdays. The feed was formulated according to the National Research Council guidelines (2001) to meet the requirement of the 650 kg lactating cow producing 45 kg/ day.

Determination of AGD

Anogenital distance, defined as the distance from the center of the anus to the base of the clitoris in dairy cows (Figure 3.1 (Chapter 3); Gobikrushanth et al., 2017b), was measured using stainless steel digital calipers (Procise, The Innovak Group, Montreal, QC, Canada). The AGD was measured thrice in quick succession and the mean of the three measurements was used in all analyses to minimize measurement error.

Study 1: Association of AMH, superovulation response, embryo yield and quality in relation to AGD in Holstein cows

Superovulation protocol and transrectal ultrasonography

Twenty - four non-pregnant cows with short AGD (≤ 124 mm; n = 12) and long AGD (> 124 mm; n = 12) in their first and second parity were randomly assigned to short- and long-AGD

categories. During initial AGD measurement, the body condition scoring (BCS) was determined on a scale of 1 (extremely thin) to 5 (obese) in quarter-point intervals (Edmonson et al., 1989). Superovulation protocol (Figure 4.2) is as described by Bó et al, (2010), with slight modifications (equal doses of the follicle-stimulating hormone were given for four days instead of decreasing doses as used by Bo and collogues and prostaglandin F2 alpha were administered with 6th and 7th dose of follicle-stimulating hormone instead of giving with last two doses of follicle-stimulating hormone). Cows were subjected to estrous synchronization using a controlled intravaginal drug release device (CIDR; EAZI-breed™ CIDR®, containing 1.9-g progesterone; Zoetis, USA) along with prostaglandin F2 alpha injection (PGF2α; Estrumate®, 500 µg i.m, cloprostenol sodium; Merck Intervet Corp. Kirkland QC, Canada) followed by gonadotropin-releasing hormone (GnRH; Fertiline®, 100 µg i.m, gonadorelin acetate; Vetoquinol N. A. Inc. Lavaltrie QC, Canada) 7 d later. After 36 hr of GnRH, superovulation protocol was initiated using follicle-stimulating hormone (FSH; Folltropin®-V, 400 mg, i.m, Porcine Pituitary Follitropin Extract; Vetoquinol N. A. Inc. Lavaltrie QC, Canada) for four days (d; 8, 9, 10, 11; 8 equal doses of 50 mg/dose every morning and evening). Prostaglandin F2α was given to induce luteolysis along with the 6th and 7th dose of FSH injection and the CIDR was removed at the last dose of PGF2α. Gonadotropin-releasing hormone administered 12 hr later (day 12) followed by artificial insemination twice, 12 h apart, with frozen-thawed semen from the same sire.

Cows were also subjected to transrectal ultrasonography (Aloka 500, Aloka Co Ltd., Tokyo, Japan) using a 7.5 MHz linear array transducer to determine ovarian cyclicity on Day 0 and presence of dominant follicle on d 7. The ovaries were scanned to determine the superovulation response based on the total number of corpora lutea from both ovaries on the day of embryo

collection (7 d after artificial insemination). The details on follicles (antral and preovulatory) and CL were recorded manually on the ovary maps.

Embryo collection and grading

Cows that showed poor superovulation response (CL ≤ 1) were not flushed. Embryos were recovered non surgically in commercial flushing media containing Dulbecco's phosphate-buffered saline (Agtech Inc, Manhattan, USA), 7 d post-insemination, under epidural anesthesia (Lidocaine HCL 2% and Epinephrine injection USP; 20mg, e/d; lidocaine hydrochloride; Bimeda, Cambridge, Ontario, Canada). A sterile Luer lock Silicone embryo flushing catheter (Minitube Canada Ltd**.** Ingersoll, Ontario, Canada) was placed into the uterine horn followed by infusion of flushing solution. The fluid recovered was collected on an embryo filter (Agtech Inc, Manhattan, USA) and then transferred into a sterile Petri dish withholding media (IVF Biosciences, Cornwall, United Kingdom) for further evaluation. The total volume of flushing medium used per uterine horn/ cow was in the range of 200 to 500 ml. Cows received two injections of PGF2α 12 h apart, 4 d after embryo collection to induce complete luteolysis thereby eliminating the risk of implantation of any embryos left in the uterus after the non-surgical embryo collection. Embryos and oocytes were searched and recovered under a stereomicroscope at 10 to 15X magnification and quality assessments were done at a higher magnification. The harvested embryos were classified according to the guidelines of the International Embryo Transfer Society manual (Stringfellow and Givens, 2010). The ideal embryo is determined by the blastomere size, color and texture. The codes for the stage of development at day 7 are morula (Stage code 3), compact morula (Stage code 4), early blastocyst (Stage code 5), blastocyst (Stage code 6), expanded blastocyst (Stage code 7) and hatched blastocyst (Stage code 8). The grades for embryo quality were assigned based on the structural integrity of the embryo and classified as Grade 1 (Good), Grade 2 (Fair) and Grade 3

(poor) and Grade 4 (dead/degenerated). The viability of embryos was determined based on the relative stage of development at 7 d after insemination.

The fertilized (Stage code ≥ 3) and good quality (Grade 1 and 2) embryos were cultured to evaluate in vitro survivability and development (Figure 4.3). The embryos that were considered transferable and at an appropriate stage of development for day 7, were washed and transferred into a petri dish containing embryo culture media (IVF Biosciences, Cornwall, United Kingdom) and placed in a CO_2 incubator at 38.5^{o} C for 24 h to determine survivability and hatching *in vitro*.

Blood sampling, AFC and AMH determination

Blood samples were collected on Day 8 before initiation of super stimulation treatment by coccygeal venipuncture into 10 mL heparinized evacuated tubes (Vacutainer®, BD life sciences, New Jersey, USA) from each cow to determine anti Müllerian hormone (AMH) concentration in plasma. The blood samples were centrifuged at 1500 x *g* for 20 min within 30 min of the collection followed by plasma separation, frozen at -20 °C for AMH quantification. Ovaries were also scanned on Day 8 to determine the antral follicular count (AFC; follicles that are >3 mm).

Serum concentrations (pg/mL) of AMH were analyzed at Ansh Labs (Webster, TX, 66, USA) using the Ansh Labs Bovine AMH enzyme-linked immunosorbent assay (Gobikrushanth et al., 2018). The assay has an analytical measurable range of 13.5 to 2240 pg/mL. The limit of detection for the AMH assay is 11 pg/mL, and the intra - assay coefficients of variation (CV) was 2.92%.

Study 2: Association between embryo yield and quality in relation to AGD in Holstein cattle based on retrospective data from commercial herds

Study 2 was conducted in two commercial dairy herds. The cows and heifers in both farms were housed in free-stall barns with free access to feed and water. The embryo donor cattle of one farm (farm 1) were inseminated with sexed semen and of the other farm (farm 2) were inseminated with either sexed or conventional semen.

Data collection and organization

Data on birthdate, calving date and parity were all collected remotely by accessing the herd database *via* DairyComp 305 herd management software (Lactanet, Guelph, ON, Canada). Embryo collection data and the date of flushing were obtained directly from the dairy farm owners. The parity at the time of flushing and AGD measurement were determined by using the calving date, flushing date and date of AGD measurement. The AGE of the cows and heifers in years on the day of flush was calculated by subtracting the birth date from the date of flush divided by 365. Animals were categorized as cows (parity ≥ 1 at AGD measurement and flushing) and heifers (parity = 0 at AGD measurement and flushing). The embryo data were organized as total structures recovered, unfertilized, fertilized, degenerated, transferable, Grade 1, Grade 2, Grade 3. The heifers were classified as Short (≤ 113 mm) and Long AGD (>113 mm), and cows were classified as Short (≤134 mm) and Long AGD (>134 mm) based on the median value, identified from the AGD data within the heifer group and cow group.

Superovulation protocol

Cows were subjected to synchronization and superovulation protocol similar to Figure. 4.2 with differences being that cows received 2mg estradiol benzoate injection (reviewed in Bó et al., 2002) followed by superovulation treatment for 4 d with FSH, with a starting dose of 60 mg, i.m on Day 7, gradually tapering to 10 mg, i.m on Day 10. The total dose for superovulation used in

cows and heifers were 400 mg and 360 mg, respectively. The cows were inseminated at detected estrus.

4.4. Statistical analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The descriptive statistics such as mean, standard error, standard deviation minimum, maximum, range of total structures recovered, fertilized eggs, transferable embryos, unfertilized eggs, degenerated embryos, AFC, preovulatory follicles and CL were determined using the MEANS procedure of SAS for each study.

Study 1 was designed as a randomized block design. The response for the superovulation treatment, embryo yield and embryo qualities in relation to AGD were the variables of interest. The cows were blocked by AGD categories and AGD was considered as a continuous variable.

$$Y_{ij} = \mu + B_i + \tau_j + \varepsilon_{ij} \qquad b_j \sim N(0, \sigma_b^2),\ \ \varepsilon_{ij} \sim N(0, \sigma^2)$$

Y_{ij} = is the response of cow i with j treatment; μ = population mean; B_i = Block effect; τ_j = effect of treatment; ε_{ij} = random error.

The association among AMH, AFC, superovulation response, embryo recovered, fertilized and transferable were analyzed using Pearson correlation coefficient (r) using PROC CORR. The difference in the proportion (%) of total structures recovered, fertilized, transferable, and Grades (1 to 3) between cows with short and long AGD were determined by using PROC FREQ and tested by the chi-square test. The difference in the proportion (%) of total structures recovered out of the total CL (percent recovery) fertilized eggs (percent fertilization) and transferable embryos (percent viability 1) out of total recovered and transferable embryos out of total structures fertilized (percent viability 2) within each AGD category were compared by using PROC GENMOD. The proportion

will be valid only if the sample size is equal. As the sample size was unequal in some of the data, the comparison of least squares means (LSM) of total structures recovered, fertilized, transferable, and Grades (1 to 3) between cows with short and long AGD were determined by using PROC MIXED. The coefficient of determination (R^2) of large follicles and fertilized eggs were estimated by linear regression analyses using PROC REG.

Significant differences were reported if $P \leq 0.05$ and considered a tendency if $P > 0.05$ and ≤ 0.10.

4.5. Results

Study 1: Three cows (one in short AGD, two in long AGD category) were not flushed because of poor superovulation response (≤ 1 CL). The AFC was moderately correlated with preovulatory follicles and CL, indicators of superovulation response. The preovulatory follicles were moderately correlated with CL, embryos recovered, fertilized and transferable embryos. The CL number was moderately correlated with embryos recovered, fertilized and transferable. The embryos recovered were highly correlated with fertilized eggs and transferable embryos. Likewise, fertilized eggs were highly correlated with transferable quality embryos (Table 4.1). The amount of variation (R^2) in preovulatory follicles explained by AFC was 34% ($P = 0.01$). Likewise, the amount of variation in fertilized eggs explained by preovulatory follicles and total structures recovered were 17% and 88%, ($P < 0.01$). In other words, the number of preovulatory follicles was influenced by AFC and the number of fertilized eggs was influenced by preovulatory follicles and total structures recovered. Body condition score did not significantly influence the fertilized eggs and total embryos recovered.

