

Exploration of anogenital distance as a novel fertility phenotype in dairy cattle

by

Jennifer E. Carrelli

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

© Jennifer E. Carrelli, 2021

Abstract

Over the last 40 years, there has been an obvious decline in reproductive performance and fertility in dairy cattle, highlighting the importance of genetic improvement in this area. With more recent technological advancements, opportunity arises to identify and incorporate novel fertility traits, with the potential to complement current traits, into breeding objectives in an effort to achieve more substantial genetic gain for fertility. Recently, anogenital distance (AGD), measured as the distance from the centre of the anus to the base of the clitoris, has shown to have an inverse association with measures of fertility in dairy cows. Two studies were conducted to explore the associations between anogenital distance and measures of fertility in a larger population of dairy heifers and cows from Western Canada and the USA.

The objectives of the first study (Chapter 3) were to (1) characterize AGD in nulliparous dairy heifers, and (2) determine if an inverse relationship between AGD and fertility, previously found in lactating dairy cows, is also evident in nulliparous heifers. AGD was normally distributed, highly variable, and inversely related with measures of fertility. Heifers with short AGD required fewer services per conception (1.5 ± 0.1 vs. 1.7 ± 0.1 ; $P < 0.01$), conceived earlier (14.9 ± 0.2 vs. 15.1 ± 0.2 mo; $P < 0.01$), and became pregnant to first artificial more often (AI; 58.3 ± 3.0 vs. 49.6 ± 3.1 %; $P < 0.001$) than their long-AGD counterparts. Moreover, heifers with long AGD had a lower relative risk for pregnancy up to 450 d of life compared with those with short AGD (hazard ratio: 0.59; $P < 0.001$). This study established that an inverse association between AGD and fertility exists in nulliparous heifers.

The second study (Chapter 4) aimed to validate findings that AGD is inversely related to measures of fertility in lactating Holstein cows. A secondary objective of this study was to

determine the association between AGD and milk yield. AGD was normally distributed, highly variable, and inversely associated with fertility measures. Cows with short AGD had improved pregnancy to first AI (35.7 ± 2.1 vs. 31.4 ± 2.0 %; $P < 0.01$) and fewer days open (136.9 ± 4.3 vs. 140.9 ± 4.3 d; $P = 0.05$) than cows with long AGD. Regardless of parity, cows with short AGD tended to require fewer services per conception (2.3 ± 0.1 vs. 2.4 ± 0.1 ; $P = 0.06$) than their long-AGD counterparts, but cumulative pregnancy risks up to 150 and 250 DIM did not differ between AGD categories. Anogenital distance had a weak positive association ($r = 0.04$; $P < 0.01$) with 305-d mature equivalent milk yield. The results of this study confirm an inverse relationship between AGD and measures of fertility in lactating cows, validating previous findings, with no evidence of parity effects. Moreover, results indicate that the phenotypic selection for AGD will not cause a substantial decline in milk production.

Overall, results from this Master's thesis research provide further insight into AGD and its associations with fertility on a larger scale, lending further support for AGD to become an indicator of fertility and a possible management tool in future selection programs.

Preface

This thesis is an original work by Jennifer E. Carrelli. The principal investigator of the studies addressed in Chapters 3 and 4 was Divakar J. Ambrose from the Department of Agricultural, Food and Nutritional Sciences, University of Alberta and the Livestock Research & Extension Branch, Alberta Agriculture and Forestry, Edmonton, AB, Canada. All animal use was approved by the University of Alberta's Animal Care and Use Committee for Livestock (AUP#00002883) as well as the University of Idaho Animal Care and Use Committee (Protocol #IACUC-2019-61).

Chapter 3 of this thesis has been submitted for publication to the *Journal of Dairy Science* (Manuscript #2020-19940; under second review at the time of writing this thesis) as “*Relationship of ano-genital distance with fertility in nulliparous Holstein heifers*” by J.E. Carrelli, M. Gobikrushanth, M. Corpron, I. Rajesh, W. Sandberg, M.G. Colazo, A. Ahmadzadeh, M. Oba, and D.J. Ambrose. The candidate was responsible for farm recruitment, performing data collection, data management, results interpretation and manuscript composition. M. Corpron, I. Rajesh, W. Sandberg aided in performing data collection. M.G. Colazo and A. Ahmadzadeh aided in data collection, reviewed, and provided important contributions with concept formation and manuscript edits. M. Gobikrushanth assisted with data collection, data analyses, and manuscript edits. M. Oba, co-supervisor, reviewed the manuscript and made contributions to strengthen the discussion. D.J. Ambrose was the supervisor and corresponding author involved in all steps of concept and objectives formation, data collection, review and interpretation of results, manuscript composition and revisions, and industry liaison.

Chapter 4 of this thesis, in a modified form, will be submitted as a complete research article for publication in the Journal of Dairy Science with the title “*Associations between anogenital distance and measures of fertility in lactating North American Holstein cows: a validation study*”. The candidate was responsible for performing data collection, data management, results interpretation and manuscript composition. The coauthors of this study will be M. Corpron, W. Sandberg for assisting in large-scale data collection, A. Ahmadzadeh for assisting in large-scale data collection and manuscript edits, M. Gobikrushanth for assisting with data collection, data analyses, and manuscript edits, and M. Oba for co-supervising and manuscript review. D.J. Ambrose will be the corresponding author and was involved in all steps of concepts formation, data collection, results review and interpretation, manuscript revisions, and industry liaison.

Dedications

This thesis is in dedication to my Grandparents, Rolph & Betty Hoefler and Ray & Pearl Carrelli, who nurtured my passion for animals and agriculture, and who have taught me the value of hard work and dedication. Thank you for always encouraging and supporting my ambitions, I am here today because of you.

In loving memory of Betty Hoefler and Jesse Haller

Acknowledgements

Firstly, I would like to acknowledge my mentor, Dr. Divakar Ambrose, for always providing me with the utmost support and encouragement. The last two years have been challenging both personally and academically, thank you for your support and understanding through these times, and for cheering me on along the way. I have grown exponentially as a professional as well as a person under your advisement, and I am very appreciative of that. I would also like to extend a special thank you to my co-supervisor, Dr. Masahito Oba, I am very thankful for your wisdoms, advice and invaluable expertise – especially in the last few months of my thesis program. I would also like to extend my deep appreciation and gratitude to Dr. Carolyn Fitzsimmons for agreeing to be my chair and arm’s length examiner.

This entire project would not have been possible without contributions from funding agencies including Alberta Agriculture and Forestry, Alberta Milk, Westgen Endowment Fund, Canadian Dairy Network, Lactanet, Holstein Canada, and the Canadian Dairy Commission. In addition, I would like to sincerely thank the twenty dairy producers who graciously opened their barn doors to myself and my colleagues, this project would not have been possible without your support.

This project would also not have been possible without members of the Ambrose Lab, Ahmadzadeh lab, and the Livestock Extension team at Alberta Agriculture and Forestry. Specifically, I’d like to thank my dear friend and colleague Dr. Mohanathas Gobikrushanth (Gobi) for his wisdom and support throughout my master’s program. He made every day and every experience a positive one and was my “go to” man for all things in dairy reproduction, statistics, and life. Thank you for all your help getting me to the finish line. I would also like to thank

Courtney Felton, Darrell Bignell and Iswarya Rajesh for helping me manage the 8,000 animals on my study! Courtney – I will never forget our trip to BC and the level of excitement you brought with you. Thank you for all of your advice throughout my program, being my confidant, and helping me clean out my road rash. Darrell, thank you for being my go-to “computer wizard”, and building me an application to efficiently sort and merge my data. Not only were you a computer wizard, but you were also first to jump in a truck and head out to a farm with me, and it was always accompanied by such enthusiasm. Thank you for being so supportive of my project and the great conversation we had along the way. Iswarya, thank you for being my road trip and travel companion to Kinsella, Idaho and Washington, and for always keeping me on my toes! I appreciate all of the effort you put in to helping me with my project. I would also like to thank Dr. Marcos Colazo for helping me on farm visits, but more importantly, for being a constant wealth of knowledge. Marcos, I admire your skill, but also your ability to teach and share your expertise. I would like to sincerely thank Dr. Amin Ahmadzadeh and his graduate students, McKenzie Corpron and Whitney Sandberg, for all of their help at 5D Farms. Thank you for all of your kindness, hospitality and humour – I wouldn’t have been able to measure 3000 cows and heifers in 2 days without your support!

I would like to extend my gratitude to Dr. Mike Dyck for his mentorship over the last 4 years – I wouldn’t be where I am today without you. Thank you for being so passionate and knowledgeable in your trade, you got me hooked on reproductive physiology and have provided me with irreplaceable opportunities, but most importantly, thank you for your utmost support along the way! Dr. Robinson, Frank, I don’t even know where to begin. You have been my biggest role model since my very first year at UofA in 2015. Your passion for Agriculture is as contagious as Covid-19! Thank you for continually checking in, thinking of me, encouraging me to do ‘bigger

and better' and providing me with the opportunities to succeed. I hope that someday I can provide the same support and encouragement to a young agricultural advocate.

To my encouraging and supportive family, I extend a huge thank you. Thank you to my parents, John and Anne Carrelli, for teaching me strong work ethic and dedication. You have always been my cheerleaders and I am so grateful for that immense level of support behind anything and everything I do. Thank you to my siblings, Bryce and Megan, for always being around when I needed you and a fun escape when I needed a break. I'll always be thankful that you two have given me the opportunity and great honour of being the best sibling.

Lastly, I would like to thank my friends. I don't think I could have made it through the last two years without you all! Thank you for being a distraction from the stress, constant source of entertainment, and for the flood of support towards my goals! I'm so thankful to have you by my side and proud to call you my friends.

Table of Contents

Abstract	ii
Preface	iv
Dedications	vi
Acknowledgements	vii
List of Tables	xiii
List of Figures	xiv
List of Abbreviations	xv
1.0 General Introduction	1
2.0 Review of Literature	4
2.1 Overview	4
2.2 Current Practices for Fertility Selection	5
2.2.1 Criterion for Selection	5
2.2.2 Categories of Reproductive Traits for Genetic Selection	7
2.3 Novel Reproductive Phenotypes	10
2.3.1 Progesterone-Based Measures	10
2.3.2 Estrus Expression and Activity Traits	12
2.3.3 Insulin-like Growth Factor 1	14
2.3.4 Anti-Müllerian Hormone	16
2.3.5 Antral Follicle Count	18
2.3.6 Ovarian Size	20
2.3.7 Reproductive Tract Size and Position Score	21
2.3.8 Anogenital Distance	22
2.4 Knowledge Gaps	25
3.0 Relationship of anogenital distance with fertility in nulliparous Holstein heifers	27

3.1 Introduction	27
3.2 Materials and Methods	29
3.2.1 Animals and Management	29
3.2.2 Determination of Anogenital Distance, Age, and Reproductive Measures	30
3.2.3 Statistical Analysis	31
3.3 Results	32
3.3.1 Descriptive Statistics	32
3.3.2 Optimum AGD Threshold to Predict Pregnancy to 1 st AI	33
3.3.3 Relationship Between AGD and Measures of Fertility	33
3.4 Discussion	34
3.5 Conclusions	39
4.0 Associations between anogenital distance and measures of fertility in lactating North American Holstein cows: a validation study	48
4.1 Introduction	48
4.2 Materials and Methods	50
4.2.1 Animals and Management	50
4.2.2 Determination of Anogenital Distance and Measures of Fertility	51
4.2.3 Statistical Analysis	52
4.3 Results	53
4.3.1 Descriptive Statistics	53
4.3.2 Association Between AGD, Parity, and 305-d Mature Equivalent Milk Yield ...	53
4.3.3 Relationship Between AGD, Parity, and Measures of Fertility	54
4.4 Discussion	54
4.5 Conclusions	60
5.0 General Discussion	67

5.1 Importance of the Current Study	67
5.2 Limitations	68
5.3 Future Research	70
5.4 Considerations	72
5.5 Conclusions	73
Literature Cited	75

List of Tables

Table 3-1: Descriptive statistics (mean \pm SD) for anogenital distance and measures of fertility in nulliparous heifers (n = 1692, from 17 dairy herds in Canada [CAN1 to CAN16] and the United States [USA1])	40
Table 3-2: Relationship between anogenital distance ¹ and various measures of fertility in nulliparous heifers of short- and long-AGD groups	42
Table 4-1: Descriptive statistics for anogenital distance (AGD) ¹ in lactating dairy cows from 19 herds in Canada and the United States.....	61
Table 4-2: Relationship between anogenital distance (AGD) and various measures of fertility in lactating cows of short- and long-AGD groups	62
Table 4-3: Relationship between parity groups and various measures of fertility in lactating cows	63

List of Figures

Figure 3-1: Distribution of anogenital distance (AGD; mm) in a population of nulliparous Holstein heifers (n = 1,692, from 17 dairy herds). Mean age at AGD measurement was 417.0 ± 45.1 d (13.9 ± 1.5 mo).....	43
Figure 3-2. Association between age at anogenital distance measurement and anogenital distance ($R^2 = 0.06$; $P < 0.01$) in nulliparous Holstein heifers (n = 1,692)	44
Figure 3-3: The estimated probability of pregnancy to first AI (P/1 st AI) plotted against anogenital distance (AGD) in nulliparous Holstein heifers (n = 1692). For every 1-unit (mm) increase in AGD, the estimated probability of pregnancy to first AI was reduced by 1.87% ($P < 0.001$)	45
Figure 3-4: Receiver operating characteristic (ROC) curve analysis for anogenital distance (AGD) that predicted probability of pregnancy to the first AI in nulliparous Holstein heifers (n = 1692; area under the curve: 0.5559; sensitivity: 66.2%; specificity: 42.5%)	46
Figure 3-5: Kaplan-Meier survival analysis to determine the proportion of nulliparous Holstein heifers that remained nonpregnant up to 450 d of age. Heifers with long-AGD had reduced ($P < 0.0001$) hazard for pregnancy up to 450 d compared with those with short-AGD (HR: 0.59)	47
Figure 4-1: Distribution of anogenital distance in first-parity (dotted bars: n = 1,920), second-parity (filled bars: n = 1,346), and third+-parity (hatched bars: n = 1,907) cows	64
Figure 4-2: Association between parity and anogenital distance ($R^2 = 0.04$; $P < 0.01$) in lactating dairy cows (n = 5,173)	65
Figure 4-3: Association between 305-d mature equivalent milk yield (ME MY) and anogenital distance ($R^2 = 0.001$; $P < 0.01$) in lactating dairy cows (n = 5,173)	66

List of Abbreviations

AFC	Antral Follicle Count
AGD	Anogenital Distance
AI	Artificial Insemination
AMH	Anti-Müllerian Hormone
AUC	Area Under the Curve
C-LA	Commencement of Luteal Activity
DIM	Days In Milk
DNB	Do Not Breed
EDCs	Endocrine Disrupting Chemicals
FTAI	Fixed Time Artificial Insemination
GnRH	Gonadotropin Releasing Hormone
GWAS	Genome-wide Association Studies
HR	Hazard Ratio
IGF-1	Insulin-like Growth Factor 1
LH	Luteinizing Hormone

LSM	Least Square Means
ME	Mature Equivalent
MY	Milk Yield
P/1stAI	Pregnancy to First Artificial Insemination
P/2ndAI	Pregnancy to Second Artificial Insemination
P/3rdAI	Pregnancy to Third Artificial Insemination
ROC	Receiver Operating Characteristic
SD	Standard Deviation
SE	Standard Error
SNP	Single Nucleotide Polymorphisms
SPS	Size and Position Score
SPS1	Size and Position Score 1
SPS2	Size and Position Score 2
SPS3	Size and Position Score 3

1.0 General Introduction

The United Nations estimates that the world population will grow from 7.7 to 10.5 billion between 2019 and 2070 (United Nations, 2019). By 2067, annual consumption of dairy products is expected to increase from 87 kg per person to 119 kg per person, based on extrapolations from Alexandratos and Bruinsma (2012). Increased dairy consumption, coupled with increased population growth, translates into a need for approximately 600 billion kilograms more milk in 2067 than the 748 billion kilograms currently produced today (FAO, 2019). In order to achieve the above target by 2067, the world average dairy cow would need to almost double its annual yield from 2,405 to 4,531 kg (Britt et al., 2018); however, the average North American Holstein already produces over 10,000 kg per year (FAO, 2019; Canadian Dairy Information Centre, 2019).

Genetic selection for important traits has aided in the transformation and advancement of the dairy industry. Specific traits considered for selection in dairy cattle have developed in response to the evolving needs of producers, consumers and society (with the aid of advances in technology and trait recording programs) in an effort to achieve improved efficiency and sustainability of milk production (Miglior et al., 2017). To date, genetic selection is responsible for about half of the perceived changes in animal performance in well-structured breeding programs, including improvements in milk production and deterioration in reproductive performance (Berry, 2015). The declining trend in reproductive performance observed in dairy cattle undoubtedly affects the future efficiency of the dairy industry (Lucy, 2001; Walsh et al., 2011). The reasons for reproductive decline are likely multifactorial, with a combination of physiological and management factors contributing to an additive effect on reproductive efficiency. The relative

influence of individual factors leading to infertility can be debated; nevertheless, the cumulative effect diminishes the efficiency and profitability of the industry (Lucy, 2001).

Due to the economic importance of reproductive performance, the relationship between fertility and production traits has received a lot of attention over the years. Early measures of female fertility were the interval from calving to first insemination, nonreturns to first service, the number of services per conception, and calving interval; however, disadvantages of these early measures of fertility exist in that they are likely influenced by farmer decisions or seasonal calving, although seasonal calving is not often practiced in North America. In addition, the potential to select for reproductive efficiency was questioned due to reports of very low heritability estimates for most of the considered fertility traits (Berry et al., 2014). Although the incorporation of traditional fertility traits, such as daughter pregnancy rate, has halted further decline in fertility among Holstein dairy cows (Garcia-Ruiz et al., 2016), genetic gain for fertility continues to be slow due to low heritability. Therefore, there is potential to incorporate novel fertility phenotypes with greater heritability estimates into selection programs in an effort to achieve improved genetic gain for fertility (Tenghe et al., 2016; Fleming et al., 2019).

Before a potential trait can be considered for selection in dairy cattle populations, it must meet several criteria. A reproductive trait that has economic value or reduces cost of production, has high variability and heritability, measurable at a low cost, and can be consistently recorded would be an ideal candidate for fertility selection in dairy cows. Moreover, a predictor or indicator trait (i.e., a trait more reflective of the underlying physiology in cattle) would be favoured if it has high genetic correlation with an economically important trait, reduces costs, has a higher heritability, or can be measured earlier in life (Shook, 1989). Therefore, the aim of this study was to further

investigate the potential of anogenital distance, a morphometric measure hypothesized to be reflective of dairy cattle fertility (Gobikrushanth et al., 2017).

The present thesis aims to initially review current practices for fertility selection in dairy cattle, and further, the use of novel phenotypes as a genetic strategy to improve fertility in dairy cattle (Chapter 2). Then, two original research studies that aimed to better understand the associations between AGD and measures of fertility in dairy cattle are presented. The general hypothesis was that AGD is inversely related with measures of fertility in dairy cattle. The first study characterized the variability of AGD and investigated the associations between AGD and fertility measures, i.e., age at conception, services per conception, pregnancy to first artificial insemination, and pregnancy risk up to 450 d of age, in nulliparous Holstein heifers (Chapter 3). The objectives of the second study were to validate previous findings that an inverse relationship exists between AGD and fertility measures, i.e., services per conception, times bred, days open, pregnancy to first, second, and third inseminations, and pregnancy risk by 150 and 250 days in milk, in a large population of lactating Holstein cows (Chapter 4). Lastly, Chapter 5 presents a general discussion, recommendations for future research, limitations, and conclusions.

2.0 Review of Literature

2.1 Overview

Reproductive efficiency plays a pivotal role in dairy farm profitability. Regardless of geographical location or production system, improvements in female fertility positively impact profitability by reducing overhead costs associated with breeding and early culling. In addition, improved reproductive efficiency will lengthen the animal's productive lifetime (Veerkamp et al., 2002; González-Recio et al., 2004; Meadows et al., 2005; De Vries, 2006). The strong association between a dairy herd's reproductive performance and farm profitability places a lot of pressure on the reproductive capacity of a heifer or cow, and the ability to successfully establish and maintain pregnancy. The timely and successful establishment of pregnancy will reduce calving intervals, while improving production and reducing management requirements (Fleming et al., 2019). Due to rapid genetic improvement and advances in management techniques, the global dairy industry has moved towards a time where a reduced number of dairy cows are capable of meeting the growing demands for dairy products (Lucy, 2001). The continuous selection for high-producing dairy cows has been associated with the rapid decline in fertility in these animals (Lucy, 2001; Pryce et al., 2004). Although accounting for the substantial selection focus on production traits, the origin of reproductive decline is likely multifactorial, where a variety of physiological, environmental, and management factors (with varying contributions and interactions) are responsible for the unfavourable, cumulative decline in reproductive efficiency (Lucy, 2001; Walsh et al., 2011). While on-farm management practices can strongly influence fertility and reproductive outcomes, amendments to these techniques have reversed some of the poor fertility observed on farm. The observed decline in reproduction has demonstrated the importance of

genetic contribution to fertility traits, despite fertility traits generally exhibiting low heritability (Seykora and McDaniel, 1983; Pryce et al., 1997; Lucy, 2001; Kadarmideen et al., 2003; Veerkamp and Beerda, 2007; Walsh et al., 2011; Peñagaricano and Khatib, 2012; Jiang et al., 2017; Ma et al., 2019). The complex nature of fertility (and obvious decline) has led to the innovation and/or development of various reproductive technologies and genetic strategies to understand and improve fertility; where reproductive technologies are intended to improve day-to-day farm-level management and genetic selection is directed to progress fertility on a long-term genetic basis. Nonetheless, the evaluation of many traits for genetic selection via on-farm recording can be costly and time intensive. In most cases, phenotypes derived from existing industry records, (e.g., gestation length and interval from calving to conception) are used as genetic selection criteria; however, such measures can be extremely variable due to random and systematic environmental effects and inaccurate recording (Dennis et al., 2017). Therefore, with more recent technological advancements, there is opportunity to identify and incorporate novel traits, with the potential to complement current traits, into breeding objectives.

2.2 Current Practices for Fertility Selection

2.2.1 Criterion for Selection

Over the last century, the collection of traits considered for genetic selection in dairy cattle populations has developed in response to the progressive needs of producers, consumers, and society. Along with technological advancements and trait recording programs, the selection of genetically important traits has transformed the dairy industry. Before a potential trait can be considered for selection in dairy populations it must fulfill certain criteria. First, the trait should

hold some economic value as a marketable product, or the cost of production should decrease as a result of its improvement (Shook, 1989). Historically, selection decisions have been highly motivated by the economic value of traits. For decades, the selection emphasis was solely on increasing milk production. Despite apprehensions that over-selecting for milk yield would result in the regression of overall fitness, the industry pushed to reach peak genetic change in the most profitable area – production. The acknowledgement that previous selection decisions resulted in a correlated genetic decline in other important traits paved the way for a more balanced breeding goal, resulting in the identification and selection of additional traits that were previously undervalued such as those pertaining to health and fertility (Miglior et al., 2005; 2017).