There was no difference in the mean AMH, AFC, superovulation response defined as the total number of preovulatory follicles (≥ 10 mm) and CL between cows with short and long AGD. Likewise, there was no difference in the proportion of AFC, and CL between short- and long-AGD

groups (Table 4.2). However, the proportion of preovulatory follicles was significantly greater (P = 0.03) in cows with short AGD than in cows with long AGD (Table 4.2).

Similarly, there was no difference in mean and proportion of embryo yield (total recovered) and quality (transferable embryos) between short- and long-AGD cows. The mean number and proportion of total structures, unfertilized eggs, degenerated, fertilized eggs and transferable embryos between short- and long-AGD cows did not differ (Table 4.2). The proportion of total recovered out of total CL, total fertilized out of total recovered, total transferable out of total recovered and total transferable out of total fertilized within short- and long-AGD category cows were not different (Table 4.2).

A subset of 19 embryos (7 from cows with short AGD and 12 from cows with long AGD) that were at the appropriate stage pertaining to the day of the collection were cultured, and all the cultured embryos advanced to the subsequent stage of development (e.g., a blastocyst developed into an expanded blastocyst or hatched after 24 h in culture. As all embryos (100%) advanced into the next stage of development, no statistical analyses were conducted. Similarly, no difference was found in hatched embryos between cows with short and long AGD categories (42.9 % vs. 57.1%, P = 0.24).

Study 2: The mean (±SE) age of cows (2.2 ± 0.0 vs. 2.7 ± 0.2; P = 0.06) and heifers (1.0 ± 0.0 vs. 1.1 ± 0.0; P = 0.2) with short AGD and long AGD categories, respectively, did not differ. The mean AGD was significantly different between short- and long-AGD categories in cows (125 ± 2.1 vs. 140 ± 2.1; P = 0.0001) and heifers (107 ± 0.78 vs. 124 ± 2.9; P = 0.0001). The mean embryo yield (total recovered) and quality (transferable) were not different between short- and long-AGD cows. As well, the mean fertilized eggs, unfertilized eggs, degenerated embryos and Grades (1 to 3) were not different between cows with short and long AGD. The proportion of total

structures recovered and unfertilized were significantly greater (P <0.05) in cows with long AGD compared with short AGD. The proportion of transferable and Grade 3 embryos tended (P <0.1) to be greater in short-AGD cows than in long-AGD cows. Degenerated embryos and Grade 2 embryos did not differ between short- and long-AGD groups (Table 4.3).

The proportions (%) of total fertilized out of total recovered, and total transferable out of total recovered were significantly greater (P <0.01) in cows with short AGD than in those with long AGD and the proportion (%) of total transferable out of total fertilized was not different between short- and long-AGD groups (Table 4.3).

There was no difference between the mean embryo yield (total embryos recovered) and quality (fertilized eggs and transferable embryos) between short- and long-AGD heifers. The fertilized embryos, unfertilized eggs, degenerated embryos and Grades (1 to 3) were not different between heifers with short and long AGD. However, the proportion of total structures recovered, fertilized eggs, transferable and Grade 1 embryos were significantly greater in heifers with short AGD (P <0.05). There was no difference in unfertilized eggs, degenerated and Grade 2 and 3 embryos between these two categories (Table 4.4). Likewise, there was no difference in the proportions (%) of total fertilized out of total recovered, total transferable out of total recovered and total transferable out of total fertilized between short- and long-AGD groups (Table 4.4).

4.6. Discussion

This study, for the first time, determined the association of embryo yield, quantity, AFC and AMH in relation to AGD to elucidate the possible answers for fertility differences in cattle with short versus long AGD.

The AFC was repeatable within cows (Burns et al., 2005; Gobikrushanth et al., 2017a) and highly correlated with the AMH, healthy follicles and oocytes in the ovary (Ireland et al., 2008).

In contrast, in our study, AFC was not associated with AMH but was significantly associated with the numbers of preovulatory follicles and CL. This association indicates that cows with a higher number of antral follicles will likely develop more preovulatory follicles in response to FSH stimulation. Though with increased healthy follicles, Ireland et al. (2007) reported that the proportion of transferable embryos was greater in beef heifers with low (≤ 15) *vs.* high antral follicular count (≥25). Souza et al. (2015) reported the correlation of AMH with superovulation response such as follicles and CL ($r = 0.65$; $P < 0.01$), total structures recovered ($r = 0.50$; $P < 0.01$) and transferable embryos ($r = 0.28$; $P < 0.02$). In the present study, however, AMH was not associated with AFC, superovulation response, total structures recovered and transferable embryos. The animal number might be small in each category (short-AGD group; n = 12; long-AGD group; n = 12) to compare the AFC and AMH, as the number of animals was determined by considering the embryos as a sample unit. Akbarinejad et al. (2019) reported that AGD tended to be positively associated with AMH (i.e., cows with short AGD had less AMH compared with long AGD). This is in contrast with the present studies where AMH had no association with AGD.

A higher proportion of follicles of large (>10mm) preovulatory stage was found in short-AGD cows compared to that of long-AGD cows in the present study. Though a similar number of antral follicles were present in cows with short and long AGD, more follicles reached the preovulatory stage in response to FSH treatment in cows with a short AGD, implying that the antral follicles are healthier and have greater competence to reach the preovulatory stage than in cows with long AGD. It could be speculated that poor competence of antral follicles to develop into a preovulatory follicle in cows with long AGD is probably because of the effect of prenatal androgen on the quality of follicular pool as studies in sheep exposed to prenatal androgenization had irregularities in the neuroendocrine pathway (Sharma et al., 2002; Savabieasfahani et al.,

2005), long-AGD (Gill and Hosking, 1995), abnormal ovarian folliculogenesis and decreased ovarian reserve in lambs (Steckler et al., 2005), and ovaries were comparable to human polycystic ovaries in ewes (Forsdike et al., 2007). Smith et al. (2009) reported that follicular recruitment was higher in Day 140 sheep fetuses leading to early depletion of the ovarian reserve during the postnatal period and severe abnormalities in antral follicles. In a study with prenatal androgenized Dorset sheep, androgen receptor protein expression increased in granulosa and stromal cells in the ovaries of fetal sheep at day 90 of gestation providing a piece of evidence that prenatal androgenization has an important role in follicle formation, steroidogenesis and polycystic ovarian syndrome (Comim et al., 2015)

Though a greater number of preovulatory follicles developed from antral follicles in cows with short AGD compared to long AGD they failed to ovulate resulting in the same ovulation rate between two AGD categories. These preovulatory follicles that have not ovulated are presumed to be present in ovaries as anovulatory follicles in cows with short AGD. Anovulation might be due to insufficient estrogen production for the stimulation of LH surge or steroid metabolism in high milk-producing cows (Wiltbank et al., 2002; Lopez et al., 2004) which is the case in this study as the animals were in peak milk production (4 – 8 weeks) at the time of flushing. The other reason might be inadequate/delayed LH surge that causes anovulatory conditions like follicular cyst (Johnson, 2008) or an early LH surge as reported by D'Occhio et al. (1999), anovulation in cows superstimulated with FSH is due to the presence of immature follicles with poor LH receptors that respond poorly to the early LH surge leading to incomplete luteolysis.

In contrast to our hypothesis, the quality of the embryo was not different between cows of short and long AGD categories. However, findings are not conclusive as the embryos recovered [mean (±SE) 2.45 ± 0.90] and of transferable quality [mean (±SE) 1.09 ± 0.56] per cow is less than

the average number (5 to 6 per cow) typically collected in superovulated cattle (Hasler, 2003; Hasler, 2010). Even with good flushing procedures and experienced flushing technicians, the lower recovery rate might occur due to several reasons like inaccessible/missed embryos that are at the anterior-most area of the uterine horns. Excess mucus and tissue debris in the recovered flushing media will also make it difficult to find embryos, affecting the recovery rate.

Flushing of the cows in this study was at a relatively early days in milk (64.5 ± 1.7). It is the period when a majority of cows would have been in negative energy balance, which in turn could have affected embryo quality. In this regard, the mean number of CL in Study 1 is comparable to the embryos recovered in Study 2; so the poor yield of embryos in Study 1 might be because of failure to recover all embryos or failure to find embryos because of excessive mucus or tissue debris. Except in a few cows, the fluid recovery was very good without any obvious issues with the flushing techniques. Searching for embryos was performed by at least two experienced individuals, with each dish searched twice. Despite good flushing technique and thorough searching, the number of embryos recovered was unexpectedly low for unexplained reasons.

Body condition has been reported to have an association with reproductive performance. As well, negative energy balance (NEB) impacts the calving to ovulation time, leads to a delayed resumption of ovarian cyclicity and oocyte quality and viability (reviewed in Butler, 2003). However, in this study, total embryos recovered and fertilized were not significantly affected by BCS.

The proportions of total embryos recovered, fertilized, transferable and degenerated were affected by the total numbers of animals used. The animal number was unequal in each category as some cows showed poor ovulation response (≤ 1 CL) despite excellent superovulation response (> 10 follicles in preovulatory stage). Some cows with excellent superovulation response could

not be flushed because of technical difficulties. Therefore, the proportion within each category was determined to make a fair comparison and to identify the probable difference in cows between two AGD categories.

In the study from the commercial herd heifers, the greater proportions of total embryos, fertilized eggs, transferable embryos, and Grade 1 embryos in the short-AGD group (Table 4.4) is probably because of a larger number of short-AGD heifers (n = 26) than long-AGD heifers (n=17). However, the per animal data (means) of the short- AGD *vs.* long-AGD groups did not differ (Table 4.4).

In the cow data, although we had the same number of animals in each AGD group, the proportions of total structures and unfertilized eggs were greater in the long-AGD group cows (Table 4.3). The proportion of transferable embryos tended to be greater in short-AGD group cows (Table 4.3), and the fertilization rate and viability rate were significantly greater in short-AGD cows (Table 4.3). We speculate that in addition to the negative effect of prenatal androgens on the follicle in long-AGD cows, the lactational stress might have also affected follicular growth thus compromising its quality as Ristic et al. (2008) reported that prenatal exposure of steroid, glucocorticoid affected the follicles in the rat. Various stressors in lactating dairy cows had impaired oocyte developmental competence and mitochondrial function (reviewed in Roth, 2018). So, it can be inferred that increased stress is associated with the release of steroids thus compromising follicular quality.

In summary, cows with short AGD had a greater proportion of healthy preovulatory follicles. Similarly, the proportions of fertilized eggs out of total structures recovered and transferable embryos out of total structures recovered were greater in cows with short AGD compared with long AGD. In heifers, the proportion of total structures recovered, fertilized,

transferable and Grade-1 were greater in heifers with short AGD compared to long AGD. Although objective 1 from chapter 2 was designed with an adequate number of cows based on a priori power analysis, the embryo yield was much lower than expected with an average of only 2 total embryos per cow and 1 transferable embryo making interpretation difficult. Therefore, this experiment must be repeated with a larger population of donor cattle, preferably in a later stage of lactation, which is not in a phase producing peak milk yield. From the results of retrospective data analysis, we infer that cows with short AGD could produce a greater yield and higher quality embryos but these findings must be validated in a much larger population. One direction for future research is to investigate whether there is differential expression of genes in the embryos derived from short- and long-AGD cattle.