Second, the genetic variation and heritability of the trait should be high, as they are fundamental in determining the threshold rate of change within a selection program (Shook, 1989). The amount of genetic and phenotypic variation in a trait varies significantly and may be more or less heritable. Traits may also be dependent on one another, where correlations can either be positive or negative and strong or weak. These correlations may be exploited by the use of an indicator trait, which may be considered preferential if the genetic correlation between itself and an economically important trait is high, has a higher heritability, has reduced cost of data collection, or can be measured at a younger age (Miglior et al., 2017).

Lastly, the consistent documentation and access to traits that are well-defined and cost effective has been a major benefit to the advancement of genetic selection. The continual influx of big-data collection throughout the industry has presented a number of traits with genetic estimates for consideration; subsequently resulting in a significant number of potential traits for inclusion in selection programs that ultimately need to be assimilated into balanced breeding objectives. Careless selection, shifting breeding objectives, or having several varying selection goals can

cause a permanent detriment on the population. The routine use of multi-trait selection indices is central to the progress and success of genetic selection in many countries (Shook, 1989; Miglior et al., 2017).

2.2.2 Categories of Reproductive Traits for Genetic Selection

Management decisions, environmental effects, and the cow's physiology all influence fertility traits measured in dairy cattle. Moreover, certain traits, such as those measured later in life or interval traits, have the potential to increase generation interval and reduce genetic progress per time unit if accurate genomic breeding values are not available to allow for early selection. The large phenotypic variation observed for reproductive traits, resulting in part from large environmental and management influences, may greatly mask the already inferior additive genetic variation (Miglior et al., 2017). In several countries the direct selection for fertility traits began during the early 21st century (Van Raden et al., 2003; Van Doormaal et al., 2004; Miglior et al., 2017). Despite the more recent attempts at direct selection for fertility, genetic gain for fertility has been slow due to low heritability and reliability of estimated breeding values. Although advancements in reproductive management tools (e.g., timed breeding programs and automated monitoring systems) have allowed for some progress, strategies for increasing reproductive performance are still at the forefront for improvement (Fleming et al., 2019). Some traits currently selected in the dairy industry are positively genetically correlated with the overall reproductive success of the cow, and thus, are contributors to the indirect genetic improvement of fertility in selection programs. Due to low heritability estimates and expansive nature of describing fertility, the present panel of fertility phenotypes has posed some challenges towards genetic gain for

fertility. Therefore, the introduction of fertility-correlated traits has been beneficial to the improvement of cow fertility; future traits currently under investigation may help further this cause.

Traditional female reproductive traits. Traditional traits are those that are regularly measured on the majority of commercial dairy (and beef) populaces. Traditional female reproductive traits can be organized into three sub-categories: (1) interval traits, (2) binary traits and (3) count traits. Perhaps some of the most commonly used reproductive traits in dairy breeding programs are those categorized under interval traits. Such traits include calving interval, interval from calving to first estrus, interval from calving to first service, interval from calving to conception (days open), and interval from first service to conception. Interval traits common in nulliparous heifers include age at first estrus, age at first service, and age at first calving. Binary traits are traits with only two outcomes, for example pregnant or open (not pregnant). Pregnant to first service (natural or artificial insemination) is a binary trait commonly used in the evaluation of female reproductive performance (Veerkamp et al., 2001; Berry et al., 2013). The last category of reproductive traits is count traits, the most common being number of services (Berry et al., 2013; Wall et al., 2003; Jamrozik et al., 2005). Number of services can either be the total number of services regardless of outcome (Veerkamp et al., 2001; Berry et al., 2003) or the number of services per conception (Wall et al., 2003; Jamrozik et al., 2005). The number of services per conception is restricted to cows and heifers that are confirmed pregnant, and therefore is favoured over the total number of services. Traditional measures of reproductive performance recorded by producers are routinely available and are now incorporated in many national dairy breeding objectives (Miglior et al., 2005); however, historically, traditional female reproductive traits have low heritability estimates across dairy breed populations, ranging from 0.02 to 0.04 (Berry et al., 2014).

Of these traditional traits, heritability tends to be greater for interval traits than both binary and count traits. For example, calving to first service interval (0.05) and calving interval (or inter-calving interval; 0.03) are the two most commonly evaluated reproductive traits in dairy cows, with mean coefficient of genetic variation 7% and 2%, respectively (Berry et al., 2014).

Detailed female reproductive traits. Deconstructing collective reproductive phenotypes into their detailed components, which are potentially less biased by management techniques, could result in improved heritability estimates and, assuming there is sufficient genetic variation, greater genetic gain for reproductive performance could be achieved. For example, calving interval is composed of many detailed reproductive components, such as the postpartum interval to the onset of estrous cyclicity, expression of estrus, conception, maintenance of pregnancy, and gestation length. Therefore, a cow with a long calving to first service interval but an acceptable pregnancy rate may have a similar phenotypic value for calving interval to a cow with short calving to first service interval but poor pregnancy rate. Although these cows are greatly dissimilar, they have the same phenotypic value and potentially the same estimated breeding value for calving interval if estimated in a univariate genetic variation model (Carthy et al., 2015). Moreover, detailed reproductive traits refer to those not routinely measured in the majority of dairy populations. Detailed traits are generally based on endocrine profiles (Royal et al., 2002a; Berry et al., 2012) or ultrasound analysis, palpation or examination of the reproductive tract (Carthy et al., 2014; Fitzgerald et al., 2014). There is an abundance of detailed reproductive phenotypes, the following traits are those that are, or can be, implemented on a large scale in national breeding indices.

2.3 Novel Reproductive Phenotypes

Enhancement of data collection and selection strategies continues in an effort to achieve considerable genetic gain in dairy cattle fertility. Current phenotypes used in genetic evaluations may be subjective to management practices, exhibit low heritability, and insufficiently describe component measures comprising overall reproductive success. Incorporating these reproduction focused traits into national selection indices has resulted in unremarkable genetic progress in these traits; however, more recent industry advancements could allow for additional traits, with the potential to complement current traits, to be incorporated. As described above, many new phenotypes have been proposed that are more reflective of the underlying reproductive physiology in cattle, with minimal influence of on-farm management techniques. Additionally, as technologies develop and thus more diverse data sources become available, the opportunity to better characterize and improve the accuracy of current traits, as well as potentially collect novel phenotypes at a national level becomes more feasible (Crowe et al., 2018; Fleming et al., 2018).

2.3.1 Progesterone-Based Measures

Endocrine-related phenotypes, especially those determined by milk or plasma progesterone levels, have been suggested as indicators of fertility in dairy cows as they are more closely associated with the physiology of the cow compared to traditional insemination- or calving-based measures (Lamming and Darwash, 1998; Darwash et al., 1999). Of these potential phenotypes, the commencement of luteal activity (C-LA) has received much of the attention. C-LA, defined as the number of days post-calving to the onset of luteal activity, is determined when milk progesterone concentration exceeds a pre-determined threshold in two consecutive samples (e.g., ≥ 3 ng/ml;

Darwash et al., 1997a; Royal et al., 2000b; Petersson et al., 2007; Pollott and Coffey, 2008; Berry et al., 2012). Length of first luteal phase, persistency of corpus luteum, delayed ovulation and percentage of animals with a milk progesterone concentration ≥ 3 ng/ml in the first 60 d post-calving are all reproductive phenotypes based on progesterone profiles in milk (Royal et al., 2002a).

Notably, C-LA and transformed C-LA have demonstrated higher heritability estimates than traditional fertility traits, ranging between 0.13 and 0.28 (Darwash et al., 1997a; Veerkamp et al., 2000; Royal et al., 2002b; Petersson et al., 2007; Nyman et al., 2014). Darwash et al. (1997b) associated an early return to cyclic activity in postpartum cows with a shortened interval from calving to conception, increased conception rate, and fewer services per conception (Darwash et al., 1997b). Furthermore, C-LA was found to be genetically correlated with interval from calving to first insemination, estimated at 0.35 (Nyman et al., 2014), where log-transformed C-LA was genetically correlated with calving interval and interval from calving to first insemination, with estimates of 0.26 and 0.37, respectively (Tenghe et al., 2015). Other fertility phenotypes, derived from the same milk progesterone measurement have been reported with varying heritability, such as length of first luteal phase, occurrence of delayed luteolysis, proportion of samples with luteal activity, interluteal interval, prolonged luteal phase, and delayed ovulation (Royal et al., 2002a; Nyman et al., 2014; Tenghe et al., 2015; Sorg et al., 2017). Delayed cyclicity has been reported to exhibit moderate heritability (0.24) whereas prolonged luteal phase, luteal phase length, interluteal interval and interovulatory interval have all demonstrated low heritability estimates (0.02, 0.08, 0.08, and 0.03, respectively; Nyman et al., 2014). More recently, Sorg et al. (2017) measured progesterone profiles using on-farm ELISA test kits and discovered low heritability estimates for delay to cyclicity and proportion of luteal activity between 15 and 63 d postpartum, 0.03 to 0.07

and 0.12 to 0.23, respectively. A recent study using milk progesterone data gathered from Herd Navigator (DeLaval International, Tumba, Sweden), a real time in-line progesterone measuring system, reported higher heritabilities for C-LA and calving to the first heat (identified by Herd Navigator) at 0.24 to 0.32 and 0.19 to 0.33, respectively (Häggman et al., 2018); however, few commercial herds have in-line systems capable of measuring progesterone, as these systems still have a high cost associated with them. Progesterone profiling remains of interest to researchers as they continue to investigate and optimize measurement and identify the most ideal traits for genetic selection. Further research into the efficient and accurate measurement of milk progesterone concentration, such as the practical use of in-line milking systems, will allow for large-scale data collection of this novel phenotype.

2.3.2 Estrus Expression and Activity Traits

Many reproductive management techniques rely on behavioural signs of estrus to determine the optimal time to inseminate as well as to predict the approximate time of ovulation; however, there are limitations to this method as failure to show or detect estrus can limit the rate at which cows become pregnant resulting in prolonged calving intervals. There are many factors that may contribute to the inability to detect estrus on-farm such as estrus detection techniques, or few cows expressing standing estrus (Roelofs et al., 2005; Palmer et al., 2010; Fricke et al., 2014). Therefore, studies have focused on assessing the ability and strength of estrus expression, a genetically controlled trait (Kommadath et al., 2011, 2013) in dairy cows, in an effort to improve reproductive efficiency.

In the past, subjective measures of strength of estrus expression have been investigated as potential traits. For example, scores for standing estrus symptoms at each insemination are included in the extensive list of data recorded for Swedish herds. Estrus intensity score was categorized as weak, normal, or strong by inseminators and farmers and heritability was evaluated; reported heritability estimates ranged from 0.01 to 0.03 (Roxström et al., 2001). The low heritability reported for heat intensity scoring demonstrates the limited efficacy of this phenotype for use in genetic selection.

In more recent years, the dairy industry has begun to adopt new technologies that more accurately detect estrus by the continuous monitoring of activity in individual cows. The use of sensor technologies, such as pedometers, activity collars, and ear-attached tags, identify secondary signs of estrus such as increased restlessness and physical activity. As these data sources objectively describe estrus behaviours in commercial herds, there is potential for these measures to be considered for phenotypic selection, thus warranting further investigation. Heritability estimates of 0.12 to 0.18 and a repeatability of 0.18 have been reported for days to first high activity, a trait representative of days to first detectable estrus (Løvendahl and Chagunda, 2009). Løvendahl and Chagunda (2009) have described other activity-derived traits such as period of increased activity and strength of estrus, although such traits exhibited low heritabilities of 0.02 to 0.08 and 0.04 to 0.08, respectively. Moreover, strong genetic correlations between interval from calving to first high activity, estrus duration, and estrus strength, with the traditional measure of interval from calving to first insemination have been reported (0.96, -0.38, and -0.50, respectively; Ismael et al., 2015).

Activity-based measurements may also be used to define traits in heifers or in dry cows preceding calving, which would not be possible with milk progesterone determined phenotypes as

described above. Silper et al. (2015) demonstrated a large variation of estrus expression existing within and between heifers. Furthermore, the time of episode onset and the category of baseline walking (low or high) were shown to influence estrus duration and increase of activity, thus suggesting that baseline steps and relative activity increase during estrus could be possible phenotypical targets to predict fertility and to assist selection for this trait (Silper et al., 2015). Moreover, Reith & Hoy (2012) demonstrated, on average, daily rumination time (time spent ruminating) at estrus was significantly reduced compared with that on nonestrus days. As activity-based measures become more readily available, further research that includes factors such as accuracy, error rate, sensitivity and specificity for various threshold values in activity-based measures will become a priority. In addition, further exploration into the variability, repeatability and heritability of activity-based traits as measured by various sensor technologies are necessary to determine whether these activity-based phenotypes are reliable indicators of estrus individually, or if a combination of multiple parameters is needed.

2.3.3 Insulin-Like Growth Factor 1

Insulin-like growth factor-1 (IGF-1) plays a vital role in the control of postnatal growth, mammary gland development, lactation and reproduction in dairy cows (Lammers et al., 1999; Jiang and Lucy, 2001; Renaville et al., 2002; Butler, 2003). Specifically, IGF-1 is essential for reproduction by functioning synergistically with gonadotropins to promote early postpartum ovarian follicular growth and ovulation (Lucy et al., 1992; Spicer et al., 1993; Beam and Butler, 1999). Thus, the high negative energy balance and low serum IGF-1 concentration, indicative of

nutrient partitioning toward milk production, risks compromising the fertility of dairy cows (Butler, 2000).

In the past, circulating IGF-1 concentration has been recognized as exhibiting high variability, moderate heritability, and positive associations with fertility, illustrating the potential of circulating IGF-1 concentration as a potential fertility trait (Velazquez et al., 2008). Reports of high variation for postpartum concentrations of circulating IGF-1 have been reported among dairy cows (Zulu et al., 2002; Moyes, 2004; Gobikrushanth et al., 2018b), with 18 to 48% of this variation attributable to genetic variation (Grochowska et al., 2001; Stirling et al., 2008; Hayhurst et al., 2009). The remaining variation in IGF-1 concentration was due to other factors such as postpartum nutrient intake, body weight and body condition score at calving, and parity (Ciccioli et al., 2003; Pushpakumara et al., 2003; Wathes et al., 2003, respectively).

In general, reports have indicated postpartum cows with increased concentrations of circulating IGF-1 have greater pregnancy to first AI (P/1stAI) than those with low concentrations of IGF-1 (Pushpakumara et al., 2003; Taylor et al., 2004; Kawashima et al., 2007; Patton et al., 2007; Falkenberg et al., 2008; Gobikrushanth et al., 2018b). Early reports suggested multiparous cows with circulating IGF-1 concentration <25.0 ng/mL were less likely to conceive to first AI, and therefore it is cost-effective to delay first service in those cows until circulating IGF-1 has increased to a concentration >50 ng/mL (Taylor et al., 2004). In a more recent study, primiparous cows required greater serum IGF-1 threshold concentration for the optimal prediction of P/1stAI than multiparous cows, where optimum serum IGF-1 threshold predictive of P/1stAI was 85.0 ng/mL and 31.0 ng/mL for primiparous and multiparous cows, respectively (Gobikrushanth et al., 2018b). However, due to the moderate relative estimates of sensitivity and specificity, the authors identify that the routine measurement of early postpartum circulating IGF-1 concentration to

predict P/1stAI, in an effort to implement enhanced nutritional management strategies for cows with low IGF-1 or to delay first service in an attempt to improve P/1stAI, is not practical or economically justifiable in either primiparous or multiparous cows (Gobikrushanth et al., 2018b) as previously suggested by Taylor et al. (2004). Further research into the diagnostic values for optimum circulating IGF-1 threshold predictive of other fertility measures of interest, such as interval from calving to conception, will be beneficial in determining the future application of IGF-1 for phenotypic selection. Therefore, future consideration into the ability of IGF-1 to predict measures of fertility, thus demonstrating the practical use of IGF-1, is necessary before the identification of SNP associated with phenotypic variation in IGF-1 concentration is feasible, and the economic feasibility of measurement and application can be evaluated.

2.3.4 Anti-Mullerian Hormone

Anti-Müllerian hormone (AMH), produced by granulosa cells of developing preantral and antral follicles (La Marca and Volpe, 2006), is indicative of antral follicle population (ovarian reserve) in dairy cows (Rico et al., 2009). In the past, circulating AMH has been associated with fertility outcomes in cows subjected to insemination at detected estrus or natural service (Ribeiro et al., 2014), but not in cows subjected to timed AI protocols (Ribeiro et al., 2014; Baruselli et al., 2015; Jimenez-Krassel et al., 2015; Gobikrushanth et al., 2018a). The association between AMH and fertility outcomes in cows subjected to timed AI is potentially masked by the use of a timed-AI management system that optimizes follicular growth, luteal regression, and synchronizes ovulation such that that variation in AMH concentrations is not associated with pregnancy response to the first insemination.

Many studies have demonstrated the high variability of AMH concentration among dairy breed populations; for example, circulating AMH concentrations for North American dairy cow populations ranged from 10 to 3,198 pg/mL (Ribeiro et al., 2014), 14 to 774 pg/mL (Gobikrushanth et al., 2017a), and 14 to 1,879 pg/mL (Gobikrushanth et al., 2018a). Furthermore, AMH concentration in North American heifers ranged from 2 to 2,000 pg/mL (Nawaz et al., 2018), in Japanese Black cows from 40 to 1,730 pg/mL (Newberry, 2016), in a mixed breed population of Irish dairy cows from 15 to 2,863 pg/mL (Gobikrushanth et al., 2019a) and Iranian Holstein cows from 98 to 2,110 pg/mL (Akbarinejad et al., 2019). Thus, findings to date are indicative that large phenotypic variation exists in circulating AMH concentrations amongst diverse populations of cattle. Studies have also demonstrated high repeatability of plasma AMH, whereby circulating AMH concentration could be measured during any stage of the estrous cycle or postpartum period to test associations with fertility outcomes (Rico et al., 2009; Ribeiro et al., 2014, Souza et al., 2015; Gobikrushanth et al., 2017a). Plasma AMH has greater heritability estimates for both Irish (0.40; Gobikrushanth et al., 2019a) and Canadian Holstein cows (0.46; Gobikrushanth et al., 2018a), as well as Holstein heifers (0.36; Nawaz et al., 2018) compared to traditional measures of fertility in Holstein populations. Berry et al. (2014) described heritability estimates for traditional measures of fertility such as first-service conception rate (0.02), number of services (0.02), and days open (0.04) in dairy cows as well as age at first service (0.13) and age at first calving (0.17) in dairy heifers. Thus, the observed moderate heritability estimates observed for plasma AMH greatly outweigh estimates for traditional fertility traits and increase the potential of circulating AMH concentration becoming a selection candidate in dairy cattle populations.

Lastly, single nucleotide polymorphisms (SNPs) have been associated with plasma AMH in Irish Holstein cows (Gobikrushanth et al., 2019a), of which many of the nearest candidate genes

identified have also been identified as candidate genes for other fertility related phenotypes in dairy cows such as genes pertaining to encoding steroidogenic factor 1 in the steroidogenic pathway (Taniguchi et al., 2009), early embryonic development and fertility (Zhang et al., 2011), and embryo production traits (Jaton et al., 2018). Recent work has suggested AMH concentration has a quadratic relationship as opposed to a linear relationship with reproductive performance of dairy cows, implying that cows with minimal and maximal levels of AMH have inferior fertility than their counterparts with moderate levels of AMH (n = 172; Akbarinejad et al., 2020). Before AMH can be considered for use in selection programs, further research into its associations with field fertility outcomes should be evaluated; however, due to its large phenotypic variation, high repeatability, moderate heritability and associated SNP, circulating AMH concentration demonstrates potential as a fertility phenotype in future breeding programs, although there are large costs associated with quantifying AMH.

2.3.5 Antral Follicle Count

During development, germ cells are surrounded by granulosa cells to form primordial follicles. As follicles begin to grow, the morphology of the granulosa cells changes, and they begin to proliferate, forming primary follicles. The granulosa cells continue to proliferate forming secondary follicles, which develop a cavity within the follicle becoming antral follicles. This developmental path explains the positive correlation between primordial and more developed follicle classes including antral follicles (Erickson, 1966). Therefore, the number of antral follicles is commonly referred to as antral follicle count (AFC) and is representative of ovarian reserve in

an individual. Antral follicles can be readily identified by ultrasonography in cattle (Cushman et al., 2009; Ireland et al., 2008) and can be used to predict ovarian reserve *in vivo*.

Antral follicle count, determined during serial ovarian ultrasonography, is defined as the average peak number of follicles ≥ 3 mm growing during consecutive follicular waves of estrous cycles (Burns et al., 2005). To date, studies have recognized AFC is highly variable among cattle, ranging from 8 to 56 follicles, but highly repeatable (repeatability of 0.85 to 0.95) within individual animals (Burns et al., 2005; Ireland et al., 2007). Moreover, the variation in AFC is associated with a number of well-established indicators of fertility in cattle, including total number of morphologically healthy follicles and oocytes in ovaries (Ireland et al., 2008), progesterone production during estrous cycles (Jimenez-Krassel et al., 2009), and responsiveness to superovulation and number of transferable embryos (Ireland et al., 2007; Rico et al., 2009). Adverse relationships between AFC and direct measures of fertility, including conception rate and services per conception, have also been reported; cows with low AFC had reduced fertility than those with high AFC (Mossa et al., 2012; Martinez et al., 2016).

The heritability of AFC in dairy cattle was first estimated in a population of North American Holstein-Friesian heifers and Irish Holstein-Friesian cows and they were 0.25 and 0.31, respectively (Walsh et al., 2014). The moderate heritability for AFC as demonstrated by Walsh et al. (2014) is significant as heritability estimates for traditional measures of fertility are less than 5% (Berry et al., 2014); however, prior to this finding, a study in beef heifers suggested the variation in follicle numbers may be highly heritable (\pm SE; 0.73 ± 0.18 , $n = 452$; Snelling et al., 2012). Moreover, the number of oocytes collected by ovum pick-up, shown to be positively associated with AFC in cattle (Ireland et al., 2008), has moderate heritability of 0.25 in cattle (Merton et al., 2009).

Therefore, during follicular waves, antral follicle count is a highly repeatable phenotypic trait positively associated with ovary size, number of morphologically healthy oocytes, ovarian function, and fertility in cattle (Burns et al., 2005; Ireland et al., 2007, 2008; Cushman et al., 2009; Mossa et al., 2010, 2012) and is also a moderately heritable genetic trait (Walsh et al., 2014). However, the collection of AFC data for genetic evaluations is not as economically feasible as other potential indicators of fertility due to limitations on the number of animals that can be examined via ultrasonography per day, and the age and size of the heifer at the time at which ultrasonography can occur. Thus, more easily measured markers predictive of AFC and associated traits, such as AMH, which is highly positively associated with AFC in cattle (Ireland et al., 2008, 2011) may be a more useful indicator of fertility.

2.3.6 Ovarian Size

Ovarian size, a simple anatomic measurement, has also been studied with relation to fertility. Studies have demonstrated a positive correlation between ovarian size and AFC, whereby animals with low AFC have smaller ovaries compared with age-matched lactating cows with a higher AFC (Ireland et al., 2008; Mossa et al., 2012). More specifically, significant reductions in ovary wet weight, ovary height, ovary length (Ireland et al., 2008), and ovarian area (Mossa et al., 2012) were observed in lactating cows with low AFC compared to those with high AFC. However, ovarian area was not associated with fertility measures such as P/1st AI, overall pregnancy rate, calving to conception interval, 21-d submission rate, or overall number of services. In addition, the total number of healthy and atretic follicles, number of healthy follicles, and number of healthy follicles per gram of ovary were lower in cattle with low compared with high AFC, although the

proportion of healthy follicles (healthy divided by the total number of healthy and atretic follicles per animal) was similar amongst AFC categories (Ireland et al., 2008). The positive association between AFC and ovarian size observed in Holstein lactating dairy cows (Ireland et al., 2008; Mossa et al., 2012) has also been observed in Angus heifers (Eborn et al., 2013). The overall positive correlation between ovarian size and AFC in the majority of studies, and the associations between AFC and fertility measures warrant further research into the suitability of ovarian size as an indicator of fertility. If ovarian size is in fact associated with fertility, the measurement of ovarian size would be another alternative to AFC as a means of predicting fertility, which requires less time and skills than those required to measure AFC in cattle (Mossa et al., 2012).

2.3.7 Reproductive Tract Size and Position Score

Recently, a reproductive tract size and position score (SPS) system was developed as a reproductive management tool to identify lactating dairy cows with decreased fertility. This system relies solely on transrectal palpation and considers the size (cervical and uterine) and position of the reproductive tract relative to the pelvis. Cows undergoing pre-breeding exams were identified as having reproductive tracts that were either: (1) small (SPS1), meaning they had small and compact uterine horns that rested within the pelvic cavity; (2) medium (SPS2), meaning they had reproductive tracts that were intermediate in cervical and uterine horn diameter, with longer uterine horns resting partially outside the pelvic cavity; or (3) large (SPS3), meaning cows had reproductive tracts that were larger and rested mostly outside the pelvic cavity (Young et al., 2017). Findings from the initial field trial (n = 1,463) demonstrated that P/1stAI was 15 percentage points less in cows with larger reproductive tracts positioned outside the pelvic cavity (SPS3) than in

cows with small reproductive tracts positioned in the pelvic cavity (SPS1; Young et al., 2017). More recently, Madureira et al. (2020) corroborated the initial findings in a population of 869 lactating Holstein cows demonstrating that SPS1 cows had greater P/1stAI when compared with SPS2 and SPS3 cows (44.4, 36.5, and 23.0%, respectively). Moreover, cows assigned as SPS1 had 1.57 greater odds to establish pregnancy within the first 300 DIM compared with SPS2 cows and had 1.76 greater odds of pregnancy compared with SPS3 cows (Madureira et al., 2020). The ease with which the relative size and position of the reproductive tract can be determined via rectal palpation as well as the association of SPS to fertility measures such as P/1stAI, highlight the potential usefulness of the size and position of the reproductive tract as an indicator of fertility in lactating dairy cows. Further research into the associations between SPS and other measures of fertility such as calving to conception interval and services per conception will be beneficial to gain valuable insights on the future application of this novel phenotype.

2.3.8 Anogenital Distance

Anogenital distance, defined as the distance between the anus and the external genitalia in both males and females, has exhibited sex-related variations in rodents. For example, excess (in females) or insufficient (in males) androgen exposure is associated with reproductive defects in rats (Clark and Galef, 1995; Drickamer et al., 1996; Bánzegi et al., 2012, 2015). In women, AGD was defined as either the distance from the center of the anus to the posterior fourchette (Salazar-Martinez et al., 2004) or the clitoris (Sathyanarayana et al., 2010), and is thought to be reflective of prenatal exposure to excess androgens resulting in androgenization of the reproductive system in utero (Bowman et al., 2003; Sadler et al., 2012). Because female reproductive tract development

is hormonally regulated, AGD serves as a biomarker for prenatal hormonal actions. Moreover, many studies have revealed that AGD was approximately twice as long in males (distance from the centre of the anus to the base of the scrotum) compared with females (Salazar-Martinez et al., 2004; Swan, 2008; Thankamony et al., 2009; Macleod et al., 2010; Sathyanarayana et al., 2010). Therefore, these studies suggest that AGD is not only a biological indicator of prenatal androgenization of the reproductive tract, but also a sexually dimorphic trait.

Gobikrushanth et al. (2017b) were the first to define and characterize AGD, defined as the distance from the center of the anus to the base of the clitoris, in dairy cattle. In this population of 921 lactating cows, AGD was normally distributed and highly variable (\pm SE [range]; 131.0 ± 12.2 mm [96.0, 170.0]; Gobikrushanth et al., 2017b), which is comparable to the pattern of distribution and variability of AGD in women (Mendiola et al., 2012). Similar patterns of distribution and variability of AGD have been reported in crossbred Black Angus x Simmental beef cows (Battista, 2019), Irish Holstein-Friesian cows (Gobikrushanth et al., 2019b) and Iranian Holstein cows (Akbarinejad et al., 2019). Moreover, overall associations between AGD and postnatal factors, such as age and height, were very weak within this population (Gobikrushanth et al., 2017b), suggesting that AGD may be largely independent of postnatal factors and thus, more heavily influenced by prenatal factors such as in utero androgen concentrations as described previously.

In dairy cows, the placenta is the primary source of androgens in dams carrying female fetuses (Mongkonpunya et al., 1975). During gestation, maternal concentrations of testosterone and androstenedione were highly variable among individual cows carrying female fetuses, 110 to 166 pg/mL and 936 to 1,400 pg/mL, respectively (Gaiani et al., 1984). However, the first report of AGD in lactating dairy cows demonstrated the relationship between AGD and testosterone was weak and nonsignificant ($R^2 = 0.02$; $P = 0.19$; Gobikrushanth et al., 2017b). The authors attribute

the lack of significance to limited sample size ($n = 93$). Interestingly, Mossa et al. (2010) evaluated AFC in dairy cattle and revealed that cows with high AFC (≥ 25) had almost double the concentrations of circulating testosterone (~ 60 vs. 30 pg/mL) throughout the estrous cycle compared with cows with low AFC (≤ 15). In addition, dairy heifers with high AFC had diminished fertility outcomes and reduced longevity (Jimenez-Krassel et al., 2017). More recent work revealed a tendency for higher concentrations of serum AMH in dairy cows with long AGD (Akbarinejad et al., 2019). As described above, AMH and AFC are highly positively correlated in dairy cattle; however, to our knowledge, no research has reported a negative relationship between AMH concentration and fertility in dairy cattle in the past. Further exploration into the association between maternal concentrations of androgens and AGD will be beneficial in understanding the physiological mechanisms behind the variation in AGD in dairy cattle.

Gobikrushanth et al. (2017b) demonstrated an inverse association between AGD and fertility in dairy cows, in which first- and second-parity cows with short AGD had greater P/1stAI than cows with long AGD. Since the first report of AGD in dairy cattle, three more studies have aimed to explore the use of AGD as a novel reproductive phenotype and have demonstrated mixed results (Akbarinejad et al., 2019; Battista, 2019; Gobikrushanth et al., 2019b). A recent study in a small subset of Iranian Holstein ($n = 86$) demonstrated cows with short AGD required fewer days to first service, improved first service conception rate, reduced number of repeat breeders, and shorter calving to conception intervals (Akbarinejad et al., 2019). In North American crossbred beef cows ($n = 578$), there has been no discernible relationship between AGD and measures of fertility including estrous expression (as a result of imposing a fixed time AI (FTAI) treatment regimen), estrous cyclicity at the time of FTAI prior to initiation of FTAI treatment regimen, pregnancies per AI at d 35 subsequent to AI, nor pregnancy loss between d 35 and d 95 after AI.

However, there was a curvilinear association between AGD and pregnancy rate at the end of the breeding season, implying that extremely long AGD were detrimental to overall pregnancy rate at the end of the breeding season (Battista, 2019). Similarly, Gobikrushanth et al. (2019b) found no association between AGD and measures of fertility in seasonally bred Irish Holstein-Friesian cows. The authors attributed the lack of observable difference to the intense selection focus for fertility in Irish populations of cattle. However, AGD was found to be moderately heritable within this same population of Irish Holstein-Friesians (Gobikrushanth et al., 2019b). As this was the first time heritability has been estimated for AGD, these results show substantial promise as traditional measures of fertility are lowly heritable (Berry et al., 2014). Therefore, further consideration should be placed on validating the inverse relationship between AGD and measures of fertility in a larger population of cattle. In addition, heritability estimates for AGD in a large population of North American Holsteins may provide valuable information on the potential future use of this novel fertility phenotype.

2.4 Knowledge Gaps

Out of the eight reproductive phenotypes addressed in this chapter, anogenital distance is one of the most recently proposed for application in dairy cattle populations. Gobikrushanth et al. (2017b) characterized AGD in North American Holstein cows and for the first time, demonstrated AGD to be highly variable, normally distributed, and inversely associated with fertility in dairy cows. Since the first report of AGD in dairy cattle, two more studies have aimed to explore the use of AGD as a novel reproductive phenotype and have demonstrated mixed results (Gobikrushanth et al., 2019b; Akbarinejad et al., 2019). Anogenital distance is a low-cost morphometric measure;

if AGD is also highly variable, highly heritable, inversely associated with fertility measures, and can be effectively measured early in life, it may become a useful reproductive phenotype for use in genetic evaluations. Therefore, to address these knowledge gaps, further research exploring the relationship between AGD and measures of fertility in large populations of heifers and cows will be beneficial in evaluating its potential for future inclusion into large-scale genetic evaluations.

3.0 Relationship of anogenital distance with fertility in nulliparous Holstein heifers

3.1 Introduction

Improving female fertility has become a key breeding goal in the dairy industry, and various measures of reproductive fitness have been incorporated into selection indices worldwide. Because of low heritability of fertility traits, genetic progress has been slow (Miglior et al., 2017; Fleming et al., 2019). Traditional measures of female reproductive performance include interval traits (e.g., calving interval), binary traits (e.g., non-return rate), and count traits (e.g., number of services per conception; Berry et al., 2014; Fleming et al., 2019), but such traits were rarely considered in genetic selection. Since the inclusion of female fertility traits in genetic selection began in North America in the early 2000's (VanRaden et al., 2004; Van Doormaal et al., 2004), the once declining trend in fertility has been reversed (Miglior et al., 2017; Ma et al., 2019). Given this, and in view of recent industry advancements in genome-wide association studies and the availability of affordable genomic testing, the collection of novel phenotypes or indicator traits that complement current traits of economic importance has been emphasized (Egger-Danner et al., 2015; Chesnais et al., 2016; Miglior et al., 2017). The advantage is that such novel phenotypes could have markedly greater heritability than previous related traits (Egger-Danner et al., 2015). In that regard, it has been proposed that the identification and use of new fertility phenotypes that are more closely related to female development, the reproductive cycle, and/or embryo survival could significantly enhance the fertility of modern dairy cows (Miglior et al., 2017). Moreover, because of the low heritability of fertility traits, novel reproductive phenotypes associated with

fertility are considered very useful to increase the accuracy of estimated breeding value for fertility (Miglior et al., 2017).

Ano-genital distance (AGD), the distance from the anus to the genitals, is a sexually dimorphic phenotype in many mammalian species, determined by exposure of the fetal reproductive system to androgens in utero (Swan and Kristensen, 2018). The AGD has been measured from the center of the anus to either the posterior fourchette (Salazar-Martinez et al., 2004) or the clitoris in women (Sathyanarayana et al., 2010) and in dairy cows (Gobikrushanth et al., 2017). Excessive prenatal androgen exposure has led to androgenization of the female reproductive system in utero resulting in greater AGD and reduced postnatal fertility outcomes in rodents (Zehr et al., 2001; Bánzegi et al., 2012; Dean et al., 2012), gilts (Drickamer et al., 1997) and women (Wainstock et al., 2017; Wu et al., 2017).

Gobikrushanth et al. (2017) first characterized AGD in 921 Canadian Holstein cows and found this phenotype to be normally distributed, highly variable, and inversely associated with fertility in first- and second-parity cows, but not in third+- parity cows. Tendencies for more days to first service, lower first service conception rate, and increased proportion of repeat breeders in long vs. short AGD groups have been reported in Iranian Holstein cows (Akbarinejad et al., 2019). In an Irish population of Holstein-Friesian cows, AGD was normally distributed and highly variable; however, none of the fertility variables evaluated differed significantly between long- and short-AGD categories (Gobikrushanth et al., 2019). The heritability estimate of 0.37 reported for AGD in the latter study was greater than that reported for most traditional female fertility traits (0.02 to 0.04; Berry et al., 2014). The authors attributed the absence of an inverse relationship between AGD and fertility in Irish Holstein-Friesian cows to the strong selection emphasis placed

on fertility traits in Ireland during the last 2 decades and aggressive culling of subfertile cows, resulting in a relatively more fertile population of dairy cows in Ireland than in Canada.

Except for a preliminary report (Carrelli et al., 2020) based on a subset of heifers used in the current study, to our knowledge, AGD has not yet been characterized in nulliparous Holstein heifers, and its association with fertility in heifers is unknown. We hypothesized that AGD in nulliparous North American heifers will have a normal distribution, high variability, and an inverse association with measures of fertility, similar to the findings of Gobikrushanth et al. (2017) in first- and second-parity Canadian Holstein cows. Therefore, our objectives were to: (1) characterize AGD in nulliparous heifers; and (2) determine if an inverse relationship exists between AGD and measures of fertility in a larger population of nulliparous Holstein heifers.

3.2 Materials and Methods

3.2.1 Animals and Management

This study was conducted using Holstein heifers from 16 dairy herds in Western Canada (Alberta and British Columbia), and one herd in the USA (State of Washington; Table 1). Heifers were housed and cared for in accordance with guidelines set out by the Canadian Council on Animal Care, and the United States Department of Agriculture. Animal use was approved by the University of Alberta's Animal Care and Use Committee for Livestock (AUP#00002883) and by the University of Idaho Animal Care and Use Committee (Protocol #IACUC-2019-61). Heifers were housed in one of: (1) outdoor dry-lot loose-housing with access to shelter (5 herds); (2) loose-housing in a dry-pack barn with access to the outdoors (3 herds); or (3) in a free-stall barn (9

herds). All heifers had ad-libitum access to water and were fed a total mixed ration (11 herds) or a partial mixed ration or dry hay top dressed with a concentrate mix (6 herds), formulated according to NRC (2001) guidelines. Primary ingredients of heifer rations were corn silage, barley silage, grass silage, haylage or the combination of two or more, dry hay, straw, concentrate and minerals. Heifers were bred via AI after visual estrus detection for primary (standing-to-be-mounted) and secondary signs of estrus (e.g., removal of tail paint, mounting, sniffing, licking, chin resting or trailing behaviors) (4 herds), automated estrus detection by activity monitoring systems (6 herds), Ovsynch/timed AI (2 herds), or a combination of techniques (5 herds).

3.22 Determination of Anogenital Distance, Age and Reproductive Measures

Anogenital distance, the distance from the center of the anus to the base of the clitoris, was measured using 8-inch stainless steel digital calipers (Pro.Point, Princess Auto Ltd., Winnipeg, Manitoba, Canada) as described by Gobikrushanth et al. (2017). A single AGD measurement was obtained by one of two experienced individuals for each heifer; however, some herds were visited up to three times if only a portion of the herd could be accessed in a single visit, thereby warranting multiple visits to measure AGD for all available heifers. Anogenital distance measurements were obtained from all breeding age heifers regardless of their insemination or pregnancy status. Initially, AGD was measured in 2,188 nulliparous heifers, after excluding any heifer that had vulvar swelling, laceration, or abnormal growth (e.g., genital wart, tumour). Only heifers in which AGD measurements were taken + or – 3 mo from date of most recent AI were considered, resulting in the exclusion of 477 heifers. Following this method of exclusion, the second exclusion criterion was applied based on date of AI. Heifers that did not receive their first AI by 18 mo (540 d) were

excluded, resulting in the exclusion of an additional 19 heifers. The final number of heifers included for statistical analyses was 1,692. Age at the time of AGD measurement was determined in days by subtracting date of birth from the date of AGD measurement. Data on fertility measures, including services per conception, age at conception, and pregnancy to first AI (P/1stAI), were retrieved for all heifers from herd management software, DairyComp305 (Lactanet, Guelph, ON, Canada; Valley Agricultural Software Inc., Tulare, CA, USA).

3.23 Statistical Analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The descriptive statistics such as minimum, maximum, mean and standard deviation for AGD were determined using MEANS procedure and normality of the data was tested by UNIVARIATE procedure.

The relationship between P/1stAI, AGD, age at AGD measurement, and herd was first evaluated by logistic regression analysis using LOGISTIC procedure. Based on stepwise regression, the effect of age at AGD measurement was excluded ($P > 0.05$). P/1stAI and herd were modelled against AGD in the final model, and the predicted probabilities of P/1stAI against AGD were determined. The relationship between AGD and predicted probabilities of P/1stAI was reported using a scatter plot.

Using the intercept and the coefficient estimates from the logistic regression equation, receiver operating characteristic (**ROC**) curve analysis was performed in SAS to compute the optimal threshold AGD to achieve greater probability of pregnancy. The ideal AGD threshold was

selected based on the greatest combined sensitivity and specificity using the maximum Youden Index value (sensitivity + [specificity – 1]) to predict pregnancy in heifers, for which sensitivity is the proportion of nulliparous heifers above the threshold that were diagnosed pregnant to first AI, and specificity is the proportion of nulliparous heifers below the threshold that were diagnosed non pregnant to first AI. Area under the curve (AUC) was used to determine the significance of the threshold AGD, where AUC ranged from 0.50 to 1.00. AUC of 0.50 is considered to be non-informative and AUC of 1.00 is considered perfect (Swets, 1988).

Based on the optimum cut-off predicted by ROC curve analysis, heifers were categorized as either short or long AGD (\leq or $>$ threshold). Associations between AGD categories and various fertility measures, including P/1st AI, age at first AI, services per conception, and age at conception were analyzed using GLIMMIX procedure, where the effect of herd was considered a random effect to account for the variation among locations.

Lastly, the Kaplan-Meier survival analysis, LIFETEST procedure, was used to determine cumulative pregnancy up to 450 d (15 mo) and 540 d (18 mo) of age. Results from the LIFETEST procedure were validated using a Cox proportional hazard model (PHREG procedure). Significant differences were declared if $P \leq 0.05$ and considered to be a tendency when $P > 0.05$ and ≤ 0.10 .

3. 3 Results

3.3.1 Descriptive Statistics

Ano-genital distance was normally distributed within a broad range of AGD estimates (Figure 1). Mean \pm standard deviation (**SD**) of AGD in heifers, age at AGD measurement, age at

first AI, age at conception, and services per conception for each farm, overall means, and minimum, maximum values are presented in Table 1. AGD was smaller ($P = 0.0073$) in heifers that were artificially inseminated within the 3 mo prior to AGD measurement compared with those artificially inseminated within the 3 mo after AGD measurement (108.1 ± 1.1 vs. 109.4 ± 1.0 mm, respectively). However, the phenotypic variation in AGD that is explainable by age at AGD measurement was small ($R^2 = 0.06$; Figure 2).

3.3.2 Optimum AGD Threshold to Predict Pregnancy to 1st AI

Results of the final regression model indicated that for every 1 mm increase in AGD, the probability of pregnancy to the first AI was reduced by 1.87% (Figure 3). The optimum AGD threshold to predict pregnancy to first AI, determined by ROC curve analysis, was 110 mm (sensitivity: 66.2%; specificity: 42.5%; Figure 4). Accordingly, based on the optimum threshold AGD value established, heifers were categorized into short (≤ 110 mm) and long (> 110 mm) AGD groups to determine associations with measures of fertility amongst AGD categories.

3.3.3 Relationship Between AGD and Measures of Fertility

Heifers with short AGD were subjected to their first AI at a younger ($P = 0.032$) age and had greater ($P = 0.00080$) pregnancy to first AI than heifers with long AGD (Table 2). In addition, heifers with short AGD required fewer ($P = 0.0032$) services per conception and were pregnant at an earlier age (5.9 days sooner; $P = 0.0018$) than those with long AGD (Table 2). Lastly, heifers with long AGD had reduced ($P < 0.0001$) hazard for pregnancy up to 450 d of life compared with

those with short AGD (HR: 0.59; Figure 5); consequently, a greater proportion of long-AGD heifers (40.3%) remained non-pregnant at 450 days of age compared with short-AGD heifers (23.6%). A reduced ($P < 0.0001$) hazard for pregnancy up to 540 d (18 mo; HR: 0.77) was also evident in heifers with long AGD compared with those with short AGD, although the difference between the two groups in cumulative pregnancy at 18 mo of age was much smaller (87.9 vs. 89.0 %, respectively).

3.4 Discussion

In the present study, we determined the distribution, variation, and association of AGD with reproductive measures in a population of 1,692 nulliparous Holstein heifers. Mean AGD for heifers, in the present study, was approximately 24, 12 and 7 mm numerically shorter than that of Canadian Holstein cows (131 mm; Gobikrushanth et al., 2017), Irish Holstein-Friesian cows (119 mm; Gobikrushanth et al., 2019), and Iranian Holstein cows (114 mm; Akbarinejad et al., 2019), respectively. Likewise, the mean AGD was 7 mm shorter than that of a subset of 671 Holstein heifers (114 mm) used in the current study, as conveyed in a preliminary report (Carrelli et al., 2020). As breeding age heifers have not yet achieved mature body weight (55-60% mature body weight by first service; Margerison and Downey, 2005), the shorter AGD in heifers compared with cows is not surprising as mean AGD differed even among first-, second- and third+-parity cows (Gobikrushanth et al., 2017). Nonetheless, the distribution and variability of AGD found in dairy heifers was comparable to the pattern of distribution and variability of AGD in dairy cows (Gobikrushanth et al., 2017; 2019; Akbarinejad et al., 2019), thus, indicating that a large phenotypic variation in AGD exists within Holstein cattle.

Although heritability estimates for traditional fertility traits are generally small (Berry et al., 2014), the negative association between production and fertility traits, as well as the decline in fertility that occurred through genetic selection (resulting in undesirable economic impacts), demonstrated the importance of genetic contribution to fertility traits (Lucy, 2001; Walsh et al., 2011; Peñagaricano and Khatib, 2012). In addition, variation in fertility between and within breeds indicate the possibility of improving fertility without severely compromising genetic gain for milk production (Lucy, 2001; Cochran et al., 2013). This notion is corroborated by the stabilization and reversal of the declining trend in daughter pregnancy rate since 2003 when daughter pregnancy rate was first included in the USDA national genetic evaluation (VanRaden et al., 2004).