Table 4. 1. Pearson correlation Coefficients denotes the correlation among AFC, AMH, follicle, CL, total embryos recovered, fertilized, and transferable between short- versus long-AGD groups.

	Pearson Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations						
	AFC	AMH	Follicle	CL	RECO	FERT	TRAN
AFC[1]	1.00000 24						
AMH[2]	0.13063 0.5429 24	1.00000 24					
Follicle[3]	0.61016 0.0015* 24	0.03546 0.8693 24	1.00000 24				
CL[4]	0.52539 0.0084* 24	0.36911 0.0759 24	0.61185 0.0015* 24	1.00000 24			
RECO[5]	0.24229 0.2900 21	0.26976 0.2370 21	0.43503 0.0487* 21	0.57759 0.0061* 21	1.00000 21		
FERT[6]	0.19690 0.3923 21	0.16033 0.4875 21	0.46842 0.0322 21	0.51781 0.0162* 21	0.94525 <.0001* 21	1.00000 21	
TRAN[7]	0.21041 0.3599 21	0.05243 0.8214 21	0.47785 0.0285* 21	0.44176 0.0450* 21	0.78175 <.0001* 21	0.79583 <.0001* 21	1.00000 21

[1]denotes antral follicular count (≥ 2 mm), assessed at the beginning of superovulation treatment

[2]denotes anti Müllerian hormone determined at the beginning of superovulation treatment

[3]denotes all preovulatory follicles ≥ 10 mm diameter assessed at the end of superovulation treatment, concurrent with the first insemination

[4]corpus luteum (CL) assessed on the day of embryo collection

[5]denotes all structures collected including unfertilized eggs and empty zona pellucida

[6]denotes fertilized embryos

[7]denotes transferable quality embryos

* Correlation value is significant

Table 4. 2. Comparison of Least Squares Means (± SE) and proportions (%) of superovulation response, embryo yield and quality in Short- and Long-AGD groups in Holstein-Friesian cows

	Short-AGD (n=12)#	Long-AGD (n=12)#		Short-AGD (n=12)#	Long-AGD (n=12)#	
	Mean/cow		*P*	Proportion of total/AGD group		*P*
Antral follicles[1]	22.25 ± 3.42 (267/12)	22.5 ± 3.42 (270/12)	0.95	49.72 (267/537)	50.28 (270/537)	0.44
Anti Müllerian hormone	132.58 ± 23.46	169.41 ± 23.46	0.27			
Preovulatory follicles[2]	11.91 ± 1.75 (143/12)	9.41 ± 1.75 (113/12)	0.32	55.86 (143/256)	44.14 (113/256)	0.03*
Corpus luteum[3]	7.16 ± 1.27 (86/12)	7.16 ± 1.27 (86/12)	1.0	50.00 (86/172)	50.00 (86/172)	0.5
Total embryos[4] recovered	2.45 ± 0.90 (27/11)	2.20 ± 0.95 (22/10)	0.84	55.10 (27/49)	44.90 (22/49)	0.23
Fertilized	1.90 ± 0.74 (21/11)	1.60 ± 0.77 (16/10)	0.77	56.76 (21/37)	43.24 (16/37)	0.20
Unfertilized	0.54 ± 0.31 (6/11)	0.60 ± 0.33 (6/10)	0.90	50.00 (6/12)	50.00 (6/12)	0.50
Degenerated	0.81 ± 0.44 (9/11)	0.50 ± 0.47 (5/10)	0.63	64.29 (9/14)	35.71 (5/14)	0.14
Transferable	1.09 ± 0.56 (12/11)	1.10 ± 0.58 (11/10)	0.99	52.17 (12/23)	47.83 (11/23)	0.41
% recovery (per ovulation basis)				31.40 (27/86)	25.58 (22/86)	0.69
% fertilized (of total embryos)				77.78 (21/27)	72.73 (16/22)	0.68
% transferable (of total embryos)				44.44 (12/27)	50.00 (11/22)	0.69
% transferable (of fertilized eggs)				57.14 (12/21)	68.75 (11/16)	0.47

[1]denotes all follicles ≥ 2 mm, assessed at the beginning of superovulation treatment

[2]denotes all preovulatory follicles ≥ 10 mm diameter assessed at the end of superovulation treatment, concurrent with first insemination

[3]corpus luteum (CL) assessed on the day of embryo collection

[4]denotes all structures collected including unfertilized eggs and empty zona pellucida

*different variables significantly differ between Short and Long AGD

Cows from short (1) and long (2) AGD were not flushed due to poor superovulation response (≤1)

Table 4.3. Comparison of Least Squares Means (± SE) per cow and proportions (%) of embryo quality and yield in Short- and Long-AGD groups in Holstein-Friesian cows in a commercial herd

	Short-AGD (n=9)	Long-AGD (n=9)		Short-AGD (n=9)	Long-AGD (n=9)	
	Mean/cow		*P*	Proportion of total		*P*
Total embryos[1] recovered	8.77 ± 3.17 (79/9)	14.0 ± 3.17 (126/9)	0.26	38.54 (79/205)	61.46 (126/205)	0.0005*
Fertilized	6.55 ± 2.08 (59/9)	5.55 ± 2.08 (50/9)	0.73	54.13 (59/109)	45.87 (50/109)	0.19
Unfertilized	2.22 ± 2.65 (20/9)	8.44 ± 2.65 (76/9)	0.11	20.83 (20/96)	79.17 (76/96)	<0.0001*
Degenerated	0.77 ± 0.54 (7/9)	1.22 ± 0.54 (11/9)	0.57	38.89 (7/18)	61.11 (11/18)	0.17
Transferable	5.77 ± 1.69 (52/9)	4.33 ± 1.69 (39/9)	0.55	57.14 (52/91)	42.86 (39/91)	0.08¥
Grade-1	4.11 ± 1.37 (37/9)	3.33 ± 1.37 (30/9)	0.69	55.22 (37/67)	44.78 (30/67)	0.19
Grade-2	1.00 ± 0.35 (9/9)	1.00 ± 0.35 (9/9)	1.00	50.00 (9/18)	50.0 (9/18)	0.50
Grade-3	0.66 ± 0.23 (6/9)	0.00 ± 0.23 (0/9)	0.06¥	100.0 (6/6)	0.0 (0/6)	0.007*
% fertilized (of total embryos)				74.68 (59/79)	39.68 (50/126)	<0.0001*
% transferable (of total embryos)				65.82 (52/79)	30.95 (39/126)	<0.0001*
% transferable (of fertilized eggs)				88.14 (52/59)	78.0 (39/50)	0.16

*,¥ different variables significantly or tended to differ between Shor and Long AGD
[1]denotes all structures collected including unfertilized eggs and empty zona pellucida

Table 4.4. Comparison of Least Squares Means (± SE) per cow and proportions (%) of embryo quality and yield in Short- and Long-AGD groups in Holstein-Friesian heifers in a commercial herd

	Short-AGD (n=26)	Long-AGD (n=17)		Short-AGD (n=26)	Long-AGD (n=17)	
	Means/cow		*P*	Proportion of total		*P*
Total embryos[1] recovered	7.38 ± 1.22 (192/26)	9.00 ± 1.52 (153/17)	0.41	55.65 (192/345)	44.35 (153/345)	0.01*
Fertilized	5.57 ± 1.04 (145/26)	6.23 ± 1.29 (106/17)	0.69	57.77 (145/251)	42.23 (106/251)	0.006*
Unfertilized	1.80 ± 0.51 (47/26)	2.76 ± 0.63 (47/17)	0.25	50.0 (47/94)	50.0 (47/94)	0.5
Degenerated	1.19 ± 0.41 (31/26)	1.47 ± 0.51 (25/17)	0.67	55.36 (31/56)	44.64 (25/56)	0.21
Transferable	4.38 ± 0.83 (114/26)	4.76 ± 1.03 (81/17)	0.77	58.46 (114/195)	41.54 (81/195)	0.0091*
Grade-1	3.61 ± 0.67 (94/26)	3.82 ± 0.83 (65/17)	0.84	59.12 (94/159)	40.88 (65/159)	0.01*
Grade-2	0.65 ± 0.21 (17/26)	0.76 ± 0.26 (13/17)	0.74	56.67 (17/30)	43.33 (13/30)	0.23
Grade-3	0.11 ± 0.09 (3/26)	0.17 ± 0.11 (3/17)	0.68	50.0 (3/6)	50.0 (3/6)	0.5*
% fertilized (of total embryos)				75.52 (145/192)	69.28 (106/153)	0.19
% transferable (of total embryos)				59.38 (114/192)	52.98 (81/153)	0.23
% transferable (of fertilized eggs)				78.62 (114/145)	76.42 (81/106)	0.67

* different variables significantly differ between Short and Long AGD
[1]denotes all structures collected including unfertilized eggs and empty zona

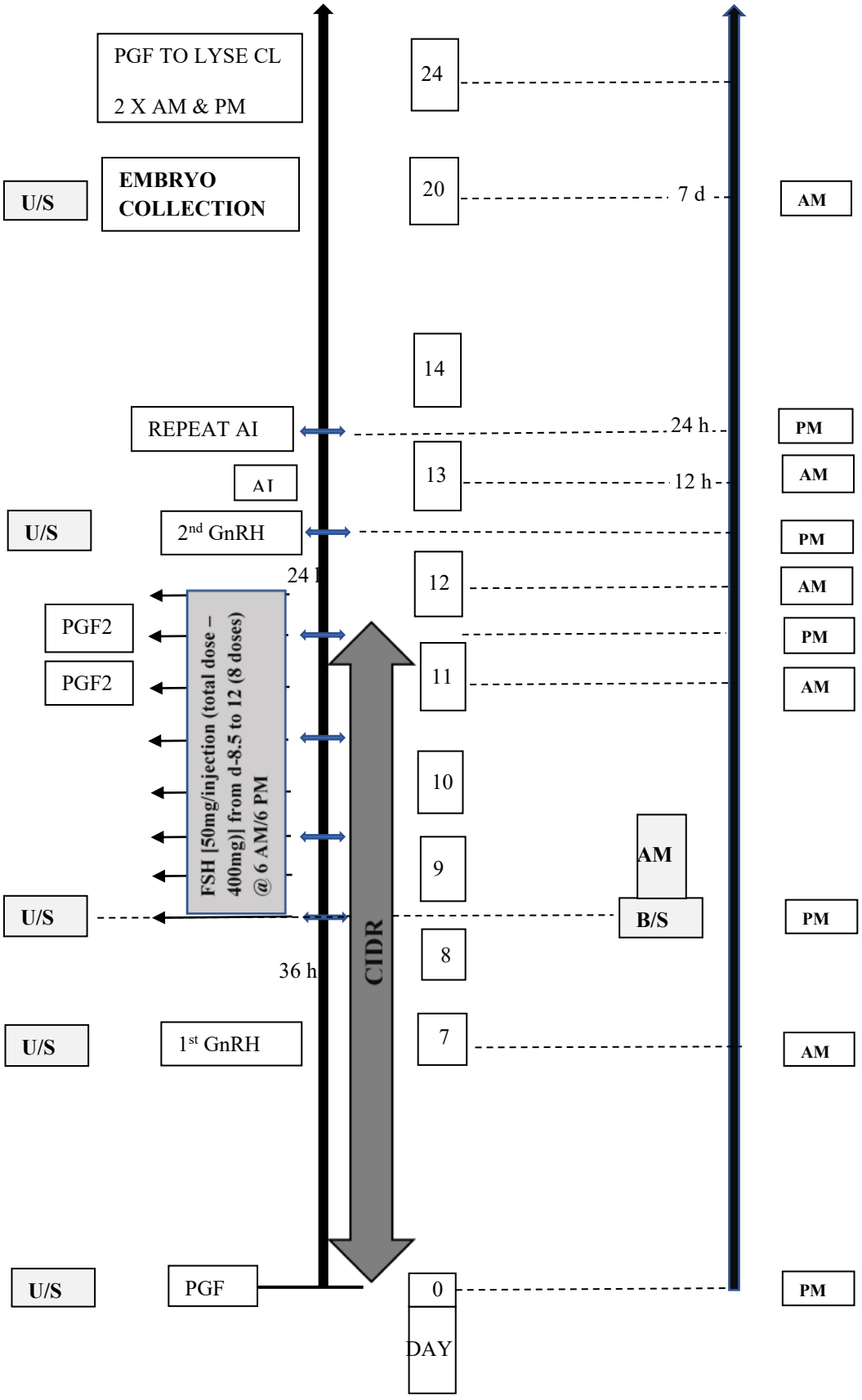

Figure 4.1. Schematic representation of the superovulation protocol used

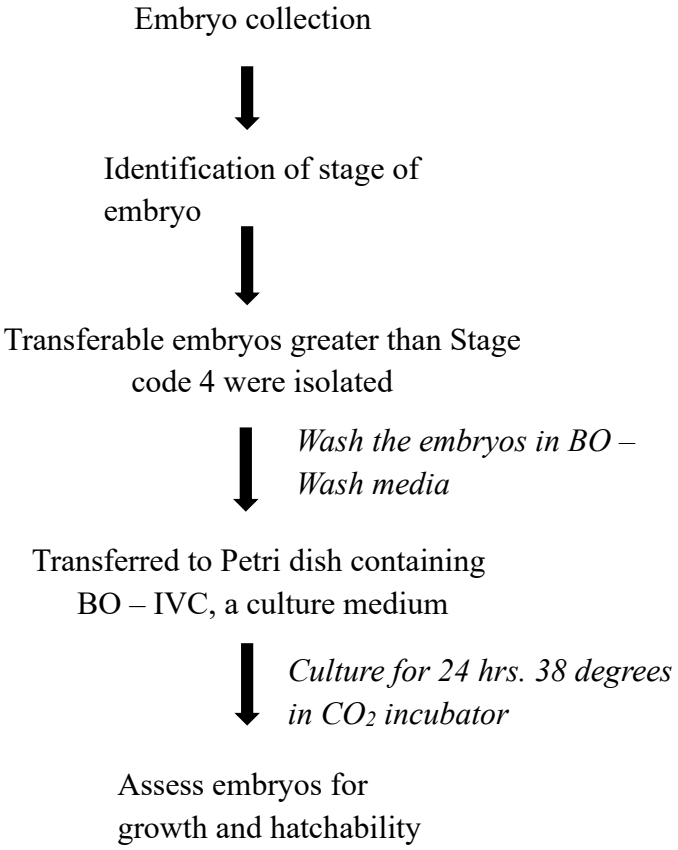

Figure 4. 2. Schematic representation of the procedure of embryo culture

CHAPTER 5

GENERAL DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS

The main reason for culling dairy cows involuntarily is reproductive issues (31%; Ontario Ministry of Agriculture, Food and Rural Affairs, 2020) resulting in economic loss to the farmers. The selection of cows for milk production has been very successful for the past 50 years partly due to moderate heritability of milk production traits and due to strong selection pressure. Likewise, high genetic variation and moderate heritability of fertility traits will provide a great opportunity for genetic selection for improved fertility. In this regard, the selection of a phenotype that is closely related to the physiology of cows and the genotype of fertility is warranted. However, due to the low heritable nature of current fertility traits, the impact of selection for fertility is of less magnitude. Novel reproductive phenotypes with moderate to high heritability, close association with fertility and simple to implement on-farm level, are helpful to increase the genetic merit for fertility. Anogenital distance (AGD), is one such novel reproductive phenotype, recently characterized as short- (≤ 127 mm) and long- (> 127 mm) AGD in first and second parity cows based on the optimal threshold of 127 mm that predicted the probability of pregnancy outcomes in 921 Holstein cows (Gobikrushanth et al., 2017). It has to be explored further for its consistency throughout the animal's life and to elucidate the mechanism associated with its inverse association to fertility. The current thesis evaluated the association of AGD measurements among the different ages, repeatability of AGD at different physiological states of life in Canadian Holstein cows. In addition, the association of anti-Müllerian hormone (AMH), antral follicular count (AFC), superovulation response, embryo yield (total structures recovered) and quality (fertilized and transferable) in Canadian Holstein cows and heifers in relation to AGD were studied.

5.1. Key findings

In chapter 3, four objectives were studied, 1) the association of AGD measured at birth and breeding age, 2) repeatability of AGD at different phases of the estrous cycle, 3) gestation and 4) lactation. The reason for designing this objective was that AGD was found to have moderate heritability in Irish Holstein cows (0.32; Gobikrushanth et al., 2019) and an inverse association with pregnancy per AI in Canadian Holsteins (Gobikrushanth et al., 2017b; cows with short AGD were found to be more fertile and vice versa). Besides, results from Akbarinejad et al. (2019) also reported that cows with short AGD are more fertile compared to long AGD. These results make AGD an acceptable phenotype to be explored further for it to be considered as a reproductive phenotype. In addition to heritability, repeatability is important as a reproductive phenotype has to be consistent in measurement to use it as a reliable indicator for fertility. The keys results are there was 1) no association between AGD at birth and breeding age. The AGD was found to be highly repeatable 2) at all phases of the estrus cycle, 3) most phases of gestation and 4) all phases of lactation. So, we have concluded that AGD measured at birth cannot be used to predict adult AGD but AGD measured at 6 mo of age were reflective of AGD at breeding age. and it can be measured at any phases of the estrus cycle, gestation except 270 days pregnant and lactation without much variation to reliably reflect the actual measurement.

In Chapter 4, two objectives were studied 1) the association of AMH, AFC, superovulation response, embryo yield and quality in relation to AGD in Holstein cows 2) the association of embryo yield and quality in heifers and cows using retrospective data. The key results from this chapter are 1) AMH was not associated with AFC, superovulation response, embryo yield and quality. The antral follicular count was moderately associated with preovulatory follicles and CL. Likewise, total structures recovered were moderately associated with fertilized and transferable

embryos. There was no difference in the proportion of AFC, superovulation response, embryo yield and quality between short- and long-AGD cows but the proportion of preovulatory follicles was higher in short–AGD cows compared to long–AGD cows. 2). The percent fertilized (total fertilized out of total recovered) and percent transferable (total transferable out of total fertilized) were higher in cows with short AGD. The proportion of total recovered, fertilized, transferable and grade 1 embryos were higher in heifers with short AGD compared to long AGD.

5.2. General discussion and limitations

In Chapter 3, we hypothesized that AGD at birth can be used to predict adult AGD, potentially the fertility status of cow and AGD measurements will be consistent without being affected by the variation in the physiological state in cows. However, in the present study, AGD at birth was not associated with adult AGD but the earliest age at which the AGD related to adult AGD was 6 mo. The limitation of this study is that study could not be completed with all 48 animals enrolled initially. The AGD measurements until the breeding age could be collected only from 22 animals as we had to stop the study because of the pandemic situation due to the coronavirus (COVID 19). Therefore, the experiment should be repeated in a much larger population of cows. In contrast to our hypothesis, AGD at birth and 6 mo were not associated. It could be speculated that this might be because of the huge variation in AGD that occurred due to rapid growth, pubertal growth of reproductive organs (Kinder et al., 1995; Desjardins and Hafs, 1969) occurred at 6 mo. It is plausible that during puberty, the rapid growth of reproductive organs occurred and grows in a steady plane until maturity. It could be speculated that this variation might be because of previous sensitization of androgens in female calves as a fetus and is further accelerated during puberty leading to a proportionate increase in length in relation to adult AGD. The AGD was found to be highly repeatable at all the physiological states except 270 DP.

In Chapter 4, we hypothesized that cows with short AGD will have greater AFC, superovulation response, higher embryo yield and quality. This study is influenced by the number of animals in each category; therefore, we have analyzed the difference in means of embryo yield and quality between the two AGD category. Though we could find the difference in proportion between short- and long–AGD cattle. We did not find any differences in the means of total embryos recovered, fertilized and of transferable quality between short- and long-AGD categories. However, in the study using retrospective data of cows, though the number of animals was equal, the percent fertilized and percent transferable within short–AGD cows were higher than long–AGD cows. As the results are not consistent among the different objectives, we could not make any firm conclusion for this study so repeating the study with a high number of cows is highly warranted.

The major limitation of study 1 of chapter 4 (study in research herd at DRTC) was that cows were in peak milk production (64.5 ± 1.7 days in milk) at the time of flushing, so the cows might have been in lactational stress and negative energy balance, which could have affected the embryo quality. The average number of embryos recovered from this study was also very low (2 per cow) despite good ovulation response (average 7.1 CL per cow), flushing procedure and technique, which could be due to embryos being missed while flushing or lost in uterine mucus.

5.3. Future Directions

It was not within the scope of this study to assess certain factors and mechanisms behind the inverse association of AGD and fertility and the carry-over effect of prenatal androgenization on cyclicity in long–AGD cows. Other knowledge gaps that need to be investigated are,

- Measuring AGD from birth to maturity and correlating it to the reproductive performance in heifers. We measured the AGD from birth to breeding age to determine the correlation

among the AGD measurements and to determine the earliest age at which AGD would be correlated to AGD of breeding age heifers. This study should be repeated with large animal numbers and determine the reproductive performance of breeding age heifers to correlate it with AGD at breeding aged heifers. Such a study will help us to determine the minimum age in which the AGD is correlated to that at breeding age and that minimum age that AGD reflects reproductive efficiency in heifers.