The unfavourable decline in fertility in combination with the low heritability of traditional fertility traits suggest that novel fertility phenotypes should remain a key focus of genetic exploration. It is notable that Gobikrushanth et al. (2019) reported a heritability estimate of 0.37 for AGD, which is much greater than that of most traditional female fertility traits, such as number of services (0.02), calving interval (0.03), and non-return rate (0.03; Berry et al., 2014). Other novel reproductive phenotypes of recent interest with heritability estimates similar to that of AGD are antral follicle count (0.31; Walsh et al., 2014) and anti-Müllerian hormone concentration (0.46; Gobikrushanth et al., 2018). These aforesaid phenotypes are much more difficult and costly to obtain and require sophisticated equipment and well-trained technicians to achieve. In contrast, AGD is a simple measurement obtained with calipers in about 30 sec per cow. Endocrine fertility traits such as circulating concentrations of progesterone (Lamming and Darwash, 1998) and GnRH-induced LH (Haley et al., 1989; Gobikrushanth et al., 2017) have been explored as alternatives for traditional fertility traits as they are more directly reflective of reproductive physiology, whereas traditional traits are more heavily influenced by management decisions and

derived from insemination and calving data (Lamming and Darwash, 1998; Darwash et al., 1999), thereby further demonstrating the potential of these novel endocrine or endocrine-influenced phenotypes. The identification of a single nucleotide polymorphism (SNP) associated with phenotypic variance of any trait may have the potential to improve the prediction accuracy of the trait if eventually incorporated into the SNP selection panel (Gobikrushanth et al., 2019). Although no significant SNPs have been documented in association with phenotypic variation in AGD in dairy cattle to date (Gobikrushanth et al., 2019), there is potential for future genome-wide association studies on AGD to identify one or more SNP that may increase the genomic prediction accuracy of the trait. The genetic associations between AGD and current fertility and production traits, however, must first be determined. Given the poor to moderate sensitivity (66.2%) and specificity (42.5%) of AGD as a predictor of pregnancy to 1st AI, further research evaluating optimum threshold AGD predictive of other reproductive measures of interest (e.g., age at conception) will be beneficial in evaluating the field application of this phenotype. If alternative measures of fertility have improved sensitivities and specificities of prediction, the practical application of AGD will have improved justification. Although we did not estimate the heritability of AGD in dairy heifers in the present study, the large phenotypic variation in combination with the strong inverse relationship between AGD and fertility measures in nulliparous heifers represent an opportunity for further exploration of the practical application of AGD.

Dairy fertility is a complex phenotype affected by various factors including environment, genetics, and management (Berry et al., 2014). Moreover, reductions in cow fertility have been linked to energy balance, nutrition, and metabolic disorders at the calving transition (Lucy, 2001). An advantage with evaluating AGD in nulliparous heifers is that the additive effects of environmental and management influences common during the transition period are removed from

the equation. From a management standpoint, heifer-rearing is often given less attention, even though it accounts for 15 to 20% of the total cost of milk production (Pirlo et al., 2000). Virgin heifers have greater first-service conception rates compared with cows (Lucy, 2001). Nevertheless, management factors during the rearing period such as poor estrus detection or lack of a systematic breeding program leading to missed or untimely breeding may negatively affect reproductive efficiency. This inefficiency manifests itself in the form of increased age at first conception, more AI services, and greater age at first calving. In the current study, the mean age at first AI in long-AGD group heifers was approximately 2 d greater than in short AGD group. While this two-day difference is statistically significant, it may not be of much relevance from a managerial perspective. In contrast, we speculate that the two-day reduction in age at first AI may be because heifers with long AGD had less pronounced signs of estrus than those with short AGD, potentially making estrus detection more challenging.

Evidently, heifers with short AGD also required fewer services per conception and became pregnant to first AI more often than heifers with long AGD. In addition, for every 1 mm increase in AGD, the estimated probability of pregnancy to first AI was reduced by 1.9%, implying that the chance of heifers becoming pregnant is reduced with increasing AGD. These results reflect our preliminary findings (Carrelli et al., 2020) with only a subset of the heifers, and indicate that heifers with long AGD are not as reproductively efficient as those with short AGD. This inverse relationship between AGD and fertility is similar to previous findings (Gobikrushanth et al., 2017; Akbarinejad et al., 2019) in Holstein cows. Given that the mean age at conception was 6 d less in short-AGD heifers in the current study, heifer rearing costs could be reduced by up to \$15.24 (USD) per head, calculated at \$2.54/d (Tranel, 2019) for a heifer weighing 360 kg at the time of conception. Notably, a greater age at conception, thereby, a greater age at first calving can cause

significant reductions to profitability during both the rearing period as well as during the first lactation (Masello et al., 2020). Given that the greatest expense in heifer rearing is attributable to feed costs, accounting for approximately 60% of total rearing costs on both conventional farms and custom heifer rearing facilities (Gabler et al., 2000), a reduction in age at first calving could improve profitability on-farm. This is of particular relevance and concern in today's environment when replacement heifers are in oversupply (Overton and Dhuyvetter, 2020). Consequently, heifers with superior fertility require less financial input during the rearing period and generate more economic gain during the first lactation period.

In the present study, heifers with long AGD had reduced hazard (HR: 0.59) for pregnancy up to 450 d (15 mo) of age, with only 59.7 % pregnant by that time, a much lower proportion than that of short-AGD heifers (76.4 % pregnant). Although long-AGD heifers had reduced hazard (HR: 0.77) for pregnancy up to 540 d (18 mo), the difference between the two groups in cumulative pregnancy at 18 mo of age was not as distinct. This indicates that the gap in cumulative pregnancy risk between long- and short-AGD heifers is more pronounced at 450 d. By 540 d, however, this gap narrows considerably. Breeding heifers at a younger age, paired with an appropriate breeding program is a promising approach to increase profitability. Results from Hare et al. (2006) demonstrated that age at first calving in Holsteins decreased from 28 mo in 1980 to 25.5 mo by 2004. During this same period, the proportion of heifers calving at 24 mo increased from 8 to 20%. Heinrichs et al. (2013) demonstrated the most efficient heifer-raising programs in Pennsylvanian herds had a mean age at first calving of 23.7 mo and first-lactation cows producing 88.4% of the milk produced by multiparous cows. As the industry shifts towards reduced age at first calving, the likelihood of heifers being culled by 540 d (18 mo) greatly increases. Considering that a significantly greater proportion of long-AGD heifers were non-pregnant at 450 d, there is a

potential opportunity to selectively remove long-AGD heifers that remain non-pregnant beyond 450 d, as a strategy to improve profitability and reproductive efficiency.

The economic value of a trait has traditionally been an important consideration for genetic selection. Moreover, an indicator trait may be favoured if it has a high genetic correlation with the economically important trait, reduces data recording costs, has a greater heritability, or can be measured earlier in life (Shook, 1989). In that regard, the potential of AGD as a novel reproductive phenotype is great because the cost of measurement is small, its heritability is greater than commonly used fertility traits, and the economic benefit of superior fertility is high.

3.5 Conclusions

In summary, the present study has demonstrated that AGD is normally distributed and highly variable in nulliparous dairy heifers. In addition, as previously reported in lactating Holstein cows (Gobikrushanth et al., 2017), strong inverse associations between AGD and measures of fertility were also found in nulliparous Holstein heifers.

Table 3-1. Descriptive statistics (mean \pm SD) for anogenital distance and measures of fertility in nulliparous heifers (n = 1692, from 17 dairy herds in Canada [CAN1 to CAN16] and the United States [USA1])

Farm	n	¹ AGD, mm	² Age at AGD, d (mo)	³ Age at 1 st AI, d (mo)	Age at conception, d (mo)	Services per conception
CAN1	275	112.4 \pm 10.1	436.5 \pm 38.8 (14.5 \pm 1.3)	455.0 \pm 16.0 (15.2 \pm 0.5)	468.0 \pm 28.2 (15.6 \pm 0.9)	1.5 \pm 0.8
CAN2	96	107.9 \pm 10.0	448.2 \pm 59.8 (14.9 \pm 2.0)	447.6 \pm 38.5 (14.9 \pm 1.3)	466.5 \pm 46.1 (15.5 \pm 1.5)	1.5 \pm 0.7
CAN3	46	115.2 \pm 10.8	446.8 \pm 38.6 (14.9 \pm 1.3)	417.3 \pm 22.6 (13.9 \pm 0.7)	431.5 \pm 27.6 (14.4 \pm 0.9)	1.6 \pm 0.7
CAN4	3	106.0 \pm 7.8	556.0 \pm 49.0 (18.5 \pm 1.7)	519.7 \pm 11.0 (17.3 \pm 0.4)	519.7 \pm 11.0 (17.3 \pm 0.4)	1.0 \pm 0.0
CAN5	17	102.2 \pm 8.8	428.4 \pm 37.2 (14.3 \pm 1.2)	407.1 \pm 30.0 (13.6 \pm 1.0)	439.0 \pm 44.0 (14.6 \pm 1.5)	1.8 \pm 0.9
CAN6	45	109.3 \pm 9.1	415.7 \pm 35.3 (13.9 \pm 1.2)	406.1 \pm 10.8 (13.5 \pm 0.4)	448.7 \pm 49.7 (15.0 \pm 1.7)	1.8 \pm 0.9
CAN7	19	100.1 \pm 6.4	480.5 \pm 59.0 (16.0 \pm 2.0)	466.7 \pm 24.7 (15.6 \pm 0.8)	479.8 \pm 40.1 (16.0 \pm 1.3)	1.4 \pm 0.8
CAN8	59	110.6 \pm 7.8	403.9 \pm 25.6 (13.5 \pm 0.9)	405.7 \pm 21.6 (13.5 \pm 0.7)	431.5 \pm 46.5 (14.4 \pm 1.6)	1.7 \pm 1.2
CAN9	40	115.5 \pm 11.2	424.5 \pm 44.8 (14.1 \pm 1.5)	403.3 \pm 20.7 (13.4 \pm 0.7)	417.5 \pm 29.1 (13.9 \pm 1.0)	1.6 \pm 0.8
CAN10	25	106.8 \pm 9.2	424.4 \pm 46.2 (14.1 \pm 1.5)	429.3 \pm 26.4 (14.3 \pm 0.9)	444.4 \pm 36.9 (14.8 \pm 1.2)	1.6 \pm 1.0
CAN11	23	108.4 \pm 7.7	433.0 \pm 56.3 (14.4 \pm 1.9)	439.4 \pm 31.1 (14.6 \pm 1.0)	475.2 \pm 50.0 (15.8 \pm 1.7)	1.6 \pm 0.9
CAN12	31	106.0 \pm 10.0	455.0 \pm 62.6 (15.2 \pm 2.1)	459.0 \pm 29.2 (15.3 \pm 1.0)	478.7 \pm 39.2 (16.0 \pm 1.3)	1.9 \pm 1.1

CAN13	42	108.4 ± 8.1	448.0 ± 22.5 (14.9 ± 0.7)	416.6 ± 7.9 (13.9 ± 0.3)	455.2 ± 50.9 (15.2 ± 1.7)	1.8 ± 1.1
CAN14	49	111.6 ± 8.8	451.6 ± 49.9 (15.1 ± 1.7)	438.0 ± 36.4 (14.6 ± 1.2)	461.1 ± 53.0 (15.4 ± 1.8)	1.4 ± 0.7
CAN15	19	112.9 ± 13.2	463.3 ± 42.0 (15.4 ± 1.4)	409.2 ± 36.9 (13.6 ± 1.2)	417.8 ± 38.2 (13.9 ± 1.3)	1.3 ± 0.5
CAN16	121	110.9 ± 9.6	447.0 ± 52.2 (14.9 ± 1.7)	423.2 ± 31.5 (14.1 ± 1.0)	457.7 ± 63.3 (15.3 ± 2.1)	1.9 ± 1.1
USA1	782	103.5 ± 9.7	391.0 ± 22.6 (13.0 ± 0.8)	388.6 ± 11.2 (13.0 ± 0.4)	398.6 ± 20.5 (13.3 ± 0.7)	1.5 ± 0.8
Overall	1,692	107.3 ± 10.5	417.0 ± 45.1 (13.9 ± 1.5)	413.6 ± 34.2 (13.8 ± 1.1)	429.8 ± 46.3 (14.3 ± 1.5)	1.5 ± 0.8
Min, Max		69, 142	323, 606 (10.8, 20.2)	349.0, 539.0 (11.6, 18.0)	349, 770 (11.6, 25.7)	1, 6

¹AGD = anogenital distance, measured from the center of the anus to the base of the clitoris;

²Age at AGD = age when AGD was measured; ³Age at 1st AI = age at first artificial insemination

Table 3-2. Relationship between anogenital distance¹ and various measures of fertility in nulliparous heifers of short- and long-AGD groups.

Description	n	Short-AGD (≤ 110 mm),	Long-AGD (> 110 mm),	<i>P</i>
		n = 1,055 (LSM \pm SE)	n = 637 (LSM \pm SE)	
Age at first AI, d	1,692	429.6 \pm 7.4	431.9 \pm 7.4	0.032
P/1 st AI, %	1,681 ²	58.3 \pm 3.0	49.6 \pm 3.1	0.00080
Services per conception	1,539 ²	1.5 \pm 0.1	1.7 \pm 0.1	0.0032
Age at conception, d	1,539 ²	448.4 \pm 6.6	454.3 \pm 6.7	0.0018

¹AGD = anogenital distance, measured from the center of the anus to the base of the clitoris

²Discrepancies in animal numbers for P/1st AI (n = 11), services per conception (n = 153), and age at conception (n = 153) can be explained by respective heifers without known conception dates

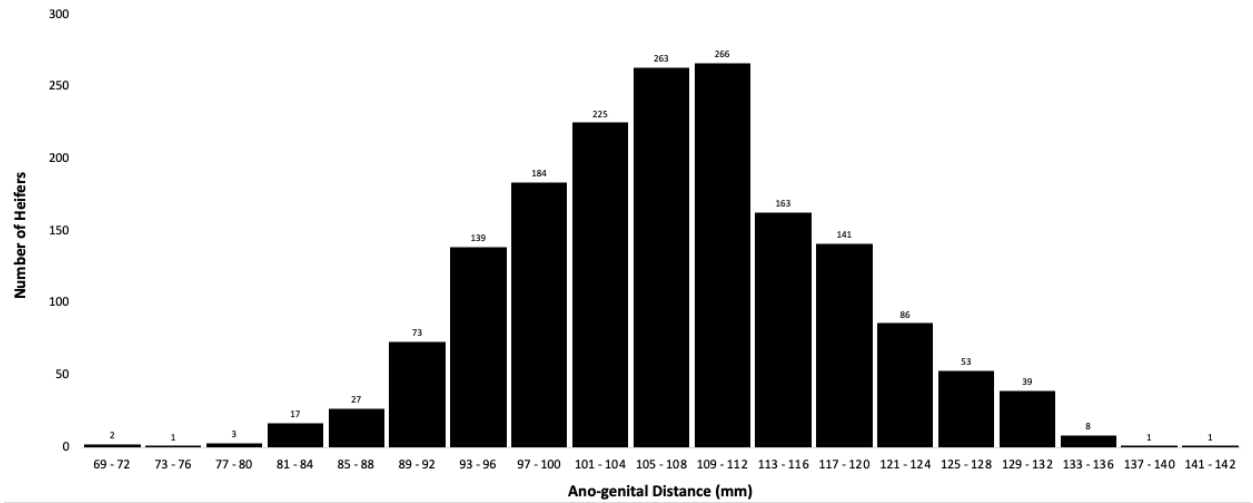


Figure 3-1. Distribution of anogenital distance (AGD; mm) in a population of nulliparous Holstein heifers (n = 1,692, from 17 dairy herds). Mean age at AGD measurement was 417.0 ± 45.1 d (13.9 ± 1.5 mo).

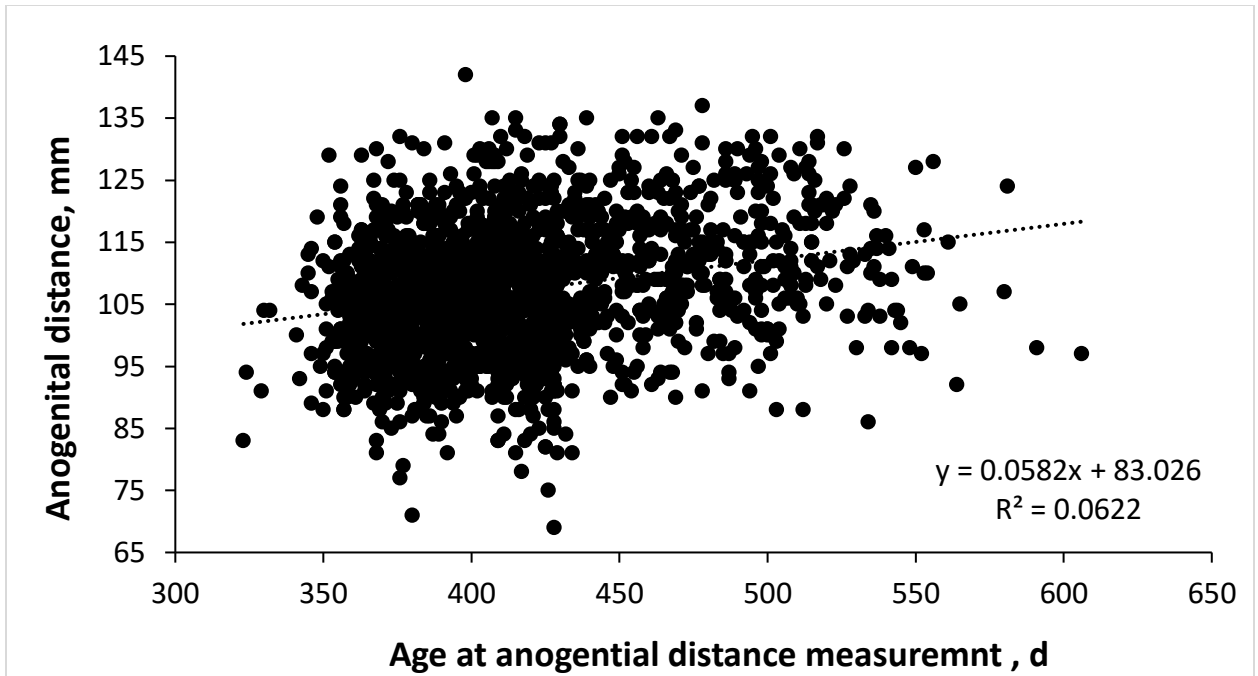


Figure 3-2. Association between age at anogenital distance measurement and anogenital distance ($R^2 = 0.06$; $P = 0.0073$) in nulliparous Holstein heifers ($n = 1,692$).

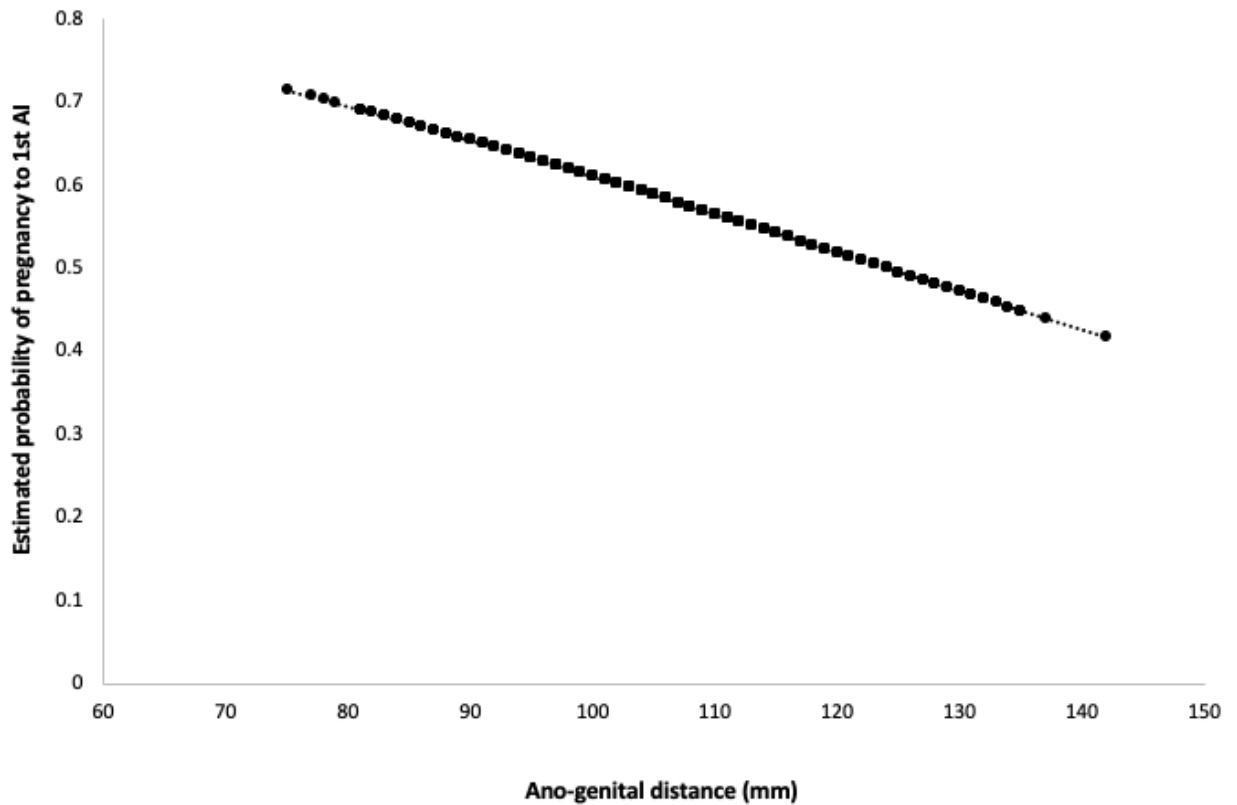


Figure 3-3. The estimated probability of pregnancy to first AI (P/1stAI) plotted according to anogenital distance (AGD) in nulliparous Holstein heifers (n = 1,692). For every 1-unit (mm) increase in AGD, the estimated probability of pregnancy to first AI was reduced (P < 0.001) by 1.87%.