- Association of AGD and testosterone at birth in calves and during gestation in dams. The variation of testosterone concentration during gestation in the dam has been discussed in chapter 4. The level of testosterone in the dam, fetus and its association with AGD at birth will determine if in utero androgen exposure is reflected as AGD and partially explain the carryover effect of in utero testosterone exposure in calves.
- To determine if heifers with prenatal testosterone exposure and resultant long-AGD, have perturbed age at puberty. Prenatal androgenization was reported to have no difference in pubertal onset in sheep (Sharma et al., 2002), delayed onset of puberty in the monkey (Zehr et al., 2005) and earlier onset in sheep (Padmanabhan et al., 2015). Testosterone and AGD at birth can be measured and its association with age at puberty in heifer calves can be determined.
- To determine if there is any difference in ovulation time and LH surge between short- and long–AGD cattle. As there are studies that reported that prenatal androgen exposure affects LH surge (Padmanabhan et al., 2015) and other neuroendocrine abnormalities (Padmanabhan et al., 2013; Padmanabhan et al., 2015), this study might explain the fertility differences in cows between AGD – categories through a better understanding of underlying mechanisms.

- To determine the repeatability of AGD through lactation (1st and 2nd) in the same cows. Though the present study (chapter 3) and a previous study (Gobikrushanth et al., 2017) found that AGD increases with parity, another study reported no change in AGD (Akbarinejad et al, 2019) in cows of different parities. In these studies, the same cows were not followed through the 1st and 2nd lactation. Therefore, investigations are warranted to determine if AGD changes over different parities in the same cows and is influenced by postnatal determinants.
- To evaluate if there are any differences in gene expression in embryos collected from cows of short vs. long AGD. Although this study was proposed it could not be carried out due to a shortage of embryos and lack of sufficient embryos in a similar stage of development within each category. Exploration of differential gene expression for embryo survivability, viability depicts the possible cause of greater pregnancy per AI in cows with short AGD (Gobikrushanth et al., 2017b).

5.4. Conclusion

The results from the present thesis indicate that AGD is highly repeatable and can be reliably measured during any physiological state except at late gestation (270 d). Thus, the high repeatability of AGD adds further strength to AGD and would be useful if it was adopted as a reproductive phenotype. The percent fertilized and percent transferable embryos were high in cows with short–AGD compared to those with long–AGD. Due to inconsistent results in this study, future studies should be done a with larger sample size and further explored for differential gene expression. The cumulative results might eventually help to bring to light a novel fertility phenotype and possibly include it in genetic selection along with traits for production, health and longevity. In conclusion, based on previous and current research findings, AGD is easy, quick and

inexpensive to measure, has moderate heritability, high repeatability, and closely associated with economically important reproductive traits such as pregnancy per AI, services per conception and calving-to-conception interval. Therefore, these results suggest that AGD could be an indicator trait for fertility in cattle.

REFERENCES

Akbarinejad. V., F. Gharagozlou, M. Vojgani, E. Shourabi, and M. J. M. Makiabadi. 2019. Inferior fertility and higher concentrations of anti-Müllerian hormone in dairy cows with longer anogenital distance. Domest. Anim. Endocrinol. 68:47-53.

Ambrose, D. J. 1999. An overview of strategies to improve reproductive efficiency. Advances in Dairy Technology. 11:87-106.

Ambrose, D. J., and M. G. Colazo. 2007. Reproductive status of dairy herds in Alberta: a closer look. In Advances in dairy technology: proceedings of the Western Canadian Dairy Seminar. 19:227-244.

Andersen-Ranberg, I. M., G. Klemetsdal, B. Heringstad, and T. Steine. 2005. Heritabilities, genetic correlations, and genetic change for female fertility and protein yield in Norwegian dairy cattle. J. Dairy Sci. 88:348–355.

Anderson, K. J., D. G. LeFever, J. S. Brinks, and K. G. Odde. 1991. The use of reproductive tract scoring in beef heifers. AgriPractice.12:19–26. cited in Holm, D. E., Thompson, P. N., and Irons, P. C. 2009. The value of reproductive tract scoring as a predictor of fertility and production outcomes in beef heifers. J. Anim. Sci. 87:1934-1940

Bánszegi, O., Szenczi, P., Dombay, K., Bilkó, Á., & Altbäcker, V. 2012. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. Physiol. Behav. 105:1226-1230. https://doi.org/10.1016/j.physbeh.2012.01.002.

Barrett, E. S., K. M. Hoeger, and S. Sathyanarayana. 2018. Anogenital distance in newborn daughters of women with polycystic ovary syndrome indicates fetal testosterone exposure. J. Dev. Orig. Health. Dis. 9:307-314.

Barry, J. A., Hardiman, P. J., Siddiqui, M. R., & Thomas, M. 2011. Meta-analysis of sex difference in testosterone levels in umbilical cord blood. J Obstet Gynaecol. 31: 697-702.

Bell, M. J., and D. J. Roberts. 2007. The impact of uterine infection on a dairy cow's performance. Theriogenology. 68:1074-9. PMID: 17869332.

Berglund, B., J. Philipsson, and O. Danell. 1987. External signs of preparation for calving and course of parturition in Swedish dairy cattle breeds. Anim. Reprod. Sci. 15:61-79. https://doi.org/10.1016/0378-4320(87)90006-6.

Berry, D. P., E. Wall, and J. E. Pryce. 2014. Genetics and genomics of reproductive performance in dairy and beef cattle. Animal. 8:105–121. https://doi.org/10.1017/S1751731114000743.

Birch RA, Padmanabhan V, Foster DL, Robinson JE. 2003. Prenatal programming of reproductive neuroendocrine function: fetal androgen exposure produces progressive disruption of reproductive cycles in sheep. Endocrinology. 144:1426–1434

Bó, G. A., P. S. Baruselli, D. Moreno, L. Cutaia, M. Caccia, R. Tríbulo, H. Tríbulo, and R. J. Mapletoft. 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology. 57:53-72.

Bó, G. A., D. C. Guerrero, A. Tríbulo, H. Tríbulo, R. Tríbulo, D. Rogan, and R. J. Mapletoft. 2010. New approaches to superovulation in the cow. Reprod Fertil Dev. 22:106-12.

Bo, G. A., and R. J. Mapletoft. 2018. Evaluation and classification of bovine embryos. Anim Reprod. 10:344-348.

Boake, C.R.B. 1989. Repeatability: Its role in evolutionary studies of mating behavior. Evol. Ecol. 3:173–182. https://doi.org/10.1007/BF02270919.

Bonneville-Hébert, A., E. Bouchard, D. D. Tremblay, and R. Lefebvre. 2011. Effect of reproductive disorders and parity on repeat breeder status and culling of dairy cows in Quebec. Can. J. Vet. Res. 75:147-151. PMID: 21731187.

Bowman, C. J., N. J. Barlow, K. J. Turner, D. G. Wallace, and P. M. Foster. 2003. Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat. Toxicol Sci. 74:393–406. PMID: 12773767.

Britt, J. H. 1985. Enhanced reproduction and its economic implications. J. Dairy Sci. 68:1585-1592.

Britt, J. H., R. A. Cushman, C. D. Dechow, H. Dobson, P. Humblot, M. F. Hutjens, G. A. Jones, P. S. Ruegg, I. M. Sheldon, and J. S. Stevenson. 2018. Invited review: Learning from the future-A vision for dairy farms and cows in 2067. J. Dairy Sci. 101:3722–3741. https://doi.org/10.3168/jds.2017-14025

Buckley, F., K. O'Sullivan, J. F. Mee, R. D. Evans, and P. Dillon. 2003. Relationships among milk yield, body condition, cow weight, and reproduction in spring-calved Holstein-Friesians. J. Dairy Sci. 86:2308–2319. PMID: 12906047.

Burns, D. S., F. Jimenez-Krassel, J. L. H. Ireland, P. G. Knight, and J. J. Ireland. 2005. Numbers of antral follicles during follicular waves in cattle evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. Biol. Reprod. 73:54–62.

Butler, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest. Prod. Sci. 83:211- 218.

Caanen, M. R., E. A. Kuijper, and P. G. Hompes. 2016. Mass spectrometry methods measured androgen and estrogen concentrations during pregnancy and in newborns of mothers with polycystic ovary syndrome. Eur. J. Endocrinol. 174:25–32. PMID: 26586837.

Canadian Council on Animal Care. 2009. Guidelines on the care and use of farm animals in research. teaching and testing. https://www.ccac.ca/en/about-the-ccac/reports-and-publications/annual-reports.html. Accessed August 29, 2020.

Canadian Dairy Information Centre 2020.Culling and replacement rates in dairy herds in Canada. https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/dairy-animal-genetics/culling-and-replacement-rates-in-dairy-herds-in-canada/?id=1502475693224. Accessed on June 11, 2020.

Canadian Dairy Information Centre. 2020. https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/dairy-animal-genetics/culling-and-replacement-rates-in-dairy-herds-in-canada/?id=1502475693224; Accessed on June 11, 2020.

Canadian Dairy Information Centre, 2020. https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/dairy-animal-genetics/culling-and-replacement-rates-in-dairy-herds-in-canada/?id=1502475693224. Accessed on November 27, 2020.

Canadian Dairy Information Center. 2020a. https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/farm-statistics/historical-milk-production/?id=1562867429638; Accessed on June 20, 2020.

Canadian Dairy Information Center. 2020b. https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/dairy-animal-genetics/culling-and-replacement-rates-in-dairy-herds-in-canada/?id=1502475693224; Accessed on June 20, 2020.

Canadian Dairy Information Centre. 2020c. https://www.dairyinfo.gc.ca/eng/about-the-canadian-dairy-information-centre/canada-s-dairy-industry-at-a-glance/?id=1502465180911; accessed on June 20, 2020.

Canadian Dairy Network. 2015. Age at first calving and profitability https://www.cdn.ca/articles.php; accessed on June 20, 2020.

Canadian Dairy Network. 2017. Genetic gain before and after genomics. Canadian Dairy Network Report. https://www.cdn.ca/document.php?id=468; accessed on June 20, 2020.

Canadian Dairy Network. 2020. https://www.cdn.ca/document.php?id=443. Accessed on June 20, 2020.

Carrelli, J.E., M. Gobikrushanth, M.G. Colazo, and D.J. Ambrose. 2020. Characterization of ano-genital distance and its relationship to fertility in Holstein heifers. J. Dairy Sci. 103 (1):245.

Chelikani, P. K., J. D. Ambrose, and J. J. Kennelly. 2003. Effect of dietary energy and protein density on body composition, attainment of puberty, and ovarian follicular dynamics in dairy heifers. Theriogenology.60:07–725. https://doi.org/10.1016/s0093-691x(03)00088-8.

Clarke, I. J., R. J. Scaramuzzi, and R. V. Short. 1976. Effects of testosterone implants in pregnant ewes on their female offspring. J. Embryol. Exp. Morphol.36:87–99. PMID: 988109.

Clemens, L. G., B. A., Gladue, and L. P. Coniglio. 1978. Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. Horm Behav.10:40-53.

Colazo, M. G., and R. J. Mapletoft. 2014. A review of current timed-AI programs for beef and dairy cattle. Can Vet J. 55:772–280. PMID: 25082993.