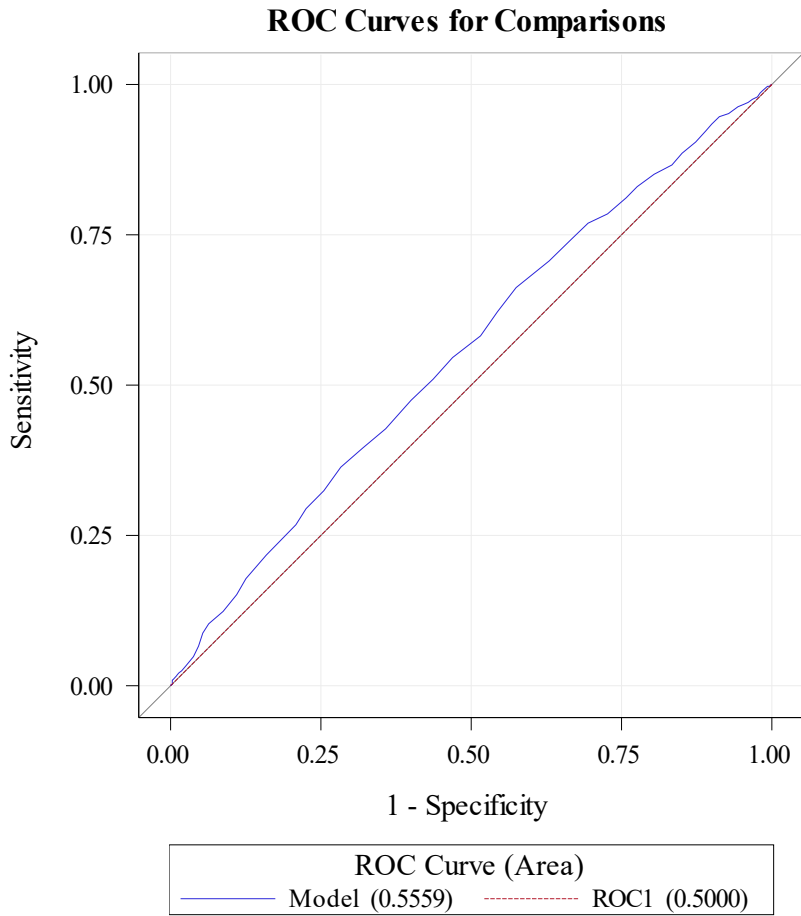


Figure 3-4. Receiver operating characteristic (ROC) curve analysis for anogenital distance (AGD) that predicted probability of pregnancy to the first AI in nulliparous Holstein heifers (n = 1,692; area under the curve: 0.5559; sensitivity: 66.2%; specificity: 42.5%).

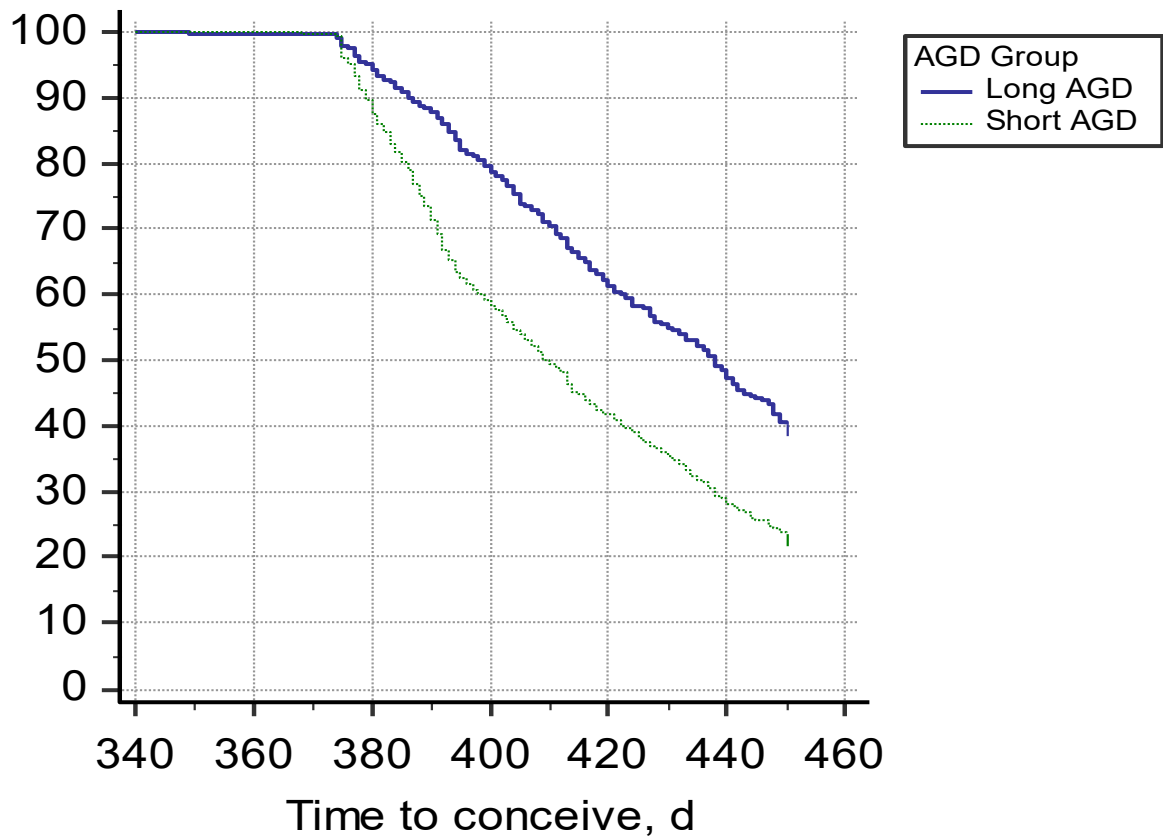


Figure 3-5. Kaplan-Meier survival analysis to determine the proportion of nulliparous Holstein heifers that remained nonpregnant up to 450 d of age. Heifers with long-AGD had reduced ($P < 0.0001$) hazard for pregnancy up to 450 d compared with those with short-AGD (HR: 0.59).

4.0 Associations between anogenital distance and measures of fertility in lactating North American Holstein cows: a validation study

4.1 Introduction

Over the last 40 years, dairy production systems where cows have been heavily selected for milk production have reported substantial declines in reproductive performance over the same relative period, despite having diverse production systems, genetic selection criteria, and climatic conditions (Walsh et al., 2011). Fertility is a multi-faceted trait and its decline has been caused by a complex framework of genetic, environmental and managerial factors as well as their interactions (Walsh et al., 2011). In addition, the genetic correlation between fertility and productive life indicates that fertility plays a significant role in the cow's longevity (VanRaden et al., 2004). As a result, the dairy industry has shifted its focus from primarily production to incorporate more comprehensive breeding objectives, such as nonproduction traits associated with improved health and fertility (Miglior et al., 2005).

Anogenital distance, defined as the distance between the anus and the external genitalia in both males and females, has exhibited sex-related variation in rodents (Clark and Galef, 1995; Drickamer et al., 1996; Bánzegi et al., 2012, 2015). In women, AGD has been defined as the distance from the center of the anus to the posterior fourchette (Salazar-Martinez et al., 2004) or clitoris in women (Sathyanarayana et al., 2010); and is said to be reflective of excess exposure of female fetuses to androgens, leading to androgenization of the reproductive system *in utero* in both rats (Bowman et al., 2003) and humans (Sadler, 2012). Anogenital distance has been more recently defined in dairy cattle as the distance from the center of the anus to the base of the clitoris (Gobikrushanth et al., 2017b), although the theory of excess androgen exposure as a determinant

of AGD has not been confirmed in dairy cattle. The first report of AGD measures in dairy cattle demonstrated the normal distribution and high variability of AGD in 921 lactating Canadian Holstein cows (Gobikrushanth et al., 2017b). Subsequent studies in Irish Holstein-Friesian (n = 1,180; Gobikrushanth et al., 2019b) and Iranian Holstein (n = 86; Akbarinejad et al., 2019) cows have demonstrated similar findings in regard to distribution and variation of AGD. In Canadian Holsteins, AGD was inversely associated with fertility in first- and second-parity cows; however, there were no apparent associations in third+-parity cows (Gobikrushanth et al., 2017b). Anogenital distance tended to be inversely related to fertility in Iranian Holstein cows by means of days to first service, first service conception rate, and proportion of repeat breeders (i.e., cows that failed to conceive after three services; Akbarinejad et al., 2019); however, there were no significant associations between AGD and fertility measures of interest in Irish Holstein-Friesian cows (Gobikrushanth et al., 2019b). The heritability estimate for AGD in the Irish study was 0.37, higher than most traditional fertility traits such as number of services (0.02), pregnancy to first service (0.02), and days open (0.06; Berry et al., 2014). The lack of an inverse relationship between AGD and fertility in Irish Holstein-Friesians was attributed to the pronounced selection emphasis on fertility traits and the aggressive culling of sub-fertile cows, leading to a relatively more fertile population of dairy cows in Ireland than in Canada and Iran. More recently, AGD has been inversely associated with measures of fertility in nulliparous heifers such as services per conception, age at conception, and pregnancy to first insemination (Carrelli et al., 2020; also, Chapter 3 of this thesis); further exemplifying the inverse relationship between AGD and fertility in North American Holstein cattle. Validating previous findings of the distribution, variability and relationship of AGD with measures of fertility in Holstein cattle with a larger sample size is necessary to evaluate the potential application of AGD for use as a novel fertility phenotype. If

AGD is successfully established as a reproductive phenotype in the future, it would be important to know whether selecting for short AGD (indicative of improved fertility) would have an inverse effect on milk production in dairy cows, as antagonistic relationships between milk production and fertility have been described in the past (Everett et al., 1966; Miller et al., 1967; Berger et al., 1981; Oltenacu et al., 1991; Dematawewa and Berger, 1998; VanRaden et al., 2004). We hypothesized that the inverse association between AGD and measures of fertility, as previously described in first- and second-parity Holstein cows, would be apparent in a larger population of North American cows. Therefore, the objective of this study was to determine if an inverse relationship exists between AGD and measures of fertility in a larger population of North American Holstein cows. A secondary objective of this study was to investigate the association between AGD and 305-d mature equivalent (ME) milk yield.

4.2 Materials & Methods

4.2.1 Animals and Management

All animal use was approved by the University of Alberta's Animal Care and Use Committee for Livestock (AUP#00002883) and by the University of Idaho Animal Care and Use Committee (Protocol #IACUC-2019-61). The study was conducted using cows from three institutional and 15 commercial dairy farms across Western Canada (Alberta and British Columbia), and one commercial dairy farm in Washington State, USA. All animal husbandry procedures were in accordance with the requirements of the Canadian Council on Animal Care and the United States Department of Agriculture. Cows were housed in either a free-stall barn (15 herds) or a tie-stall barn (4 herds), provided a total mixed ration (primarily composed of barley or

corn silage, alfalfa silage, alfalfa hay, and concentrates) formulated according to NRC (2001) guidelines and had ad-libitum access to water. Cows received fresh feed up to twice daily and were milked up to three times daily. Cows were bred based on activity monitoring systems, ovulation synchronization protocols, or a combination of two or more techniques.

4.2.2 Determination of Anogenital Distance and Measures of Fertility

Anogenital distance, the distance from the center of the anus to the base of the clitoris, was measured using 8-inch stainless steel digital calipers (Pro.Point, Princess Auto Ltd., Winnipeg, Manitoba, Canada) as described by Gobikrushanth et al. (2017b). A single AGD measurement was obtained for each cow, measured by one of two experienced individuals; however, each herd may have been visited up to two times if only a portion of the herd could be accessed in a single visit. Anogenital distance measurements were obtained from all cows that had no apparent perineal abnormalities such as inflamed or lacerated vulva as indicators of trauma at parturition. All animals that were ± 14 DIM at the time of AGD measurement, designated as “do not breed” (DNB) before their first insemination, or had left the herd (i.e., dead or sold) with no fertility data were excluded from analyses, resulting in a final population of 5,173 cows. Data on 305-d mature-equivalent milk yield and fertility measures [pregnancy to first AI (P/1st AI), pregnancy to second AI (P/2nd AI), pregnancy to third AI (P/3rd AI), times bred, services per conception, days open (interval from calving until subsequent conception), and cumulative pregnancy by 150 DIM and 250 DIM] were retrieved for all cows using DairyComp 305 herd management software (Lactanet, Guelph, ON, Canada; Valley Agricultural Software Inc., Tulare, CA, USA).

4.2.3 Statistical Analyses

Data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Descriptive statistics such as mean, standard deviation, minimum and maximum for AGD as well as normality of the data were determined using UNIVARIATE procedure of SAS. Descriptive data were analyzed for all cows ($n = 5,173$), and by parity group (first-parity: $n = 1,920$; second-parity: $n = 1,346$; third+-parity: $n = 1,907$). For calculation of mean DIM at AGD measurement, data from 38 cows were removed from this analysis because they were over 500 DIM and considered outliers in this population.

MIXED procedure of SAS was used to determine if AGD differed between Canadian and US populations of dairy cows; based on this analysis, we were able to keep Canadian and US datasets combined because AGD did not differ between the two populations ($P > 0.10$). With AGD as a continuous variable, associations between AGD, parity and 305-d ME milk yield (kg) were analyzed for all cows ($n = 5,173$) using CORR and REG procedures of SAS to determine if AGD differed among parity groups and whether 305-d ME milk yield was influenced by AGD. The association between AGD and P/1stAI, P/2ndAI, and P/3rdAI were analyzed using LOGISTIC procedure of SAS to determine the estimated probability of pregnancy to first, second and third AI for all cows and by parity group. No significant relationships were observed ($P > 0.10$); therefore, cows were classified into short- (\leq mean) and long-AGD ($>$ mean) groups based on mean AGD for each parity group [first- (129 mm), second- (133 mm), and third+-parity (136 mm)]. GLIMMIX procedure was used to analyze reproductive outcomes by AGD group, parity group, and their interaction, with herd treated as a random effect. The interaction between AGD group and parity was not significant ($P > 0.10$) and was removed from the model. Therefore, the final GLIMMIX model analyzed the relationships between AGD group, parity group, and fertility

measures of interest such as P/1st AI, P/2nd AI, P/3rd AI, times bred, services per conception, and days open, with the effect of herd treated as random. Differences in cumulative pregnancy at 150 DIM and 250 DIM were analyzed using the LIFETEST procedure of SAS in a subset of cows, excluding 534 cows without conception data (pregnancy outcomes were not available at time of analysis or cows had left the herd before confirmed pregnant).

4.3 Results

4.3.1 Descriptive Statistics

Anogenital distance was normally distributed within a wide range of AGD estimates (Figure 4-1). The overall mean \pm standard deviation and range of AGD by parity group are as presented in Table 4-1. Mean days in milk at AGD measurement was 169.1 ± 103.4 ranging from 15 to 498 DIM.

4.3.2 Association between AGD, Parity and 305-d Mature Equivalent Milk Yield

In the present study, AGD was positively correlated with parity group (Figure 4-2); however, the strength of the relationship was weak among the population ($r = 0.21$; $P < 0.01$). The AGD was also positively, albeit poorly, correlated with 305-d ME milk yield (Figure 4-3; $r = 0.04$; $P < 0.01$). In addition, the phenotypic variation in AGD that was explainable by these two variables was small (coefficient of determination $R^2 = 0.04$ and 0.001 , respectively).

4.3.3 Relationship between AGD, Parity, and Measures of Fertility

Cows with short AGD had greater P/1st AI than cows with long AGD ($P < 0.01$); however, there was no difference in P/2nd AI and P/3rd AI between AGD categories ($P > 0.10$). In addition, cows with short AGD had fewer days open and a tendency to require fewer services per conception than cows with long AGD (Table 4-2). There was no interaction observed between parity and AGD group; however, fertility measures of interest did vary significantly by parity group (Table 4-3). Pregnancy to first AI was greater for first-parity cows compared to cows in both second- ($P = 0.05$) and third+-parity ($P < 0.01$). There was no difference in P/1st AI between cows in second and third lactation ($P > 0.10$). In addition, P/2nd AI tended to vary between parity groups, where first parity cows conceived to their second insemination more often than third+-parity cows ($P = 0.07$). There was no difference in P/3rd AI among parity groups. Similar to findings above for P/1st AI, first-parity cows were subjected to fewer inseminations overall and required fewer services per conception than second-parity cows ($P = 0.04$ and 0.01 , respectively). Moreover, cows in their first lactation were also subjected to fewer inseminations overall and tended to require fewer services per conception than cows in their third lactation or greater ($P = 0.02$ and 0.07 , respectively). Days open tended to differ among parity groups ($P = 0.09$), with first-parity cows conceiving 5.1 d sooner than third+-parity cows. Lastly, cumulative pregnancy risk up to 150 and 250 DIM did not differ between AGD categories ($P = 0.12$ and 0.30 , respectively).

4.4 Discussion

Anogenital distance was previously characterized in a population of 921 Canadian Holstein cows and found to be normally distributed and highly variable, with mean (\pm SD) of 131.0 ± 12.2

mm, ranging from 96 to 170 mm (Gobikrushanth et al., 2017b). Overall mean AGD, as observed in the current study, is 1.5 mm greater than the overall mean AGD observed in Canadian Holsteins (Gobikrushanth et al. 2017b), 13 mm greater than Irish Holstein-Friesians (Gobikrushanth et al., 2019b), and 19 mm greater than Iranian Holstein cows (Akbarinejad et al., 2019). The AGD was normally distributed and highly variable in all described populations, suggesting that there is a relatively larger population of cows with short AGD in Ireland and Iran than in North America. Moreover, mean (\pm SD) AGD for first-, second-, and third+-parity cows as observed in the current study were 2 mm, 1 mm, and 1 mm greater, respectively, than that observed in Canadian Holsteins previously (Gobikrushanth et al., 2017b). Despite AGD being highly variable, similarities in mean AGD and range across these two North American populations of Holstein cattle suggest that measurement of AGD is highly repeatable within the North American population.

The correlation between measures of fertility and milk production has received a lot of attention in the past, where an antagonistic relationship between female fertility and milk production has been reported in several studies over the last 50 years (Everett et al., 1966; Miller et al., 1967; Berger et al., 1981; Oltenacu et al., 1991; Dematawewa and Berger, 1998; VanRaden et al., 2004). If AGD is successfully established as a reproductive phenotype for consideration in future genetic selection programs, it would be desirable to know whether selecting for short AGD (indicative of improved fertility) would have an inverse effect on milk production in dairy cows. Thus, the association between AGD and 305-d mature equivalent (ME) milk yield was investigated; The present study demonstrates that only 0.1% of the variation in AGD was explainable by 305-d ME milk yield, indicating that the phenotypic selection for AGD will not cause a substantial decline in milk production. Similar results have been described in the past, where even if an antagonistic relationship between fertility and milk yield existed, the response on

fertility due to selection on milk production would not be significant (Everett et al., 1966; Miller et al., 1967; Shanks et al., 1978). Regardless, it became accepted in the industry that due to unfavourable genetic correlations, selection for higher milk yields in dairy cattle, combined with little or no selection emphasis on fertility traits, has possibly contributed to a decline in fertility. In addition, traditional fertility traits such as number of services, pregnancy to first service, calving interval, days open, tend to have low heritability estimates (0.02 to 0.04; Berry et al., 2014) and difficulties related to their measurement, suggesting that indicator traits could be very useful for increasing accuracy of estimated breeding values for fertility (Miglior et al., 2017). Endocrine fertility traits, based on milk progesterone concentrations, have been suggested as indicators for fertility as they are more directly reflective of a cow's reproductive physiology than insemination- and calving-based measures of fertility and less influenced by on-farm management decisions (Lamming and Darwash, 1998; Darwash et al., 1999). The collection of detailed phenotypes for a sufficiently large reference population, paired with the corresponding genotypic information for those reference animals, allows accurate estimation of marker effects for a specific trait. The benefit being that those detailed phenotypes could have noticeably higher heritability than traditional measures of fertility (Miglior et al., 2017). Although heritability of AGD was not estimated in the present study's population of North American Holsteins, a heritability estimate of 0.37 was reported for AGD in Irish Holstein-Friesians (Gobikrushanth et al., 2019b). Measuring AGD and genotyping animals in a sufficiently large reference population will allow estimation of marker effects, which can then be used to direct genomic breeding values in a group of selection candidates. In addition, combining genomic predictions for endocrine and traditional fertility traits may provide a more accurate means of improving dairy cow fertility.

In a first report by Gobikrushanth et al. (2017b), AGD and P/1st AI had a significant inverse relationship in first- and second- parity Holstein cows; however, AGD was not associated with P/1st AI in third+-parity cows. Unlike the latter study, the present study as well as that of Akbarinejad et al. (2019) found the interaction between parity and AGD to have no significance on measures of fertility. The odds of first service conception rate in the overall population of Iranian Holsteins tended to be 18 percentage units less in cows with long AGD (30.2%) compared to those with short AGD (48.8%; Akbarinejad et al., 2019). Similarly, first- and second-parity Canadian Holstein cows with long AGD became pregnant to first insemination 23% and 16% less frequently than cows with short AGD, respectively (30.9 vs. 53.6 % and 28.3 vs. 44.4%, respectively; Gobikrushanth et al., 2017b). Results of the present study corroborate the latter studies, albeit less pronounced, in that cows with long AGD became pregnant to first AI 4% less often than those with short AGD. The lack of relationship between AGD groups and pregnancy to second and third inseminations may be attributable to the notion that cows advancing to 2nd or 3rd service were in positive energy state such that fertility differences were not that evident between AGD groups. Moreover, the number of repeat breeders in the Iranian Holstein population varied significantly between AGD groups, where the percentage of repeat breeders among long-AGD cows (32.6%) was twice that of short-AGD cows (16.3%; Akbarinejad et al., 2019). In the present study, cows with short AGD were subject to fewer inseminations overall and tended to require fewer services per conception than cows with long AGD. These results differ from those of a previous report (Akbarinejad et al., 2019) where services per conception were not different between AGD groups.

In Iranian Holstein cows, the hazard of interval from calving to first service tended to be reduced in long-AGD cows than short-AGD cows (HR: 0.69) leading to a tendency for longer days

to first service in cows with long AGD. In addition, the interval between calving to conception was 38 days longer for cows with long AGD compared to those with short AGD (Akbarinejad et al., 2019). Although the interval from calving to conception was not as prominent as reported by the latter authors, a similar positive relationship between AGD and days open, was evident in the present study, where long-AGD cows remained non-pregnant an average of 4 days longer than cows with short AGD. Although the relationship between AGD and days open is comparable between the present study and that by Akbarinejad et al. (2019), the 34 d reduction in the interval from calving to conception in the present study may be attributable to the increased population size of the present study (n = 5,173) compared to the study in Iranian cattle (n = 86).

Previous work has demonstrated first-parity cows with long AGD have a decreased likelihood (hazard ratio: 0.68) of pregnancy by 250 DIM compared to first-parity short-AGD cows (Gobikrushanth et al., 2017b). Additionally, maiden heifers with long AGD have reduced hazard for pregnancy up to 450 d (15 mo) of age compared with those with short AGD (59.7 vs. 76.4%, respectively; Carrelli et al., 2020). In the present study, however, pregnancy risk by 150 and 250 DIM did not significantly differ by AGD category. These results may explain why P/2nd AI and P/3rd AI did not differ between AGD categories. Differences among pregnancy risk may have been more apparent earlier in lactation (~100 DIM) when significant differences for P/1st AI between short- and long-AGD categories were detected. Cows with short AGD may have conceived to 1st AI more readily, despite negative energy balance, because they were inherently more fertile.