Cole, J. B., and P. M. VanRaden. 2018. Symposium review: Possibilities in an age of genomics: The future of selection indices. J. Dairy Sci. 101:3686–3701. https: / / doi .org/ 10 .3168/ jds .2017 -13335.

Comim, F. V., K. Hardy, J. Robinson, and S. Franks. 2015. Disorders of follicle development and steroidogenesis in ovaries of androgenised foetal sheep. J. Endocrinol. 225:39–46.

Cooper, T. A., G. Wiggans, D. Null, J. Hutchison, and J. Cole. 2014. Genomic evaluation, breed identification, and discovery of a haplotype affecting fertility for Ayrshire dairy cattle. J. Dairy Sci. 97:3878–3882.

Curran, S., J. P. Kastelic, and O. J. Ginther. 1989. Determining sex of the bovine fetus by ultrasonic assessment of the relative location of the genital tubercle. Anim. Reprod Sci. 19:217-227. https://doi.org/10.1016/0378-4320(89)90095-X.

Cushman, R. A., M. F. Allan, L. A. Kuehn, W. M. Snelling, A. S. Cupp, and H. C. Freetly. 2009. Evaluation of antral follicle count and ovarian morphology in crossbred beef cows: investigation of influence of stage of the estrous cycle, age, and birth weight. J. Anim Sci. 87:1971– 1980. PMID: 19286826.

Dairy Farmers of Canada. 2018 Canadian dairy sector overview. https://www.dairyfarmers.ca/content/download/5731/51857/version/1/file/DFC+201+Dairy+Sector+Overview.pdf. Accessed on June 2020.

Darwash, A. O., Lamming, G. E, & Wooliams, J. A. 1997. The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. Anim. Sci. 65: 9-16.

Day, M. L., K. Imakawa, P. L. Wolfe, R. J. Kittok, and J. E. Kinder. 1987. Endocrine mechanisms of puberty in heifers. Role of hypothalamo-pituitary estradiol receptors in the negative feedback ofestradiol on luteinizing hormone secretion. Biol. Reprod. 37:1054–1065. https://doi.org/10.1095/biolreprod37.5.1054.

De Vries, M. J., and R. F. Veerkamp. 2000. Energy balance of dairy cattle in relation to milk production variables and fertility. J. Dairy Sci. 83:62-69.

Desjardins, C., and H. D. Hafs. 1969. Maturation of bovine female genitalia from birth through puberty. J. Anim. Sci. 28:502-507.

Diskin, M. G., and J. M. Sreenan. 2000. Expression and detection of oestrus in cattle. Reprod. Nutr. Dev. 40:481-491.

Domenici, L., A. Musella, C. Bracchi, F. Lecce, M. C. Schiavi, V. Colagiovanni, V. D. Donato, C. Marchetti, F. Tomao, I. Palaia, L. Muzii, and P. B. Panici. 2018. Comparison of anogenital distance and correlation with vulvo-vaginal atrophy: A pilot study on premenopausal and postmenopausal. Women. J. Menopausal Med. 24:108–112.

Drickamer, L. C., R. D. Arthur, and T. L. Rosenthal. 1997. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. J. Anim. Sci. 75:2192–2196. https://doi.org/10.2527/1997.7582192x.

Dušek, A., and L. Bartoš. 2012. Variation in Ano-Genital Distance in Spontaneously Cycling Female Mice. Reprod. Domest. Anim. 47:984-987.

Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68–78.

Eisenberg, M. L., Jensen, T. K., Walters, R. C., Skakkebaek, N. E., & Lipshultz, L. I. 2012. The relationship between anogenital distance and reproductive hormone levels in adult men. J Urol. 187:594-598.

Evans, A. C., F. Mossa, S. W. Walsh, D. Scheetz, F. Jimenez-Krassel, J. L. Ireland, G. W. Smith, and J. J. Ireland. 2012. Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. Reprod Domest Anim. 47:31–37.

Faber, K. A., and C. L. Jr. Hughes. 1992. Anogenital distance at birth as a predictor of volume of the sexually dimorphic nucleus of the preoptic area of the hypothalamus and pituitary responsiveness in castrated adult rats, Biol. Reprod. 46:101-104. PMID: 1547306.

Felton, C. A., M. G. Colazo, P. Ponce-Barajas, C. J. Bench, and D. J. Ambrose. 2012. Dairy cows continuously-housed in tie-stalls failed to manifest activity changes during estrus. Can. J. Anim. Sci. 92:189-196. https://doi.org/10.4141/cjas2011-134.

Ferguson, J. D., and A. Skidmore. 2013. Reproductive performance in a select sample of dairy herds. J. Dairy Sci. 96:1269-1289. https://doi.org/10.3168/jds.2012-5805.

Ferris, C. P., D. C. Patterson, F. J. Gordon, S. Watson, and D. J. Kilpatrick. 2014. Calving traits, milk production, body condition, fertility, and survival of Holstein-Friesian and Norwegian Red dairy cattle on commercial dairy farms over 5 lactations. J. Dairy Sci. 97:5206-5218. https://doi.org/10.3168/jds.2013-7457.

Fetrow, J., and S. Eicker. 2003. High Production and Health- A Curious Paradox. Bov. Practitioner 37:128–136.

Fleming, A., C. F. Baes, A. A. A. Martin, T. C. S. Chud, F. Malchiodi, L. F. Brito, and F. Miglior. 2019. Symposium review: The choice and collection of new relevant phenotypes for fertility selection. J. Dairy Sci. 102:3722-3734.

Food and Agricultural Organization of the United Nations. 2020a. Gateway to dairy production and products. http://www.fao.org/dairy-production-products/production/en/. Accessed on December 24, 2020.

Food and Agricultural Organization of the United Nations. 2020b. Overview of global dairy market developments in 2019, 2020. Accessed on December 11, 2020 http://www.fao.org/3/ca8341en/CA8341EN.pdf.

Forsdike, R. A., K. Hardy, L. Bull, J. Stark, L. J. Webber, S. Stubbs, J. E. Robinson, and S. Franks. 2007. Disordered follicle development in ovaries of prenatally androgenized ewes. J. Endocrinol. 192:421–8.

Fortune, J. E. 1986. Bovine Theca and Granulosa Cells Interact to Promote Androgen Production, Biol. Reprod.35:292–299.https://doi.org/10.1095/biolreprod35.2.292.

Gaiani, R., F. Chiesa, M. Mattioli, G. Nannetti, and G. Galeati. 1984. Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. J. Reprod. Fert. 70:55–59. PMID: 6694152.

García-Ruiz, A., J. B. Cole, P. M. VanRaden, G. R. Wiggans, F. J. Ruiz-López, and C. P. Van Tassell. 2016. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proc. Natl. Acad. Sci. 113:E3995–E4004. https://doi.org/10.1073/pnas.1519061113.

Garmo, R. T., A. O. Refsdal, K. Karlberg, E. Ropstad, A. Waldmann, J. F. Beckers, and O. Reksen. 2008. Pregnancy Incidence in Norwegian Red Cows Using Nonreturn to Estrus, Rectal

Palpation, Pregnancy-Associated Glycoproteins, and Progesterone. J. Dairy Sci. 91:3025-3033. https://doi.org/10.3168/jds.2007-0778.

Garmo, R. T., Ropstad, E., Havrevoll, Ø., Thuen, E., Steinshamn, H., Waldmann, A., and Reksen, O. 2009. Commencement of luteal activity in three different selection lines for milk yield and fertility in Norwegian Red cows. J. Dairy Sci. 92: 2159-2165.

Gill, J. W., and B. J. Hosking, 1995. Acute prenatal androgen treatment increases birth weights and growth rates in lambs. J. Anim. Sci. 73:2600-2608.

Gobikrushanth, M., P. A. Dutra, T. C. Bruinje, M. G. Colazo, S. T. Butler, and D. J. Ambrose. 2017a. 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. Theriogenology. 92:90–94.

Gobikrushanth, M., T. C. Bruinje, M. G. Colazo, S. T. Butler, and D. J. Ambrose. 2017b. Characterization of anogenital distance and its relationship to fertility in lactating Holstein cows. J. Dairy Sci. 100:9815–9823.

Gobikrushanth, M., D. C. Purfield, M. G. Colazo, S. T. Butler, Z. Wang, and D. J. Ambrose. 2018. The relationship between serum anti-Müllerian hormone (AMH) concentrations and fertility, and genome wide associations for AMH in Holstein dairy cows. J. Dairy Sci. 101:7563–7574.

Gobikrushanth, M., D. C. Purfield, J. Kenneally, R. C. Doyle, S. A. Holden, P. M. Martinez, E. R. Canadas, T. C. Bruinjé, M. G. Colazo, D. J. Ambrose, and S. T. Butler. 2019. The relationship between anogenital distance and fertility, and genome-wide associations

for anogenital distance in Irish Holstein-Friesian cows. J. Dairy Sci. 102:1702–1711. https://doi.org/10.3168/jds.2018-15552.

Gutierrez, K., R. Kasimanickam, A. Tibary, J. M. Gay, J. P. Kastelic, J. B. Hall, and W. D. Whittier. 2014. Effect of reproductive tract scoring on reproductive efficiency in beef heifers bred by timed insemination and natural service versus only natural service. Theriogenology. 81:918-24.

Häggman, J., J. M. Christensen, E. A. Mäntysaari, and J. Juga. 2019. Genetic parameters for endocrine and traditional fertility traits, hyperketonemia and milk yield in dairy cattle. Animal. 13:248-255. https://doi.org/10.1017/S1751731118001386.

Hansen, L. B. 2000. Consequences of selection for milk yield from a geneticist's viewpoint. J. Dairy Sci. 83:1145–1150. PMID: 10821591.

Hasler, J. 2010. Bovine embryo transfer: are efficiencies improving? Applied Reproductive Strategies Conference Proceedings August 5th and 6th, 2010 Nashville, TN in the cow. Biol. Reprod. 80:50–9.

Hasler, J. F. 2003. The current status and future of commercial embryo transfer in cattle. Anim. Reprod. Sci. 79:245-264. https://doi.org/10.1016/S0378-4320(03)00167-2.

Hayes, B. J., P. J. Bowman, A. J. Chamberlain, and M. E. Goddard. 2009. Genomic selection in dairy cattle: Progress and challenges. J. Dairy Sci. 92:433-443. PMID: 19164653.

Henricks, D. M., J. F. Dickey, and J. R. Hill. 1971. Plasma estrogen and progesterone levels in cows prior to and during estrus. Endocrinology. 89:1350-1355.

Holm, D. E., P. N. Thompson, and P. C. Irons. 2009. The value of reproductive tract scoring as a predictor of fertility and production outcomes in beef heifers. J. Anim. Sci. 87:1934–1940. https://doi.org/10.2527/jas.2008-1579.

Hotchkiss, A. K., C. S. Lambright, J. S. Ostby, L. Parks-Saldutti, J. G. Vandenbergh, and L. E. Gray Jr. 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol. Sci. 96:335–345. https://doi.org/10.1093/toxsci/kfm002.