Irrespective of AGD, first-parity cows became pregnant to 1st AI more often than those in second- and third+-parity. Therefore, results of the present study corroborate findings of Gobikrushanth et al. (2017b) that P/1st AI is higher in short-AGD cows; however, no differences were observed by parity. P/2nd AI tended to differ among parity groups, where first-parity cows

tended to conceive to their second AI more often than cows in third+-parity, likely due to reduced fertility as a consequence of age (Norman et al., 2009). Notably, the number of overall inseminations and the number of services per conception varied by parity-group, where first-parity cows were subject to fewer overall inseminations and required fewer services per conception than cows in both second- and third+-parities. Increased number of services per conception is often indicative of complications with the individual animal's reproductive system, which has a negative impact on farm profitability (LeBlanc, 2007; Honarvar et al., 2010) and often results in culling from the herd (Sewalem et al., 2008). Moreover, first-parity cows remained open an average of 5 d less than cows in third+-parity. These results are in alignment with findings of Norman et al. (2009), where days between calving and first breeding, days between calving and last breeding, and the interval between first and last breeding generally increased with increasing parity.

Inferior reproductive performance of long-AGD cows in the present study could be attributed to carryover effects of prenatal exposure to androgens on various reproductive organs and regulatory systems including the ovary as well as neuroendocrine and metabolic systems (Padmanabhan and Veiga-Lopez, 2013). Excessive prenatal androgen exposure disrupts expression of steroid receptors, cytokines, and factors regulating apoptosis in the ovary (Ortega et al., 2009; Salvetti et al., 2012). Additionally, prenatal androgenization could result in neuroendocrine defects by lessening the negative and positive feedback effects of estradiol on the hypothalamus, thereby disrupting the tonic and surge secretion of LH, respectively (Padmanabhan and Veiga-Lopez, 2013; Veiga-Lopez et al., 2009). Although the source of excess androgens is easily explained in litter bearing species (male litter mates being a major source; Bánzegi et al., 2012; Dean et al., 2012), the source of excess androgens in non-litter bearing species (i.e., dairy

cattle) is not well known; therefore, investigations into the primary source of androgens in utero may be beneficial to understanding the phenotypic variation of AGD described in dairy cattle.

4.5 Conclusions

In conclusion, the present study validates earlier findings that AGD is inversely associated to measures of fertility in a large population of North American Holstein cows. Future research into the biological origin of excess androgens, as well as estimated heritability of AGD, will provide valuable insight into the potential of AGD as an indicator of dairy cow fertility for incorporation into comprehensive selection programs.

Table 4-1. Descriptive statistics for anogenital distance (AGD)¹ in lactating dairy cows from 19 herds in Canada and the United States.

Item	n	Mean ± SD (mm)	Minimum (mm)	Maximum (mm)
Overall AGD, all parities	5,173	132.5 ± 11.8	94.0	177.0
AGD in first-parity cows	1,920	128.9 ± 11.0	95.0	174.0
AGD in second-parity cows	1,346	133.3 ± 11.3	99.0	177.0
AGD in third+-parity cows	1,907	135.6 ± 12.1	94.0	176.0

¹AGD = the distance from the center of the anus to the base of the clitoris

Table 4-2. Relationship between anogenital distance (AGD) and various measures of fertility in lactating cows of short- and long-AGD groups.

Fertility Measure	n	Short-AGD ¹ (LSM ± SE)	Long-AGD ² (LSM ± SE)	P
P/1 st AI, % ³	5,119	35.7 ± 2.1 (n = 2,629)	31.4 ± 2.0 (n = 2,490)	<0.01
P/2 nd AI, %	3,474	37.4 ± 2.3 (n = 1,751)	34.9 ± 2.2 (n = 1,723)	0.15
P/3 rd AI, %	2,216	41.8 ± 2.4 (n = 1,115)	43.4 ± 2.4 (n = 1,101)	0.49
Times bred	5,173	2.5 ± 0.1 (n = 2,656)	2.6 ± 0.1 (n = 2,517)	0.04
Services per conception	4,639	2.3 ± 0.1 (n = 2,403)	2.4 ± 0.1 (n = 2,236)	0.06
Days open, d	4,639	136.9 ± 4.3 (n = 2,403)	140.9 ± 4.3 (n = 2,236)	0.05

¹Cows were considered short AGD if \leq mean of their respective parity group, i.e., \leq 129 mm (1st parity), 133 mm (2nd parity), 136 mm (3rd+parity)

²Cows were considered long AGD if $>$ mean of their respective parity group, i.e., $>$ 129 mm (1st parity), 133 mm (2nd parity), 136 mm (3rd+parity).

³Note: Cows that received a single insemination, but conception data was not yet available (n = 54) were excluded from this analysis as we could not verify number of services per conception.

Table 4-3. Relationship between parity groups and various measures of fertility in lactating cows.

Fertility Measure	n	First-parity (LSM ± SE)	Second-parity (LSM ± SE)	Third+-parity (LSM ± SE)	<i>P</i>
P/1 st AI, % ¹	5,119	36.5 ± 2.2 ^a (n = 1,903)	32.4 ± 2.2 ^b (n = 1,332)	31.7 ± 2.1 ^b (n = 1,884)	<0.01
P/2 nd AI, %	3,474	38.8 ± 2.4 ^a (n = 1,244)	35.3 ± 2.4 ^{ab} (n = 904)	34.5 ± 2.3 ^b (n = 1,326)	0.07
P/3 rd AI, %	2,216	44.5 ± 2.6 (n = 770)	43.5 ± 2.7 (n = 575)	39.9 ± 2.5 (n = 871)	>0.10
Times bred	5,173	2.4 ± 0.1 ^a (n = 1,920)	2.6 ± 0.1 ^b (n = 1,346)	2.6 ± 0.1 ^b (n = 1,907)	<0.01
Services per conception	4,639	2.2 ± 0.1 ^a (n = 1,754)	2.4 ± 0.1 ^b (n = 1,207)	2.4 ± 0.1 ^b (n = 1,678)	<0.01
Days open, d	4,639	136.4 ± 4.3 ^a (n = 1,754)	138.9 ± 4.4 ^{ab} (n = 1,207)	141.5 ± 4.4 ^b (n = 1,678)	0.09

¹Note: Cows that received a single insemination but conception data was not yet available (n = 54) were excluded from this analysis as we could not verify number of services per conception.

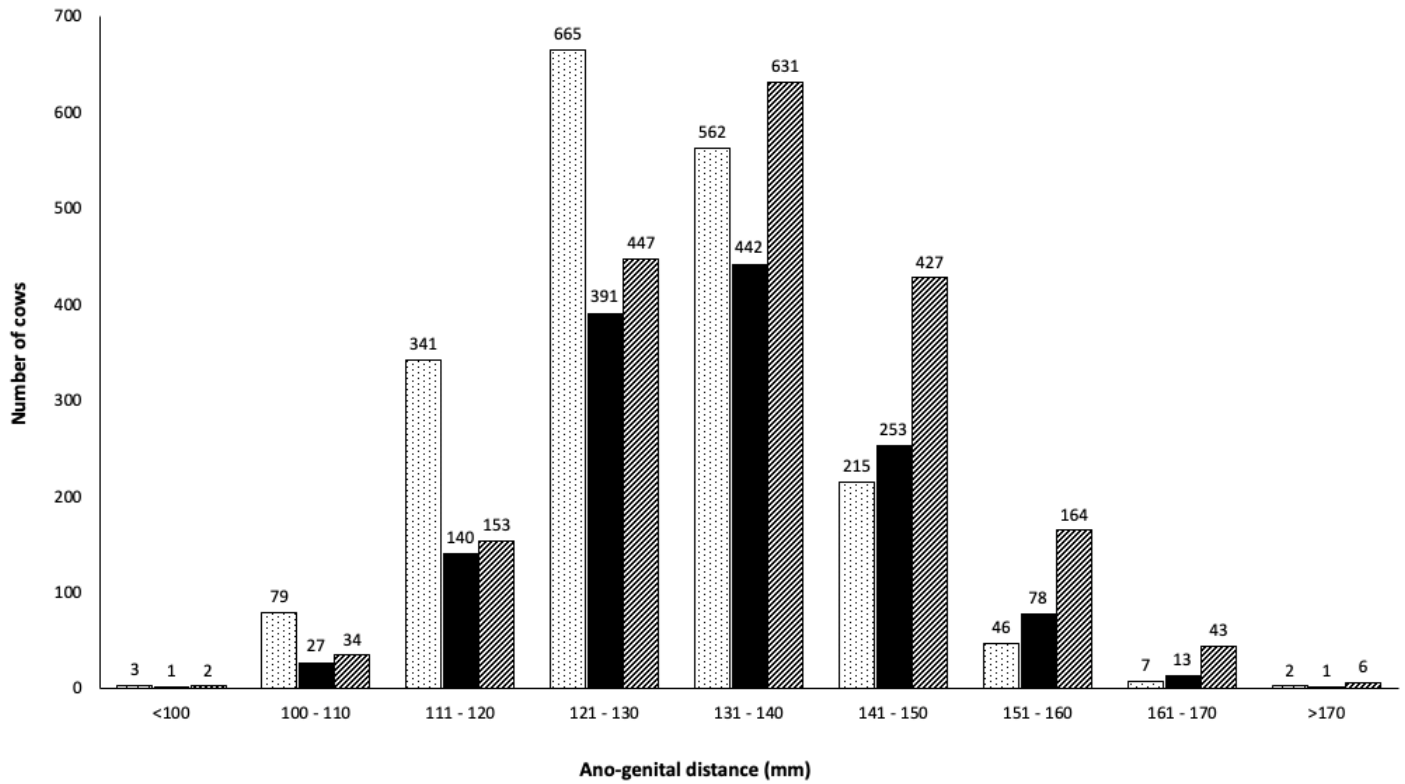


Figure 4-1. Distribution of anogenital distance in first-parity (dotted bars: n = 1,920), second-parity (filled bars: n = 1,346), and third+ parity (hatched bars: n = 1,907) cows.

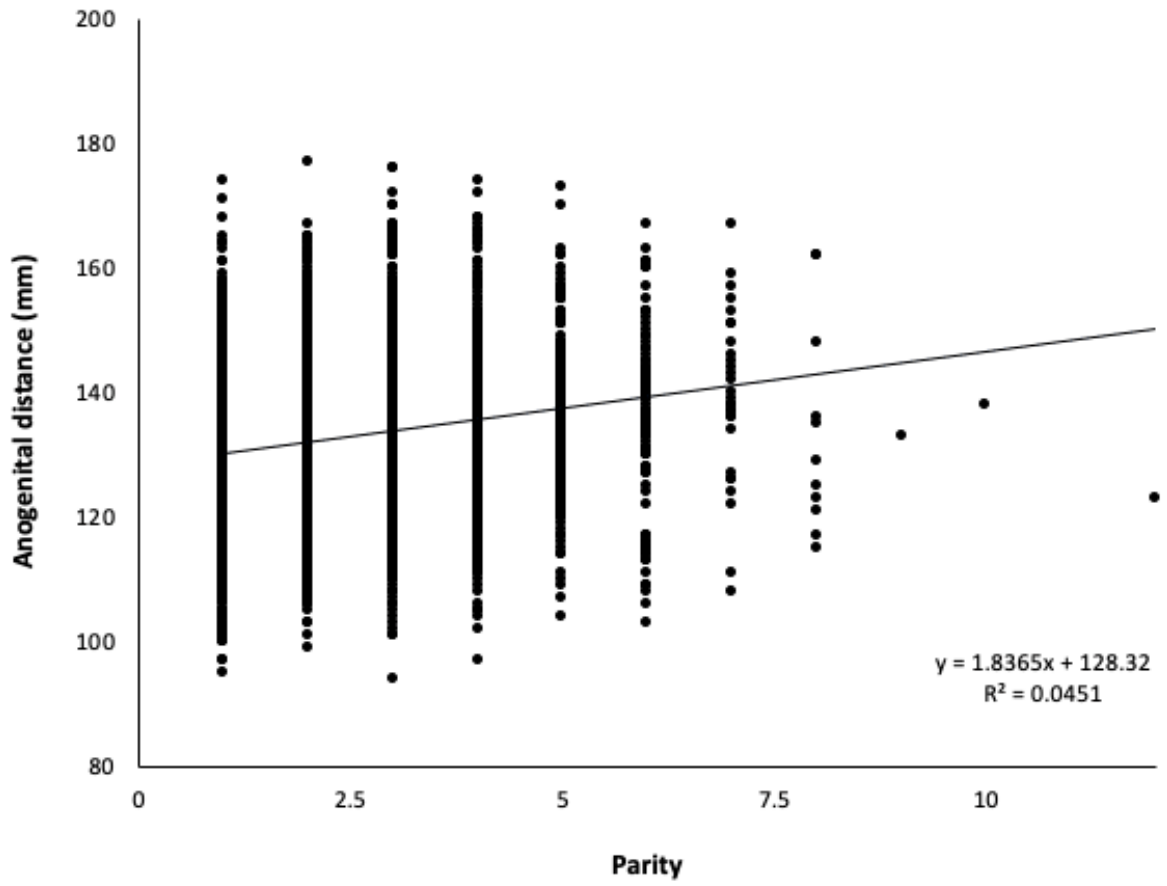


Figure 4-2. Association between parity and anogenital distance ($R^2 = 0.04$; $P < 0.01$) in lactating dairy cows ($n = 5,173$).

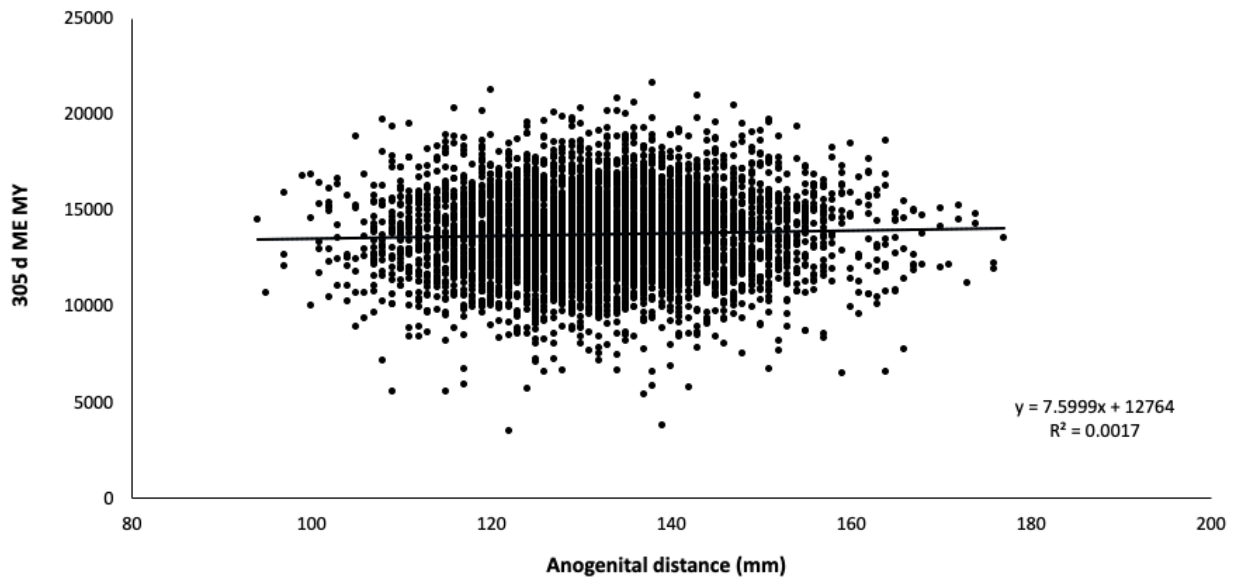


Figure 4-3. Association between 305-d mature equivalent milk yield (ME MY) and anogenital distance ($R^2 = 0.001$; $P < 0.01$) in lactating dairy cows ($n = 5,173$).

5.0 General Discussion

5.1 Importance of the current study

For a long time, selection in dairy cattle focused on the improvement of highly heritable production and conformation traits such as milk yield (0.2 to 0.55), milk fat (0.15 to 0.55), mammary conformation (0.15 to 0.45), and body conformation (0.15 to 0.45; Miglior et al., 2017). Selection goals have since developed to incorporate economically important traits with low heritability (e.g., interval from first service to conception, interval from calving to conception, services per conception, days open). These developments are partially attributable to the realization that such traits can be genetically improved and partially due to the fact that technological advancements have improved quantity and quality of data, in that more accurate reproduction data is readily available for analyses (Miglior et al., 2017). Since the inclusion of functional traits in selection indices, the detrimental effects of narrow selection goals have been counteracted (e.g., the absence of fertility traits), and we are currently making genetic progress in all traits of economic interest. To ensure continued progress and to develop breeding goals more in line with producer and consumer expectations, novel traits and phenotypes are now being considered for inclusion in national selection indices. The advantage being that such novel phenotypes could have markedly greater heritability than previously described traits (Egger-Danner et al., 2015). Anogenital distance, a simple morphologic phenotype, has demonstrated an inverse association with fertility in large populations of North American Holstein cows and heifers. As high-throughput genomic selection tools have advanced greatly in precision and now offer a platform for much more rapid genetic gains, the incorporation of newly discovered traits of

particular relevance to fertility, such as anogenital distance, are likely to bring significant benefits to the industry through improvements to reproductive performance.

5.2 Limitations

A reproductive phenotype that has high variability, repeatability, and heritability, in combination with a strong association with measures of fertility has the potential to become an ideal candidate trait in genetic selection for the overall improvement of fertility in dairy cattle populations. Characterization of AGD and its association with measures of dairy cattle fertility is a relatively new concept, with the first reports of this novel phenotype in 2017 using 921 Canadian Holstein cows (Gobikrushanth et al., 2017b). Since then, reports of AGD in dairy cows have corroborated findings of the original authors who indicated the novel phenotype is normally distributed and highly variable (Gobikrushanth et al., 2019b; Akbarinejad et al., 2019), with inverse associations with varying measures of fertility (Akbarinejad et al., 2019). The current studies measured AGD in large populations of North American Holstein cows and heifers and found AGD to be normally distributed and highly variable, with significant inverse relationships between AGD and measures of fertility in first-parity cows and nulliparous heifers. However, repeatability of AGD measure (consistency of AGD measurement for the same subject from multiple measures) was not evaluated for the current populations of cows and heifers. This is perhaps a limitation as 323 d old (minimum age in present study) heifers could have drastic proportional differences in size compared to the 606 d old (maximum age) heifers. However, filters were applied to remove data pertaining to heifers that were outliers in terms of age at 1st AI in the population, in addition, heifers where AGD was measured outside of ± 3 months of AI were

removed to provide some homogeneity to the data and account for these differences. Moreover, height and age measurements were not highly correlated to AGD in previous work in Holstein cows (Gobikrushanth et al., 2017b).

Functional demands fluctuate during stage of lactation, as high producing cows require increased nutrient intake to sustain production. In addition, nutrient demands further increase when a cow successfully establishes pregnancy and continues to increase with each stage of gestation. Therefore, evaluating repeatability of measurement during various periods of physiological demand will be important in evaluating the appropriate timing of AGD measurement and will lend further insight into the practical application of the AGD phenotype in selection indices. Therefore, not measuring repeatability of AGD at different stages of lactation as well as different stages of gestation are potential limitations to this study. Although a limitation of the current study, these questions have been addressed in another study from our lab group (Rajesh, 2021).

To date, heritability of AGD has only been estimated in a population of Irish Holstein-Friesian cows and found to be moderately heritable (0.37). This provides valuable insight to the use of AGD as a fertility indicator trait as the reported estimate is significantly higher than estimates reported for any traditional fertility traits (0.02 to 0.04; Berry et al., 2014). Heritability estimates were not completed for the given populations, and thus, a limitation of this study and an avenue for future exploration.

Lastly, our understanding of the physiological mechanisms controlling AGD in cattle is limited. This study did not aim to evaluate pre- and post-natal factors influencing AGD and should be considered a limitation of this study and an opportunity for future investigation.

5.3 Future Research

To improve our understanding of anogenital distance and its associations with measures of fertility in both lactating dairy cows and nulliparous heifers, future studies should focus on a few key knowledge gaps that still exist. Firstly, the theory of excess androgen exposure as a determinant of AGD has not been confirmed in dairy cattle, where the relationship between AGD and plasma testosterone concentration in a subset of 93 cows was weak and nonsignificant ($R^2 = 0.02$; $P = 0.19$; Gobikrushanth et al., 2017b); however, has been described in polytocous species such as rodents (Zehr et al., 2001; Bánzegi et al., 2012; Dean et al., 2012) and pigs (Drickamer et al., 1997), as well as monotocous species such as humans (Wainstock et al., 2017; Wu et al., 2017). Interestingly, the inferior reproductive performance of ewes has been at least partially explained by carryover effects of prenatal exposure to androgens on various reproductive organs and regulatory systems including the ovary as well as neuroendocrine and metabolic systems (Padmanabhan and Veiga-Lopez, 2013). This is of particular interest as the fetal development and reproductive physiology of sheep most closely compares to that of cattle, compared to any of the other aforementioned species. Future exploration into the primary source of androgens in utero is necessary to unraveling the underlying physiological mechanisms leading to poor fertility in cows and heifers with long AGD, and will be beneficial to understanding what proportion of the phenotypic variation observed for AGD is attributable to the animal's pre- and post-natal environment. Looking at ovine models for the physiological mechanisms behind the adverse effects of prenatal androgen exposure on reproductive pathways and future performance, may be valuable in determining associations between prenatal androgen exposure and AGD in cattle. Moreover, the determination of amniotic fluid and placental blood concentrations of androgens during a heifer's prenatal reproductive programming window will perhaps allow us to better

understand if the theory of excess androgen exposure as a determinant of AGD holds true in dairy cattle. To this effect, it may also be beneficial to measure concentrations of endocrine disrupting chemicals in maternal feed and water sources.

Endocrine disrupting chemicals (EDCs) are broadly used in an agricultural context by means of pesticides, fungicides and herbicides. Early life exposures to EDCs have been linked to changes in early sexual dimorphic markers such as AGD in both male and female offspring (Nelson et al., 2019; Bowman et al., 2003; Swan et al., 2005 from Nelson et al., 2020). Studies in humans suggest that EDCs can be transferred through the placenta and breast milk to developing offspring (Chen et al., 2014), either by simple diffusion or primary active transport (Molsa et al., 2005). Some EDCs have been documented to have adverse effects of the hypothalamo-pituitary gland complex in a range of different adult, pre-pubertal (Adewale et al., 2009) and fetal animals. Thus, the adverse epigenetic effects of EDCs on reproduction are apparent both pre- and postnatally and could be contributing to reduced fertility. Moreover, determining the associations between AGD at birth and later stages of life will determine repeatability of AGD measurement in young stock, and can potentially aid in the selection process of replacement heifers in combination with various traits of interest.

Thirdly, determining the influence of stage of lactation and gestation on AGD will offer further understanding on the repeatability of measure in primiparous and multiparous cows. If the associations between AGD, and stages of lactation and gestation are negligible, the potential use of AGD as a novel fertility phenotype in complex selection programs would be strengthened. Moreover, we can ignore the functional demands of lactation and gestation when deciphering the underlying physiological mechanisms controlling AGD. Although this is an important area of

research, the relationship between AGD and stages of lactation and gestation are currently being studied in our lab (Rajesh, 2021).

Lastly, the major directive for measuring AGD is its ability to accurately predict reproductive performance. Given the poor to moderate sensitivity (66.2%) and specificity (42.5%) of prediction of pregnancy to 1st AI, further research evaluating optimum threshold values of AGD predictive of other reproductive measures of interest (such as age at conception) will be beneficial to determine the feasibility of future application. If other measures of fertility have higher sensitivities and specificities of prediction, the practical application of AGD will have more merit. If this is the case, the estimation of heritability for a larger population of dairy cows and heifers, as well as genome-wide association studies (GWAS) to locate potential single nucleotide polymorphisms (SNP) associated with AGD will be necessary to assess the value of AGD as a novel fertility phenotype for use in national selection indices. Moving forward, studies investigating the associations between AGD and measures of fertility should consider measuring AGD in a large population of cows where genomic information is readily available and accessible in order to conduct GWAS and SNP panels.

5.4 Considerations

Developed technologies may have applications that could provide benefits to the dairy industry and considered integral and revolutionary by scientists but may bring elements of concern from the public perspective. The improvement of reproductive efficiency of dairy cows is a key goal within the dairy industry. The improvement of reproductive fitness in dairy breed populations will allow for reduction in exogenous hormone use, increasing animal welfare, longevity and

sustainability (Garnsworthy, 2004), which will be valuable in the eyes of the consumer. As traditional fertility traits have low heritability estimates associated with them, the use of novel reproductive phenotypes with greater heritability are now being sought to facilitate selection for improved fertility. Although the measurement of AGD is non-invasive, measurement of other proposed phenotypic indicators of fertility, such as circulating IGF-1 or AMH concentrations, may not be as well perceived by the general public due to the invasive nature of (blood) sampling. The public's support of emerging technologies will ultimately decide how commonplace these practices are in the industry, and with an expanding world population to feed, demands will be placed on the agricultural sector.

5.5 Conclusions

The objectives of this thesis were to (1) characterize AGD in nulliparous heifers and (2) validate if the previously described inverse relationship between AGD and measures of fertility was present in large populations of dairy cows and heifers. The AGD was normally distributed with high variability in the large populations of North American Holstein cows and heifers. Moreover, AGD was inversely associated with measures of fertility in nulliparous heifers including age at first AI, pregnancy to first AI, services per conception, age at conception, and pregnancy risk up to 450 d. AGD was also found to be inversely related to measures of fertility in first-parity cows such as pregnancy to first AI, times bred, services per conception (tendency), and days open (interval from calving to conception). In summary, this thesis further strengthens the potential of AGD for use as a novel fertility phenotype in complex selection programs as it is normally distributed, highly variable, and inversely associated with measures of fertility in both

nulliparous heifers and first-parity cows. In addition, the cost of measuring this novel phenotype is low, and has the potential to improve cost of production by decreased rearing costs and improved reproductive lifespan.

Literature Cited

- Akbarinejad, V., F. Gharagozlou, M. Vojgani, and A. Ranji. 2020. Evidence for quadratic association between serum anti-Müllerian hormone (AMH) concentration and fertility in dairy cows. *Anim. Reprod. Sci.* 218. (Abstr.)
<https://doi.org/10.1016/j.anireprosci.2020.106457>
- 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.
<https://doi.org/10.1016/j.domaniend.2019.01.011>
- Bánszegi, O., P. Szenczi, D. Kriszta, A. Bilko, and V. Altbacker. 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>
- Bánszegi, O., P. Szenczi, A. Dúcs, R. Hudson, and V. Altbäcker. 2015. Long-term under-masculinization in male rabbits due to maternal stress is reversed by prenatal administration of testosterone. *Behav. Processes.* 115:156-162.
<https://doi.org/10.1016/j.beproc.2015.03.013>
- Baruselli, P.S., E.O.S. Batista, L.M. Vieira, and A.H. Souza. 2015. Relationship between follicle population, AMH concentration and fertility in cattle. *Anim. Reprod.* 12:487-497

- Battista, S. 2019. Associations between morphometric characteristics of the reproductive tract and fertility in beef cattle. MSc Thesis. Department of Animal Science, Ohio State University, Columbus, OH.
- Berry, D. P., J. Bastiaansen, R. Veerkamp, S. Wijga, E. Wall, B. Berglund, and M. Calus. 2012. Genome-wide associations for fertility traits in Holstein-Friesian dairy cows using data from experimental research herds in four European countries. *Animal* 6:1206–1215. <https://doi.org/10.1017/S1751731112000067>
- Berry, D., J. Kearney, K. Twomey, and R. Evans. 2013. Genetics of reproductive performance in seasonal calving dairy cattle production systems. *Ir. J. Agric. Food Res.* 52:1–16.
- Berry, D., E. Wall, and J. Pryce. 2014. Genetics and genomics of reproductive performance in dairy and beef cattle. *Anim.* 8 (Suppl. 1):105–121. <https://doi.org/10.1017/S1751731114000743>
- Berry D.P., F. Buckley, P. Dillon, R.D. Evans, M. Rath, and R.F. Veerkamp. 2003. Genetic relationships among body condition score, body weight, milk yield and fertility in dairy cows. *J. Dairy Sci.* 86:2193–2204. [https://doi.org/10.3168/jds.S0022-0302\(03\)73809-0](https://doi.org/10.3168/jds.S0022-0302(03)73809-0)
- 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. <https://doi.org/10.1093/toxsci/kfg128>
- Burns, D. S., F. Jimenez-Krassel, J. L. 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. <https://doi.org/login.ezproxy.library.ualberta.ca/10.1095/biolreprod.104.036277>

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

[https://doi.org/10.1016/S0301-6226\(03\)00112-X](https://doi.org/10.1016/S0301-6226(03)00112-X)

Canadian Dairy Information Centre. 2019. Average Milk Production by Breed. Accessed Dec.

21, 2020. <https://www.dairyinfo.gc.ca/eng/dairy-statistics-and-market-information/dairy-animal-genetics/average-milk-production-by-breed/average-milk-production-by-breed/?id=1574186422369>

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 (Suppl. 1): 245. (Abstr.)

Carthy, T. R., D. P. Berry, A. Fitzgerald, S. McParland, E. J. Williams, S. T. Butler, A. R.

Cromie, and D. Ryan. 2014. Risk factors associated with detailed reproductive phenotypes in dairy and beef cows. *Animal* 8:695–703.

<https://doi.org/10.1017/S1751731114000354>

Carthy, T.R., D.P. Ryan, A.M. Fitzgerald, R.D. Evans, and D.P. Berry. 2015. Genetic parameters of ovarian and uterine reproductive traits in dairy cows. *J. Dairy Sci.* 98:4095-4106.

<http://dx.doi.org/10.3168/jds.2014-8924>

- Chen, Z.J., H.Y. Liu, Z. Cheng, Y.B. Man, K.S. Zhang, W. Wei, J. Du, M.H. Wong, and H.S. Wang. 2014. Polybrominated diphenyl ethers (PBDEs) in human samples of mother-newborn pairs in South China and their placental transfer characteristics. *Environ. Int.* 73:77-84. <https://doi.org/10.1016/j.envint.2014.07.002>
- Chesnais J.P., T.A. Cooper, G.R. Wiggans, M. Sargolzaei, J.E. Pryce, and F. Miglior. 2016. Using genomics to enhance selection of novel traits in North American dairy cattle. *J. Dairy Sci.* 99:2413-2427. <https://doi.org/10.3168/jds.2015-9970>
- Ciccioli, N. H., R. P. Wettemann, L. J. Spicer, C. A. Lents, F. J. White, and D. H. Keisler. 2003. Influence of body condition at calving and postpartum nutrition on endocrine function and reproductive performance of primiparous beef cows. *J. Anim. Sci.* 81:3107–3120. <http://doi.org/10.2527/2003.81123107x>
- Clark, M.M., B.G. Galef Jr. 1995. Prenatal influences on reproductive life-history strategies. *Trends Ecol. Evol.* 10:151–153. [https://doi.org/10.1016/S0169-5347\(00\)89025-4](https://doi.org/10.1016/S0169-5347(00)89025-4)
- Cochran, S. D., J. B. Cole, D. J. Null, and P. J. Hansen. 2013. Discovery of single nucleotide polymorphisms in candidate genes associated with fertility and production traits in Holstein cattle. *BMC Genet.* 14:49. <https://doi.org/10.1186/1471-2156-14-49>
- Crowe, M. A., M. Hostens, and G. Opsomer. 2018. Reproductive management in dairy cows – the future. *Ir. Vet. J.* 71:1. [https://doi.org/10.1016/S0169-5347\(00\)89025-4](https://doi.org/10.1016/S0169-5347(00)89025-4)
- 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.* 87:1971–80. <http://doi.org/10.2527/jas.2008-1728>
- Darwash, A. O., G. L. Ward, G. E. Lamming, and J. A. Woolliams. 1999. The effects of raising post-oestrus progesterone concentrations on luteal activity in post-partum dairy cows. *Anim. Sci.* 68:527–532. <https://doi.org/10.1017/S1357729800050554>
- Darwash, A. O., G. E. Lamming, and J. A. Woolliams. 1997a. Estimation of genetic variation in the interval from calving to postpartum ovulation of dairy cows. *J. Dairy Sci.* 80:1227–1234. [https://doi.org/10.3168/jds.S0022-0302\(97\)76051-X](https://doi.org/10.3168/jds.S0022-0302(97)76051-X)
- Darwash, A. O., G. E. Lamming, and J. A. Woolliams. 1997b. The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. *Anim. Sci.* 65:9–16. <https://doi.org/10.1017/S1357729800016234>
- Dean, A., L. B. Smith, S. Macpherson, and R. M. Sharpe. 2012. The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. *Int. J. Androl.* 35:330–339. <https://doi.org/10.1111/j.1365-2605.2011.01236.x>
- Dennis, N.A., K. Stachowicz, B. Visser, F.S. Hely, D.K. Berg, N.C. Friggens, P.R. Amer, S. Meier, and C.R. Burke. 2017. Combining genetic and physiological data to identify predictors of lifetime reproductive success and the effect of selection on these predictors on underlying fertility traits. *J. Dairy Sci.* 101:3176-3192. <https://doi.org/10.3168/jds.2017-13355>

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. *J. Dairy Sci.* 89:3876–3885.

[https://doi.org/10.3168/jds.S0022-0302\(06\)72430-4](https://doi.org/10.3168/jds.S0022-0302(06)72430-4)

Drickamer, L.C. 1996. Intra-uterine position and anogenital distance in house mice: consequences under field conditions. *Anim. Behav.* 51:925–934.

<https://doi.org/10.1006/anbe.1996.0096>

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>

Egger-Danner, C., J.B. Cole, J.E. Pryce, N. Gengler, B. Heringstad, A. Bradley, and K.F. Stock. 2015. Invited review: overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. *Animal* 9:191-207.

<https://doi.org/10.1017/s1751731114002614>

Erickson, B.H. 1966. Development and senescence of the postnatal bovine ovary. *J. Anim. Sci.* 25:800–805. <http://doi.org/10.2527/jas1966.253800x>

Falkenberg, U., J. Haertel, K. Rotter, M. Iwersen, G. Arndt, and W. Heuwieser. 2008.

Relationships between the concentration of insulin-like growth factor-1 in serum in dairy cows in early lactation and reproductive performance and milk yield. *J. Dairy Sci.*

91:3862–3868. <https://doi.org/10.3168/jds.2007-0887>

Food and Agriculture Organization (FAO) of the United Nations. 2019. FAOSTAT: Livestock Primary. Accessed Dec. 20, 2020. <http://www.fao.org/faostat/en/#data/QL>

- Fitzgerald, A. M., D. P. Ryan, T. R. Carthy, R. D. Evans, and D. P. Berry. 2014. Ovarian structures and uterine environment are associated with phenotypic and genetic merit for performance in lactating dairy cows. *Theriogenology* 82:1231–1240. <https://doi.org/10.1016/j.theriogenology.2014.07.037>
- Fleming, A., E.A. Abdalla, C. Maltecca, and C.F. Baes. 2018. Invited review: Reproductive and genomic technologies to optimize breeding strategies for genetic progress in dairy cattle. *Arch. Anim. Breed.* 61:43-57. <https://doi.org/10.5194/aab-61-43-2018>
- 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. <https://doi.org/10.3168/jds.2018-15470>
- Fricke, P.M. 2002. Scanning the future – ultrasonography as a reproductive management tool for dairy cattle. *J. Dairy Sci.* 85:1918–1926. [https://doi.org/10.3168/jds.S0022-0302\(02\)74268-9](https://doi.org/10.3168/jds.S0022-0302(02)74268-9)
- Fricke, P.M., P.D. Carvalho, J.O. Giordano, A. Valenza, G. Lopes Jr, and M.C. Amundson. 2014. Expression and detection of estrus in dairy cows: The role of new technologies. *Animal.* 8s1:134–143. <https://doi.org/10.1017/s1751731114000299>
- 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. Fertil.* 70:55–59. <https://doi.org/10.1530/jrf.0.0700055>

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.

<https://doi.org/10.1016/j.theriogenology.2017.01.018>

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. <https://doi.org/10.3168/jds.2017-13033>

Gobikrushanth, M., D. C. Purfield, M. G. Colazo, S. T. Butler, Z. Wang, and D. J. Ambrose.

2018a. 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. <https://doi.org/10.3168/jds.2017-13940>

Gobikrushanth, M., D.C. Purfield, M.G. Colazo, Z. Wang, S.T. Butler, and D.J. Ambrose.

2018b. The relationship between serum insulin-like growth factor-1 (IGF-1) concentration and reproductive performance, and genome-wide associations for serum IGF-1 in Holstein cows. *J. Dairy Sci.* 101:9154-9167. [https://doi.org/10.3168/jds.2018-](https://doi.org/10.3168/jds.2018-14535)

[14535](https://doi.org/10.3168/jds.2018-14535)

Gobikrushanth, M. D.C. Purfield, E.R. Canadas, M.M. Herlihy, J. Kenneally, M. Murray, F.J.

Kearney, M.G. Colazo, D.J. Ambrose, and S.T. Butler. 2019a. Anti-Müllerian hormone in grazing dairy cows: Identification of factors affecting plasma concentration, relationship

- with phenotypic fertility, and genome-wide associations. *J. Dairy Sci.* 102:11622-11635.
<https://doi.org/10.3168/jds.2019-16979>
- Gobikrushanth, M., D.C. Purfield, J. Kenneally, R.C. Doyle, S.A. Holden, P.M. Martinez, E. Rojas Canadas, T.C. Bruinjé, M.G. Colazo, D.J. Ambrose, and S.T. Butler. 2019b. 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>
- González-Recio, O., M. A. Pérez-Cabal, and R. Alenda. 2004. Economic value of female fertility and its relationship with profit in Spanish dairy cattle. *J. Dairy Sci.* 87:3053–3061.
[https://doi.org/10.3168/jds.S0022-0302\(04\)73438-4](https://doi.org/10.3168/jds.S0022-0302(04)73438-4)
- Grochowska, R., P. Sorensen, L. Zwierzchowski, M. Snochowski, and P. Lovendahl. 2001. Genetic variation in stimulated GH release and in IGF-I of young dairy cattle and their associations with the leucine/valine polymorphism in the GH gene. *J. Anim. Sci.* 79:470–476. <https://doi.org/10.2527/2001.792470x>
- Häggman, J., J. M. Christensen, E. A. Mäntysaari, and J. Juga. 2018. Genetic parameters for endocrine and traditional fertility traits, hyperketonemia and milk yield in dairy cattle. *Animal*. <https://doi.org/10.1017/S1751731118001386>
<https://doi.org/10.1017/S1751731118001386>
- Haley, C. S., G. J. Lee, M. Fordyce, G. Baxter, R. B. L., and R. Webb. 1989. Study of LH response to GnRH in the young male as a criterion of genetic merit for female

reproduction in sheep. *J. Reprod. Fert.* 86:119–133. PMID: 2666648.

<http://doi.org/10.1530/jrf.0.0860119>

Hare, E., H. D. Norman, and J. R. Wright. 2006. Trends in calving ages and calving intervals for dairy cattle breeds in the United States. *J. Dairy Sci.* 89:365–370.

[https://doi.org/10.3168/jds.S0022-0302\(06\)72102-6](https://doi.org/10.3168/jds.S0022-0302(06)72102-6)

Hayhurst, C., A. P. F. Flint, P. Løvendahl, J. A. Woolliams, and M. D. Royal. 2009. Genetic variation of metabolite and hormone concentration in UK Holstein-Friesian calves and the genetic relationship with economically important traits. *J. Dairy Sci.* 92:4001–4007.

<https://doi.org/10.3168/jds.2008-1130>

Heinrichs, A. J., C. M. Jones, S. M. Gray, P. A. Heinrichs, S. A. Cornelisse, and R. C. Goodling. 2013. Identifying efficient dairy heifer producers using production costs and data envelopment analysis. *J. Dairy Sci.* 96:7355–7362. <https://doi.org/10.3168/jds.2012-6488>

Honarvar, M., A. Nejati Javaremi, S.R. Miraei Ashtiani, and M. Dehghan Banadaki. 2010. Effect of length of productive life on genetic trend of milk production and profitability: A simulation study. *Afr. J. Biotechnol.* 9:3000–3010. [https://doi.org/10.5897/AJB2010.000-](https://doi.org/10.5897/AJB2010.000-3135)

[3135](https://doi.org/10.5897/AJB2010.000-3135)

Hooijer, G.A., R.B. Lubbers, B.J. Ducro, J.A. van Arendonk, L.M. Kaal-Lansbergen, and T. van der Lende. 2001. Genetic parameters for cystic ovarian disease in Dutch black and white dairy cattle. *J. Dairy Sci.* 84:286–291. [https://doi.org/10.3168/jds.S0022-0302\(01\)74478-](https://doi.org/10.3168/jds.S0022-0302(01)74478-)

[5](https://doi.org/10.3168/jds.S0022-0302(01)74478-)

- Ireland, J. J., G. W. Smith, D. Scheetz, F. Jimenez-Krassel, J. K. Folger, J. L. H. Ireland, F. Mossa, P. Lonergan, and A. C. O. 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-Mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reprod. Fertil. Dev.* 23:1–14. <https://doi.org/10.1071/rd10226>
- Ireland, J. J., F. Ward, F. Jimenez-Krassel, J. L. Ireland, G. W. Smith, P. Lonergan, and A. C. 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. <https://doi.org/10.1093/humrep/dem071>
- Ireland, J. L., D. Scheetz, F. Jimenez-Krassel, A. P. 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.* 79:1219–1225. <https://doi.org/10.1095/biolreprod.108.071670>
- Ismael, A., E. Strandberg, M. Kargo, A. Fogh, and P. Løvendahl. 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>
- Jamrozik, J., J. Fatehi, G.J. Kistemaker, and L.R. Schaeffer. 2005. Estimates of genetic parameters for Canadian Holstein female reproduction traits. *J. Dairy Sci.* 88:2199–2208. [https://doi.org/10.3168/jds.s0022-0302\(05\)72895-2](https://doi.org/10.3168/jds.s0022-0302(05)72895-2)

- Jaton, C., F.S. Schenkel, M. Sargolzaei, A. Canova, F. Malchiodi, C.A. Price, C. Baes, F. Miglior. 2018. Genome-wide association study and in silico functional analysis of the number of embryos produced by Holstein donors. *J. Dairy Sci.* 101:7248-7257.
<https://doi.org/10.3168/jds.2017-13848>
- Jiang, H., and M. C. Lucy. 2001. Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: Isolation, expression and effects on translational efficiency. *Gene* 265:45–53. [https://doi.org/10.1016/S0378-1119\(01\)00356-0](https://doi.org/10.1016/S0378-1119(01)00356-0)
- Jiang, J., B. Shen, J. R. O’Connell, P. M. VanRaden, J. B. Cole, and L. Ma. 2017. Dissection of additive, dominance, and imprinting effects for production and reproduction traits in Holstein cattle. *BMC Genomics* 18:425. <https://dx.doi.org/10.1186%2Fs12864-017-3821-4>
- Jimenez-Krassel, F., J. K. Folger, J. L. Ireland, G. W. Smith, X. Hou, J. S. Davis, P. Lonergan, A. C. Evans, and J. J. Ireland. 2009. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biol. Reprod.* 80:1272–1281.
<https://doi.org/10.1095/biolreprod.108.075093>
- Jimenez-Krassel, F., D. Scheetz, L. Neuder, J. Pursley, and J. J. Ireland. 2017. A single ultrasound determination of ≥ 25 mm follicle ≥ 3 mm in diameter in dairy heifers is predictive of a reduced productive herd life. *J. Dairy Sci.* 100:5019–5027.
<https://doi.org/10.3168/jds.2016-12277>

- Jimenez-Krassel, F., D. Scheetz, L. Neuder, J. Ireland, J. Pursley, G. Smith, R. Tempelman, T. Ferris, W. Roudebush, and F. Mossa. 2015. Concentration of anti-Müllerian hormone in dairy heifers is positively associated with productive herd life. *J. Dairy Sci.* 98:3036-3045. <https://doi.org/10.3168/jds.2014-8130>
- Kadarmideen, H. N., R. Thompson, M. P. Coffey, and M. A. Kossaibati. 2003. Genetic parameters and evaluations from single- and multiple-trait analysis of dairy cow fertility and milk production. *Livest. Prod. Sci.* 81:183–195. [https://doi.org/10.1016/S0301-6226\(02\)00274-9](https://doi.org/10.1016/S0301-6226(02)00274-9)
- Kawashima, C., M. Sakaguchi, T. Suzuki, Y. Sasamoto, Y. Takahashi, M. Matsui, and A. Miyamoto. 2007. Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. *J. Reprod. Dev.* 53:113–120. <https://doi.org/10.1262/jrd.18105>
- Kommadath, A., M.F.W. te Pas, and M.A. Smits. 2013. Gene coexpression network analysis identifies genes and biological processes shared among anterior pituitary and brain areas that affect estrous behavior in dairy cows. *J. Dairy Sci.* 96:2583-2595. <https://doi.org/10.3168/jds.2012-5814>
- Kommadath, A., H. Woelders, B. Beerda, H.A. Mulder, A.A.C. de Wit, R.F. Veerkamp, M.F.W. te Pas, and M.A. Smits. 2011. Gene expression patterns in four brain areas associate with quantitative measure of estrous behavior in dairy cows. *BMC Genom.* 12:200. <https://dx.doi.org/10.1186%2F1471-2164-12-200>