Hotchkiss, A. K., C. S. Lambright, J. S. Ostby, L. Parks-Saldutti, J. G. Vandenbergh, and L. E. Gray. 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague–Dawley rats. Toxicol. Sci. 96:335– 345. PMID: 17218470.

Ireland, J. J., F. Ward, F. Jimenez-Krassel, J. L. Ireland, G. W. Smith, P. Lonergan, and A. C. O. Evans. 2007. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. Hum. Reprod. 22:1687–1695. PMID: 17468258.

Ireland, J. L. H., D. Scheetz, F. Jimenez-Krassel, A. P. N. Themmen, F. Ward, P. Lonergan, G. W. Smith, G. I. Perez, A. C. Evans, and J. J. Ireland. 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. Biol. Reprod. 79:1219–25. https://doi.org/10.1095/biolreprod.108.071670.

Ireland, J. J., G. W. Smith, D. Scheetz, F. Jimenez-Krassel, J. K. Folger, J. L. Ireland, F. Mossa, P. Lonergan, and A. C. Evans. 2011. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. Reprod Fertil Dev. 23:1–14.

Ismael, A., E. Strandberg, M. Kargo, A. Fogh, and P. Lovendahl. 2015. Estrus traits derived from activity measurements are heritable and closely related to the time from calving to first insemination. J. Dairy Sci. 98:3470–3477. https://doi.org/10.3168/jds.2014-8940.

Jainudeen, M. R., and S. E. Hafez. 1965. Attempts to induce bovine freemartinism experimentally. J. Reprod. Fertil. 10:281–283. https://doi.org/10.1530/jrf.0.0100281.

Johnson, H. W. 2008. Managing Anovulation and Cystic Ovaries in Dairy Cows. WCDS Advances in Dairy Technology. 20:311-326.

Kamimura, S., S. Kondoh, S. Enomoto, H. Sameshima, K. Goto, and K. Hamana.1994. Ultrasonic diagnosis of bovine fetal sex by the relative location of the genital tubercle in early pregnancy. J Reprod Dev. 40:343-347. https://doi.org/10.1262/jrd.40.343.

Kanova, N., and M. Bicikova. 2011. Hyperandrogenic states in pregnancy. Physiol Res. 60:243–252. PMID: 21114372.

Kinder, J. E., E. G. M. Bergfeld, M. E. Wehrman, K. E. Peters, and F. N. Kojima. 1995. Endocrine basis for puberty in heifers and ewes. J. Reprod. Fertil. 49:393-407.

Kobayashi, A., and R. R. Behringer. 2003. Developmental genetics of the female reproductive tract in mammals. Nat Rev Genet. 4:969–980. PMID: 14631357.

Kordić, B., S. Pribićević, M. Muntanola-Cvetković, P. Nikolić, and B. Nikolić. 1992. Experimental study of the effects of known quantities of zearalenone on swine reproduction. J Environ Pathol. Toxicol. Oncol. 11:53–55.

Koyama, K., T. Koyama, and M. Sugimoto. 2018. Repeatability of antral follicle count according parity in dairy cows. J.Reprod Devel. 64:535-539. https://doi: 10.1262/jrd.2018-062

Kuhn, M. T., J. L. Hutchison, and G. R. Wiggans. 2006. Characterization of Holstein Heifer fertility in the United States. J. Dairy. Sci. 89:4907– 4920.

Lacroix, E., W. Eechaute, and I. Leusen. 1974. The biosynthesis of estrogens by cow follicles. Steroids. 23:337-356.

Lamm, C. G., P. M. Hastie, N. P. Evans, and J. E. Robinson. 2012. Masculinization of the distal tubular and external genitalia in female sheep with prenatal androgen exposure. Vet Pathol. 49:546-551.

LeBlanc, S. 2010. Assessing the association of the level of milk production with reproductive performance in dairy cattle. J. Reprod. Dev. 56:1–7. PMID: 20629210.

LeBlanc, S. 2013. Is a high level of milk production compatible with good reproductive performance in dairy cows? Animal Frontiers. 3:84–91.

Lillie, F.R. 1917. The free-martin; a study of the action of sex hormones in the foetal life of cattle. J. Exp. Zool. 23:371-452. https://doi.org/10.1002/jez.1400230208.

Lim, J. J., Y. C. Han, R. D. Lee, and K. B. Tsang. 2017. Ring Finger Protein 6 Mediates Androgen-Induced Granulosa Cell Proliferation and Follicle Growth via Modulation of Androgen Receptor Signaling. Endocrinology. 158:993–1004.

Lima, F. S., F. T. Silvestre, F. Peñagaricano, and W. W. Thatcher. 2020. Early genomic prediction of daughter pregnancy rate is associated with improved reproductive performance in Holstein dairy cows. J. Dairy Sci. 103:3312-3324.

Lopez, H., L. D. Satter, and M. C. Wiltbank. 2004. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim. Reprod. Sci. 81:209–223. PMID: 14998648.

Lucy, M. C. 2001. Reproductive loss in high producing dairy cattle: Where will it end? J. Dairy Sci. 84:1277-1293. PMID: 11417685.

Matt, D. W., & Macdonald, G. J. 1984. In vitro progesterone and testosterone production by the rat placenta during pregnancy. Endocrinology. 115:741-747.

Ma, L., J. B. Cole, Y. Da, and P. M. VanRaden. 2019. Symposium review: Genetics, genome-wide association study, and genetic improvement of dairy fertility traits. J Dairy Sci. 102:3735-3743. https://doi.org/10.3168/jds.2018-15269.

Maculan, R., T. L. C. Pinto, G. M. Moreira, G. L. de Vasconcelos, J. A. Sanches, R. C. Rosa, R. R. Bonfim, T. deM. Goncalves, and J. C. de Souza. 2018. Anti-Müllerian hormone (AMH), antral follicle count (AFC), external morphometrics and fertility in Tabapuã cows. Anim. Reprod. Sci. 189:84–92.

Meisel, R. L., and I. L. Ward. 1981. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science, 213: 239- 242.

Mendiola, J., M. Roca, L. Mınguez-Alarcon, M. P. Mira-Escolano, J. J. Lopez-Espın, E. S. Barrett, S. H. Swan, and A. M. Torres-Cantero. 2012. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. Environ. Health. 11:90. https://doi.org/10.1186/1476-069X-11-90.

Miglior, F. 2007. Genetic evaluation of reproductive performance in Canadian dairy cattle. Ital. J. Anim. Sci. 6:29–37.

Miglior, F., A. Fleming, F. Malchiodi, L. F. Brito, P. Martin, and C. F. Baes. 2017. A 100-year review: Identification and genetic selection of economically important traits in dairy cattle. J. Dairy Sci. 100:10251–10271. PMID: 29153164.

Mira-Escolano, M. P., Mendiola, J., Mínguez-Alarcón, L., Melgarejo, M., Cutillas-Tolín, A., Roca, M., López-Espín, J. J., Noguera-Velasco, J. A., and Torres-Cantero, A. M. 2014. Longer anogenital distance is associated with higher testosterone levels in women: a cross-sectional study. BJOG. 121:1359–1364.

Mongkonpunya, K., Y. C. Lin, P. A. Noden, W. D. Oxender, and H. D. Hafs. 1975. Androgens in the Bovine Fetus and Dam. Proceedings of the society for experimental biology and medicine. 148:489–493. https://doi.org/10.3181%2F00379727-148-38567.

Mossa, F., S. Walsh, S. Butler, D. Berry, F. Carter, P. Lonergan, G. Smith, J. Ireland, and A. Evans. 2012. Low numbers of ovarian follicles ≥3mm in diameter are associated with low fertility in dairy cows. J. Dairy Sci. 95:2355–2361. PMID: 22541464.

Najdi, N., F. Safi, S. Hashemi-Dizaji, G. Sahraian, and Y. Jand. 2019. First trimester determination of fetal gender by ultrasonographic measurement of anogenital distance: A cross-sectional study. Int. J. Reprod. Biomed. 17:51–56.

National Research Council 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, 198, Washington, DC.

Norman, H. D., J. L. Hutchison, J. R. Wright, and M. T. Kuhn. 2007. Selection of yield and fitness traits when culling Holsteins during the first three lactations. J. Dairy Sci. 90:1008–1020.

Norman, H. D., J. R. Wright, S. M. Hubbard, R. H. Miller, and J. L. Hutchison. 2009. Reproductive status of Holstein and Jersey cows in the United States. J. Dairy Sci. 92:3517–3528. https://doi.org/10.3168/jds.2008-1768.

Nowicki, A., W. Barański, A. Baryczka, and T. Janowski. 2017. OvSynch protocol and its modifications in the reproduction management of dairy cattle herds -an update. J. Vet. Res. 61:329-336.

Ontario Ministry of Agriculture, Food and Rural affairs. 2020. http://www.omafra.gov.on.ca/english/livestock/dairy/facts/cullcowwelfare.htm. Accessed on January 24, 2021

Opsomer, G., Y. T. Gröhn, J. Hertl, M. Coryn, H. Deluyker, and A. de Kruif. 2000. Risk factors for post-partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. Theriogenology. 53:841-857.

Padmanabhan, V., M. Manikkam, S. Recabarren, and D. Foster. 2006. Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. Molec. Cell. Endocrinol. 246:165-174.

Padmanabhan V., Veiga-Lopez A. 2013. Sheep models of polycystic ovary syndrome phenotype. Mol. Cell. Endocrinol. 373:8–20. https://doi.org/10.1016/j.mce.2012.10.005.

Padmanabhan, V., A. Veiga-Lopez, C. Herkimer, B. Abi Salloum, J. Moeller, E. Beckett, and R. Sreedharan. 2015. Developmental programming: prenatal and postnatal androgen antagonist and insulin sensitizer interventions prevent advancement of puberty and improve LH surge dynamics in prenatal testosterone-treated sheep. Endocrinology. 156:2678–92. https://doi.org/10.1210/en.2015-1235.

Papadopoulou, E., M. Vafeiadi, S. Agramunt, X. Basagaña, K. Mathianaki, P. Karakosta, A. Spanaki, A. Koutis, L. Chatzi, M. Vrijheid, and M. Kogevinas. 2013. Anogenital distances in newborns and children from Spain and Greece. Paediatr. Perinat. Epidemiol. 27:89-99. https://doi.org/10.1111/ppe.12022.

Petersson, K. J., Berglund, B., Strandberg, E., Gustafsson, H., Flint, A. P. F., Woolliams, J. A., & Royal, M. D. 2007. Genetic analysis of postpartum measures of luteal activity in dairy cows. J. Dairy Sci. 90: 427-434.

Philipsson, J. 1981. Genetic aspects of female fertility in dairy cattle. Livest. Prod. Sci. 8:307–319. https://doi.org/10.1016/0301-6226(81)90049-X

Priskorn, L., J. H. Petersen, N. Jørgensen, H. B. Kyhl, M. S. Andersen, K. M. Main, A. M. Andersson, N. E. Skakkebaek, and T. K. Jensen. 2018. Anogenital distance as a phenotypic signature through infancy. Pediatr. Res. 83:573–579.

Pryce, J. E., S. Bolormaa, A. J. Chamberlain, P. J. Bowman, K.Savin, M. E. Goddard, and B. J. Hayes. 2010. A validated genome-wide association study in 2 dairy cattle breeds for milk production and fertility traits using variable length haplotypes. J. Dairy Sci. 93:3331–3345. PMID: 20630249.

Rabiee, A. R., K. L. Macmillan, and F. Schwarzenberger. 2002. Plasma, milk and faecal progesterone concentrations during the oestrus cycle of lactating dairy cows with different milk yields. Anim Reprod Sci. 74:121– 131. PMID: 12417115.

Redden, K. D., A. D. Kennedy, J. R. Ingalls, and T. L. Gilson. 1993. Detection of estrus by radiotelemetric monitoring of vaginal and ear skin temperature and pedometer measurements of activity. J Dairy Sci. 76:713-721.

Rico, C., S. Fabre, C. Médigue, N. di Clemente, F. Clément, M. Bontoux, J. L. Touzé, M. Dupont, E. Briant, B. Rémy, J. F. Beckers, and D. Monniaux. 2009. Anti-müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. Biol. Reprod. 80:50–59.

Ristic, N., N. Nestorovic, M. Manojlovic-Stojanoski, B. Filipovic, B. Sosic-Jurjevic, V. Milosevic, and M. Sekulic. 2008. Maternal dexamethasone treatment reduces ovarian follicle number in neonatal rat offspring. J. Microsc. 232:549–557.

Robertson, H. A. 1972. Sequential changes in plasma progesterone in the cow during the estrous cycle, pregnancy, at parturition, and postpartum. Can. J. Anita. Sci. 52:645.

Roth, Z. 2018. Symposium review: Reduction in oocyte developmental competence by stress is associated with alterations in mitochondrial function. J. Dairy Sci. 101:3642-3654.

Salazar-Martinez, E., P. Romano-Riquer, E. Yanez-Marquez, M. P. Longnecker, and M. Hernandez-Avila. 2004. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. Environ. Health. 3:8.

Sangsritavong, S., D.K. Combs, R. Sartori, and M.C. Wiltbank. 2002. High feed intake increases blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. J. Dairy Sci. 85:2831–2842.

Santos, M. H., E. P. Moraes, and F. Q. Bezerra. 2007. Early fetal sexing of Saanen goats by use of transrectal ultrasonography to identify the genital tubercle and external genitalia. Am J Vet Res. 68:561-564. https://doi.org/10.2460/ajvr.68.5.561.

Savabieasfahani, M., J. S. Lee, C. Herkimer, T. P. Sharma, D. L. Foster, and V. Padmanabhan. 2005. Fetal programming: testosterone exposure of the female sheep during midgestation disrupts the dynamics of its adult gonadotropin secretion during the periovulatory period. Biol. Reprod.72: 221-229.

Schuler, G., R. Fürbass, and K. Klisch. 2018. Placental contribution to the endocrinology of gestation and parturition. Anim Reprod. 15:822-842. https://doi.org/10.5167/uzh-167451.

Sharma. T. P., C. Herkimer, C. West, W. Ye, R. Birch, J. E. Robinson, D. L. Foster, and V. Padmanabhan. 2002. Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. Biol Reprod. 66:924–933.

Shook, G. E. 1989. Selection for disease resistance. J. Dairy Sci. 72:1349–1362.

Smith, J. F., R. J. Fairclough, E. Payne, and A. J. Peterson. 1975. Plasma hormone levels in the cow. New Zeal J. Agr. Res. 18:123-129.

Smith, P., T. L. Steckler, A. Veiga-Lopez, and V. Padmanabhan. 2009. Developmental programming: differential effects of prenatal testosterone and dihydrotestosterone on follicular recruitment, depletion of follicular reserve, and ovarian morphology in sheep. Biol. Reprod. 80:726–36. https://doi.org/10.1095/biolreprod.108.072801.

Souza, A. H., P. D. Carvalho, A. E. Rozner, L. M. Vieira, K. S. Hackbart, R. W. Bender, A. R. Dresch, J. P. Verstegen, R. D. Shaver, and M. C. Wiltbank. 2015. Relationship between circulating anti-Müllerian hormone (AMH) and superovulatory response of high-producing dairy cows. J. Dairy Sci. 98:169–178.

Spencer, T. E., P. J. Hansen, J. B. Cole, J. Dalton, and H. Neibergs. 2014. Genomic selection and reproductive efficiency in dairy cattle. In: Proc. Dairy Cattle Reproduction Council Annual Conference, Salt Lake City, UT. 16-31.

Statista, 2019. Average net operating income of dairy farms in Canada 2013 – 2018. https://www.statista.com/statistics/468327/average-net-operating-income-of-dairy-farms-in-canada/; accessed on January 22, 2021.

Steckler, T., J. Wang, F. F. Bartol, S. K. Roy, and V. Padmanabhan. 2005. Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. Endocrinology. 146:3185–93.

Steckler, T. L., E. K. Roberts, D. D. Doop, T. M. Lee, and V. Padmanabhan. 2007. Developmental programming in sheep: administration of testosterone during 60–90 days of pregnancy reduces breeding success and pregnancy outcome. Theriogenology. 67:459-467.

Stringfellow, D. A., and M. D. Givens. 2010. Manual of the International Embryo Transfer Society (IETS). 4th ed.Champaign, IL: IETS.

Swan, S. H. 2008. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ. Res. 108:177–184. https://doi.org/10.1016/j.envres.2008.08.007.

Taylor, V. J., Z. Cheng, P. G. Pushpakumara, D. E. Beever, and D. C. Wathes. 2004. Relationships between the plasma concentrations of insulin-like growth factor-1 in dairy cows and their fertility and milk yield. Vet. Rec. 155:583–588.

Tenghe, A. M. M., A. C. Bouwman, B. Berglund, E. Strandberg, J.Y. Blom, and R. F.Veerkamp. 2015. Estimating genetic parameters for fertility in dairy cows from in-line milk progesterone profiles. J. Dairy Sci. 98:5763–73. https://doi.org/10.3168/jds.2014-8732.

Thankamony, A., K. K. Ong, D. B. Dunger, C. L. Acerini, and L. A. Hughes. 2009. Anogenital distance from birth to 2 years: a population study. Environ. Health Perspect. 117:1786-90. https://doi.org/10.1289/ehp.0900881.

USDA. 2019. Daughter pregnancy rate evaluation of cow fertility. https: / / aipl .arsusda .gov/ reference/ fertility/ DPR_rpt.htm. Accessed November 25, 2020.

VanRaden, P., M. A. H. Sanders, M. E. Tooker, R. H. Miller, H. D. Norman, M. T. Kuhn, and G. R. Wiggans. 2004**.** Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285-2292. https://doi.org/10.3168/jds.S0022-0302(04)70049-1.

VanRaden, P. M., K. Olson, D. Null, and J. Hutchison. 2011. Harmful recessive effects on fertility detected by absence of homozygous haplotypes. J. Dairy Sci. 94:6153–6161.

Veerkamp, R. F., and B. Beerda. 2007. Genetics and genomics to improve fertility in high producing dairy cows. Theriogenology. 68:S266-S273.

Veiga-Lopez, A., O. I. Astapova, E. F. Aizenberg, J. S. Lee, and V. Padmanabhan. 2009. Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep.Biol Reprod. 80:718-725. https://doi.org/10.1095/biolreprod.108.074781.

Veiga-Lopez, A., J. Moeller, D. Patel, W. Ye, A. Pease, J. Kinns, and V. Padmanabhan. 2013. Developmental programming: impact of prenatal testosterone excess on insulin sensitivity, adiposity, and free fatty acid profile in postpubertal female sheep. Endocrinology. 154:1731–1742. PMID: 23525243.

Velazquez, M. A., L. J. Spicer, and D. C. Wathes. 2008. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. Domest. Anim. Endocrinol. 35:325–342.

Veyssiere, G., M. Berger, C. Jean-Faucher, D. M. Turckheim, and C. Jean. 1985. Androgen receptor in genital tubercle of rabbit fetuses and newborns. Ontogeny and properties. J. Steroid Biochem. 23:399-404. DOI: https://doi.org/10.1016/0022-4731(85)90185-2.

vom Saal, F. S. 1981. Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. Reproduction. 62:633-650.

vom Saal, F. S., and F. H. Bronson. 1978. In utero proximity of female mouse fetuses to males: Effect on reproductive performance during later life. Biol. Reprod. 19:842-853. PMID: 743525.

Walsh, S.W., E. J. Williams, and A. C. O. Evans. 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. 123: 127-138. PMID: 2125520.

Walsh, S. W., F. Mossa, S. T. Butler, D. P. Berry, D. Scheetz, F. Jimenez-Krassel, R. J.Tempelman, F. Carter, P. Lonergan, A. C. Evans, and J. J. Ireland. 2014. Heritability and impact of environmental effects during pregnancy on antral follicle count in cattle. J. Dairy Sci. 97:4503–4511. PMID: 24835969.

Wiggans, G. R., P. M. VanRaden, and T. A. Cooper. 2011. The genomic evaluation system in the United States: Past, present, future. J. Dairy Sci. 94:3202–3211. https: / / doi .org/ 10 .3168/ jds .2010-3866.

Wiltbank, M. C., A. Gümen, and R. Sartori, 2002. Physiological classification of anovulatory conditions in cattle. Theriogenology. 57:21–52.

Wiltbank, M., H. Lopez, R. Sartori, S. Sangsritavong, and A. Gumen. 2005. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology 65:17–29.

Wolak, M. E., Fairbairn, D. J., & Paulsen, Y. R. 2012. Guidelines for estimating repeatability. Ecol. Evol. 3:129-137.

Wolfenson, D., Z. Roth, and R. Meidan. 2000. Impaired reproduction in heat-stressed cattle: basic and applied aspects. Anim. Reprod. Sci. 60–61:535–547.

Wolfenson, D., and Z. Roth 2019. Impact of heat stress on cow reproduction and fertility. Animal Frontiers. 9:32-38.

Wu, Y., G. Zhong, S. Chen, C. Zheng, D. Liao, and M. Xie. 2017. Polycystic ovary syndrome is associated with anogenital distance, a marker of prenatal androgen exposure. Hum. Reprod. 32:937–943. https://doi.org/10.1093/humrep/dex042.

Young, C. D., F. N. Schrick, K. G. Pohler, A. M. Saxton, F. A. Di Croce, D. A. Roper, J. B. Wilkerson, and J. L. Edwards. 2017. Short communication: A reproductive tract scoring system to manage fertility in lactating dairy cows. J. Dairy Sci.100:5922–5927.

Zehr, J. L., S. E. Gans, and M. K. McClintock. 2001. Variation in reproductive traits is associated with short anogenital distance in female rats. Dev. Psychobiol. 38:229–238. PMID: 11319729.

Zhao, F., and H. H. Yao. 2019. A tale of two tracts: history, current advances, and future directions of research on sexual differentiation of reproductive tracts. Biol. Reprod. 101:602-616. https://doi.org/10.1093/biolre/ioz079.