- La Marca A., and A. Volpe. 2006. Anti-Müllerian hormone (AMH) in female reproduction: is measurement of circulating AMH a useful tool?. *Clin. Endocrinol. (Oxf.)*. 64:603-610. <https://doi.org/10.1111/j.1365-2265.2006.02533.x>
- Lamming, G. E., and A. O. Darwash. 1998. The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. *Anim. Reprod. Sci.* 52:175–190. [https://doi.org/10.1016/S0378-4320\(98\)00099-2](https://doi.org/10.1016/S0378-4320(98)00099-2)
- Lammers, B. P., A. J. Heinrichs, and R. S. Kensinger. 1999. The effects of accelerated growth rates and estrogen implants in prepubertal Holstein heifers on growth, feed efficiency, and blood parameters. *J. Dairy Sci.* 82:1746–1752. [https://doi.org/10.3168/jds.S0022-0302\(99\)75405-6](https://doi.org/10.3168/jds.S0022-0302(99)75405-6)
- LeBlanc, S. 2007. Economics of improving reproductive performance in dairy herds. *WCDS Advances in Dairy Technology* 19:201 – 214.
- Løvendahl, P., and M. G. G. Chagunda. 2009. Short communication: Genetic variation in estrus activity traits. *J. Dairy Sci.* 92:4683– 4688. <https://doi.org/10.3168/jds.2008-1736>
- Løvendahl, P., and M. G. G. Chagunda. 2010. On the use of physical activity monitoring for estrus detection in dairy cows. *J. Dairy Sci.* 93:249–259. <https://doi.org/10.3168/jds.2008-1721>
- Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? *J. Dairy Sci.* 84:1277-1293. [https://doi.org/10.3168/jds.S0022-0302\(01\)70158-0](https://doi.org/10.3168/jds.S0022-0302(01)70158-0)

- 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>
- Macleod, D. J., R. M. Sharpe, M. Welsh, M. Fiskens, M. Scott, G. R. Hutchison, A. J. Drake, and S. van den Driesche. 2010. Androgen action in the masculinization programming window and development of male reproductive organs. *Int. J. Androl.* 33:279–287. <https://doi.org/10.1111/j.1365-2605.2009.01005.x>
- Madureira, A.M.L., R.K. Poole, T.A. Burnett, T.G. Guida, J.L. Edwards, F.N. Schrick, J.L.M. Vasconcelos, R.L.A. Cerri, and K.G. Pohler. 2020. Size and position of the reproductive tract impacts fertility outcomes and pregnancy losses in lactating dairy cows. *Theriogenology* 158:66-74. <https://doi.org/10.1016/j.theriogenology.2020.08.022>
- Margerison, J., and N. Downey. 2005. Guidelines for optimal dairy heifer rearing and herd performance. In *Calf and heifer rearing: principles of rearing the modern dairy heifer from calf to calving*, pp. 307-338. Edited by P.C. Garnsworthy. Nottingham University Press, Nottingham.
- Martinez, M.F., N. Sanderson, L.D. Quirke, S.B. Lawrence, and J.L. Juengel. 2016. Association between antral follicle count and reproductive measures in New Zealand lactating dairy cows maintained in a pasture-based production system. *Theriogenology* 85:466-475. <https://doi.org/10.1016/j.theriogenology.2015.09.026>

- Masello, M., M.M. Perez, G.E. Granados, M.L. Stangaferro, B. Ceglowski, M.J. Thomas, and J.O. Giordano. 2020. Association between age at first calving and economic performance of replacement dairy heifers. *J. Dairy Sci.* 103(Suppl. 1):243. (Abstr.)
- Meadows, C., P. J. Rajala-Schultz, and G. S. Frazer. 2005. A spreadsheet-based model demonstrating the nonuniform economic effects of varying reproductive performance in Ohio dairy herds. *J. Dairy Sci.* 88:1244–1254. [https://doi.org/10.3168/jds.S0022-0302\(05\)72791-0](https://doi.org/10.3168/jds.S0022-0302(05)72791-0)
- Mendiola, J., M. Roca, L. Mi'nguez-Alarco'n, M. P. Mira-Escolano, J. J. Lo'pez-Esp'i'n, 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>
- Merton, J. S., B. Ask, D. C. Onkundi, E. Mullaart, B. Colenbrander, and M. Nielen. 2009. Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology* 72:885–893. <https://doi.org/10.1016/j.theriogenology.2009.06.003>
- Miglior, F., B.L. Muir, and B.J. Van Doormaal. 2005. Selection indices in Holstein cattle of various countries. *J. Dairy Sci.* 88:1255–1263. [https://doi.org/10.3168/jds.S0022-0302\(05\)72792-2](https://doi.org/10.3168/jds.S0022-0302(05)72792-2)
- 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. <https://doi.org/10.3168/jds.2017-12968>

- Molsa, M. T. Heikkinen, J. Hakkola, K. Hakala, O. Wallerman, M. Wadelius, C. Wadelius, and K. Laine. 2005. Functional role of P-glycoprotein in the human blood-placental barrier. *Pharmacokinet. Drug Dispos.* 78:123-131. <https://doi.org/10.1016/j.clpt.2005.04.014>
- Mongkonpunya, K., Y. C. Lin, P. A. Noden, W. D. Oxender, and H. D. Hafs. 1975. Androgens in the Bovine Fetus and Dam. *Proc. Soc. Exp. Biol. Med.* 148:489–493. <https://doi.org/10.3181/00379727-148-38567>
- Mossa, F., F. Jimenez-Krassel, J. K. Folger, J. L. H. Ireland, G. W. Smith, P. Lonergan, A. C. O. Evans, and J. J. Ireland. 2010. Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle. *Reproduction* 140:713–720. <https://doi.org/10.1530/rep-10-0214>
- Mossa, F., S. W. Walsh, S. T. Butler, D. P. Berry, F. Carter, P. Lonergan, G. W. Smith, J. J. Ireland, and A. C. Evans. 2012. Low numbers of ovarian follicles ≥ 3 mm in diameter are associated with poor fertility in dairy cows. *J. Dairy Sci.* 95:2355–2361. <https://doi.org/10.3168/jds.2011-4325>
- Moyes, T. E. 2004. Variation in concentrations of insulin-like growth factor-1 in pasture-fed Holstein-Friesian cows. PhD Thesis. Department of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia.
- Nawaz, M.Y., F. Jimenez-Krassel, J.P. Steibel, Y. Lu, A. Baktula, N. Vulkasinovic, L. Neuder, J.L.H. Ireland, J.J. Ireland, and R.J. Tempelman. 2018. Genomic heritability and genome-wide association analysis of anti-Müllerian hormone in Holstein dairy heifers. *J. Dairy Sci.* 101:8063-8075. <https://doi.org/10.3168/jds.2018-14798>

- Nelson, W., Y.X. Wang, G. Sakwari, and Y.B. Ding. 2019. Review of the effects of perinatal exposure to endocrine-disrupting chemicals in animals and humans. *Rev. Environ. Contam. Toxicol.* 251:131–184. https://doi.org/10.1007/398_2019_30
- Newberry, H. 2016. Use of anti-Müllerian hormone to select for fertility in beef heifers. *Animal Science Undergraduate Honors Theses*. University of Arkansas Fayetteville
<https://scholarworks.uark.edu/anscuht/9>
- Nyman, S., K. Johansson, D. J. de Koning, D. P. Berry, R. F. Veerkamp, E. Wall, and B. Berglund. 2014. Genetic analysis of atypical progesterone profiles in Holstein-Friesian cows from experimental research herds. *J. Dairy Sci.* 97:7230–7239.
<https://doi.org/10.3168/jds.2014-7984>
- Ortega, H.H., N.R. Salvetti, and V. Padmanabhan. 2009. Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance. *Reprod.* 137:865–77.
<https://dx.doi.org/10.1530%2FREP-08-0491>
- Overton M.W. and K. C. Dhuyvetter. 2020. Symposium review: An abundance of replacement heifers: What is the economic impact of raising more than are needed? *J. Dairy Sci.* 103:3828-3837. <https://doi.org/10.3168/jds.2019-17143>
- Padmanabhan V., and A. Veiga-Lopez. 2013. Sheep models of polycystic ovary syndrome phenotype. *Mol. Cell Endocrinol.* 373:8–20. <https://doi.org/10.1016/j.mce.2012.10.005>

- Palmer, M. A., G. Olmos, L. A. Boyle, and J. F. Mee. 2010. Estrus detection and estrus characteristics in housed and pastured Holstein- Friesian cows. *Theriogenology* 74:255–264. <https://doi.org/10.1016/j.theriogenology.2010.02.009>
- Patton, J., D. A. Kenny, S. McNamara, J. F. Mee, F. P. O’Mara, M. G. Diskin, and J. J. Murphy. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein–Friesian cows. *J. Dairy Sci.* 90:649–658. [https://doi.org/10.3168/jds.S0022-0302\(07\)71547-3](https://doi.org/10.3168/jds.S0022-0302(07)71547-3)
- Peñagaricano, F., and H. Khatib. 2012. Association of milk protein genes with fertilization rate and early embryonic development in Holstein dairy cattle. *J. Dairy Res.* 79:47–52. <https://doi.org/10.1017/S0022029911000744>
- Petersson, K.-J., B. Berglund, E. Strandberg, H. Gustafsson, A. P. F. Flint, J. A. Woolliams, and M. D. Royal. 2007. Genetic analysis of postpartum measures of luteal activity in dairy cows. *J. Dairy Sci.* 90:427–434. [https://doi.org/10.3168/jds.S0022-0302\(07\)72644-9](https://doi.org/10.3168/jds.S0022-0302(07)72644-9)
- Pirlo, G., F. Miglior, and M. Speroni. 2000. Effect of age at first calving on production traits and on difference between milk yield returns and rearing costs in Italian Holsteins. *J. Dairy Sci.* 83:603-608. [https://doi.org/10.3168/jds.S0022-0302\(00\)74919-8](https://doi.org/10.3168/jds.S0022-0302(00)74919-8)
- Pollott, G.E., and M.P. Coffey. 2008. The effect of genetic merit and production system on dairy cow fertility, measured using progesterone profiles and on-farm recording. *J. Dairy Sci.* 91:3649–3660. <https://doi.org/10.3168/jds.2007-0913>

- Pryce, J. E., M. D. Royal, P. C. Garnsworthy, and I. L. Mao. 2004. Fertility in the high-producing dairy cow. *Livest. Prod. Sci.* 86:125–135. [https://doi.org/10.1016/S0301-6226\(03\)00145-3](https://doi.org/10.1016/S0301-6226(03)00145-3)
- Pryce, J. E., R. F. Veerkamp, R. Thompson, W. G. Hill, and G. Simm. 1997. Genetic aspects of common health disorders and measures of fertility traits in Holstein Friesian dairy cattle. *Anim. Sci.* 65:353– 360. <https://doi.org/10.1017/S1357729800008559>
- Pushpakumara, P. G. A., N. H. Gardner, C. K. Reynolds, D. E. Beever, and D. C. Wathes. 2003. Relationships between transition period diet, metabolic parameters and fertility in lactating dairy cows. *Theriogenology* 60:1165–1185. [https://doi.org/10.1016/s0093-691x\(03\)00119-5](https://doi.org/10.1016/s0093-691x(03)00119-5)
- Rajesh, I. 2021. Repeatability of anogenital distance in Holstein cattle and its association with embryo yield and quality. MSc Thesis. Department of Agricultural, Food & Nutritional Sciences, University of Alberta, Edmonton, AB.
- Reith, S., and S. Hoy. 2012. Relationship between daily rumination time and estrus of dairy cows. *J. Dairy Sci.* 95:6416-6420. <https://doi.org/10.3168/jds.2012-5316>
- Renaville, R., M. Hammadi, and D. Portetelle. 2002. Role of the somatotrophic axis in the mammalian metabolism. *Domest. Anim. Endocrinol.* 23:351–360. [https://doi.org/10.1016/S0739-7240\(02\)00170-4](https://doi.org/10.1016/S0739-7240(02)00170-4)
- Ribeiro, E.S., R.S. Bisinotto, F.S. Lima, L.F. Greco, A. Morrison, A. Kumar, W.W. Thatcher, and J.E.P. Santos. 2014. Plasma anti-Müllerian hormone in adult dairy cows and

- associations with fertility. *J. Dairy Sci.* 97:6888-6900. <https://doi.org/10.3168/jds.2014-7908>
- 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. <https://doi.org/10.1095/biolreprod.108.072157>
- Roelofs, J. B., F. J. C. M. van Eerdenburg, N. M. Soede, and B. Kemp. 2005. Various behavioral signs of estrous and their relationship with time of ovulation in dairy cattle. *Theriogenology* 63:1366–1377. <https://doi.org/10.1016/j.theriogenology.2004.07.009>
- Roxström, A., E. Strandberg, B. Berglund, U. Emanuelson, and J. Philipsson. 2001. Genetic and environmental correlations among female fertility traits, and between the ability to show oestrus and milk production in dairy cattle. *Acta Agric. Scand. A Anim. Sci.* 51:192–199. <https://doi.org/10.1080/09064700118617>
- Royal, M. D., J. Pryce, J. Woolliams, and A. Flint. 2002a. The genetic relationship between commencement of luteal activity and calving interval, body condition score, production, and linear type traits in Holstein-Friesian dairy cattle. *J. Dairy Sci.* 85:3071–3080. [https://doi.org/10.3168/jds.S0022-0302\(02\)74394-4](https://doi.org/10.3168/jds.S0022-0302(02)74394-4)
- Royal, M. D., A. P. F. Flint, and J. A. Woolliams. 2002b. Genetic and phenotypic relationships among endocrine and traditional fertility traits and production traits in Holstein-Friesian dairy cows. *J. Dairy Sci.* 85:958–967. [https://doi.org/10.3168/jds.S0022-0302\(02\)74155-6](https://doi.org/10.3168/jds.S0022-0302(02)74155-6)

Sadler, T. W. 2012. Chapter 16: Urogenital system. Pages 232–259 in Langman’s Medical Embryology. 12th ed. The Williams & Wilkins Company, Baltimore, MD.

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. <https://doi.org/10.1186/1476-069X-3-8>

Salvetti, N.R., H.H. Ortega, A. Veiga-Lopez, and V. Padmanabhan. 2012. Developmental programming: impact of prenatal testosterone excess on ovarian cell proliferation and apoptotic factors in sheep. *Biol. Reprod.* 87:1–10. <https://doi.org/10.1095/biolreprod.112.100024>

Sathyanarayana, S., L. Beard, C. Zhou, and R. Grady. 2010. Measurement and correlates of anogenital distance in healthy, newborn infants. *Int. J. Androl.* 33:317–323. <https://doi.org/10.1111/j.1365-2605.2009.01044.x>

Sewalem, A., F. Miglior, G.J. Kistemaker, P. Sullivan, and B.J. Van Doormaal. 2008. Relationship between reproduction traits and functional longevity in Canadian dairy cattle. *J. Dairy Sci.* 91:1660–1668. <https://doi.org/10.3168/jds.2007-0178>

Seykora, A. J., and B. McDaniel. 1983. Heritabilities and correlations of lactation yields and fertility for Holsteins. *J. Dairy Sci.* 66:1486–1493. [https://doi.org/10.3168/jds.S0022-0302\(83\)81963-8](https://doi.org/10.3168/jds.S0022-0302(83)81963-8)

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

[https://doi.org/10.3168/jds.S0022-0302\(89\)79242-0](https://doi.org/10.3168/jds.S0022-0302(89)79242-0)

Silper, B.F., I. Robles, A.M.L. Madureira, T.A. Burnett, M.M. Reis, A.M. de Passillé, J. Rushen, and R.L.A. Cerri. 2015. Automated and visual measurements of estrous behaviour and their sources of variation in Holstein heifers. I: Walking activity and behaviour frequency.

Theriogenology 84:312-320. <https://doi.org/10.1016/j.theriogenology.2014.12.029>

Snelling, W. M., R. A. Cushman, M. R. Fortes, A. Reverter, G. L. Bennett, J. W. Keele, L. A. Kuehn, T. G. McDanel, R. M. Thallman, and M. G. Thomas. 2012. Physiology and Endocrinology Symposium: How single nucleotide polymorphism chips will advance our knowledge of factors controlling puberty and aid in selecting replacement beef females. *J. Anim. Sci.* 90:1152–1165.

<https://doi.org/10.2527/jas.2011-4581>

Sorg, D., M. Wensch-Dorendorf, K. Schöpke, G. Martin, R. Schafberg, N. Reinhold, S. Pache, and H. Swalve. 2017. Genetic analysis of new progesterone-based fertility traits in dairy cows measured on-farm. *J. Dairy Sci.* 100:8205–8219.

<https://doi.org/10.3168/jds.2016-11865>

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, 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. <https://doi.org/10.3168/jds.2014-8182>

- Stirling, T. E., C. R. Stockdale, and K. L. Macmillan. 2008. Heritability of IGF-1 and its genetic correlation with milk yield in Holstein- Friesian cows in selected Victorian herds. *Proc. N.Z. Soc. Anim. Prod.* 68:98–99.
- 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>
- Swan, S. H., and D. M. Kristensen. 2018. Anogenital distance: a marker of steroidal endocrine disruption. Pages 588-593 in *Encyclopedia of Reproduction (2nd ed)*. Vol. 1. Male Reproduction. Skinner M. K, ed. Elsevier Inc.
- Swan, S.H., K.M. Main, F. Liu, S.L. Stewart, R.L. Kruse, A.M. Calafat, C.S. Mao, J.B. Redmon, C.L. Ternand, S. Sullivan, J.L. Teague, E.Z. Drobnis, B.S. Carter, D. Kelly, T.M. Simmons, C. Wang, L. Lumbreras, S. Villanueva, M. Diaz-Romero, M.B. Lomeli, E. Otero-Salazar, C. Hobel, B. Brock, C. Kwong, A. Muehlen, A. Sparks, A. Wolf, J. Whitham, M. Hatterman-Zogg, and M. Maifield. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ. Health Perspect.* 113:1056-1061. <https://doi.org/10.1289/ehp.8100>
- Swets, J. A. 1988. Measuring the accuracy of diagnostic systems. *Science* 240:1285–1293.
<https://doi.org/10.1126/science.3287615>
- Taniguchi, H., J. Komiyama, R.S. Viger, and K. Okuda. 2009. The expression of the nuclear receptors NR5A1 and NR5A2 and transcription factor GATA6 correlated with

steroidogenic gene expression in the bovine corpus luteum. *Mol. Reprod. Dev.* 76:873-880. <https://doi.org/10.1002/mrd.21054>

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.

<https://doi.org/10.1136/vr.155.19.583>

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.* 98:5763–5773. [https://doi.org/10.3168/jds.2014-](https://doi.org/10.3168/jds.2014-8732)

[8732](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–

1790. <https://doi.org/10.1289/ehp.0900881>

United Nations. 2019. Department of Economic and Social Affairs, Population Division (2019).

World Population Prospects: The 2019 Revision. Accessed Dec. 21, 2020.

<https://population.un.org/wpp/Download/Probabilistic/Population/>

Tranel, L. Heifer raising costs in 2019. ISU Publication LT-18–01. Accessed Nov. 12, 2020.

https://www.extension.iastate.edu/dairyteam/files/page/files/whats_it_cost_to_raise_heifers_2019_0.pdf

- Van Doormaal, B.J., G. Kistemarker, J. Fatehi, F. Miglior, J. Jamrozik, and L.R. Schaeffer. 2004. Genetic evaluation of female fertility in Canadian dairy breeds. Proc. 2004 Interbull Meeting, Sousse, Tunisia 32:86-89.
<https://journal.interbull.org/index.php/ib/article/view/844>
- VanRaden, P. M., and A. H. Sanders. 2003. Economic merit of crossbred and purebred US dairy cattle. J. Dairy Sci. 86:1036–1044. [https://doi.org/10.3168/jds.S0022-0302\(03\)73687-X](https://doi.org/10.3168/jds.S0022-0302(03)73687-X)
- VanRaden, P. M., A. H. Sanders, M. E. Tooker, R. H. Miller, H. D. Norman, M. T. Kuhn, and G. R. Wiggins. 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](https://doi.org/10.3168/jds.S0022-0302(04)70049-1)
- Veerkamp, R. F., and B. Beerda. 2007. Genetics and genomics to improve fertility in high producing dairy cows. Theriogenology 68:S266–S273.
<https://doi.org/10.1016/j.theriogenology.2007.04.034>
- Veerkamp, R. F., P. Dillon, E. Kelly, A. R. Cromie, and A. F. Groen. 2002. Dairy cattle breeding objectives combining yield, survival and calving interval for pasture-based systems in Ireland under different milk quota scenarios. Livest. Prod. Sci. 76:137–151.
[https://doi.org/10.1016/S0301-6226\(02\)00006-4](https://doi.org/10.1016/S0301-6226(02)00006-4)
- Veerkamp, R.F., E.P.C. Koenen, and G. De Jong. 2001. Genetic correlations among body condition score, yield, and fertility in first-parity cows estimated by random regression models. J. Dairy Sci. 84:2327–2335. [https://doi.org/10.3168/jds.S0022-0302\(01\)74681-4](https://doi.org/10.3168/jds.S0022-0302(01)74681-4)

Veerkamp, R. F., J. K. Oldenbroek, H. J. Van Der Gaast, and J. H. J. Van Der Werf. 2000.

Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weights. *J. Dairy Sci.* 83:577–583. [https://doi.org/10.3168/jds.s0022-0302\(00\)74917-4](https://doi.org/10.3168/jds.s0022-0302(00)74917-4)

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.* 80:718–25. <https://dx.doi.org/10.1095%2Fbiolreprod.108.074781>

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. <https://doi.org/10.1016/j.domaniend.2008.07.002>

Wainstock, T., I. Shoham-Vardi, E. Sheiner, and A. Walfisch. 2017. Fertility and anogenital

distance in women. *Reprod. Toxicol.* 73:345-349.
<https://doi.org/10.1016/j.reprotox.2017.07.009>

Wall, E., S. Brotherstone, J.A. Woolliams, G. Banos, and M.P. Coffey. 2003. Genetic evaluation

of fertility using direct and correlated traits. *J. Dairy Sci.* 86:4093–4102.
[https://doi.org/10.3168/jds.S0022-0302\(03\)74023-5](https://doi.org/10.3168/jds.S0022-0302(03)74023-5)

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.
<https://doi.org/10.1016/j.anireprosci.2010.12.001>

- 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. <https://doi.org/10.3168/jds.2013-7758>
- Wathes, D. C., V. J. Taylor, Z. Cheng, and G. E. Mann. 2003. Follicle growth, corpus luteum function and their effects on embryo development in postpartum dairy cows. *Reprod. Suppl.* 61:219–237.
- 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. <https://doi.org/10.3168/jds.2016-12288>
- 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. <https://doi.org/10.1002/dev.1017>
- Zhang, B., F. Penagaricano, A. Driver, H. Chen, and H. Khatib. 2011. Differential expression of heat shock protein genes and their splice variants in bovine preimplantation embryo. *J. Dairy Sci.* 94:4174-4182. <https://doi.org/10.3168/jds.2010-4137>

Zulu, V. C., N. Toshihiko, and Y. Sawamukai. 2002. Insulin-like growth factor 1 as a possible hormonal mediator of nutritional regulation of reproduction in cattle. *J. Vet. Med. Sci.* 64:657–665. <https://doi.org/10.1292/jvms.64.657>