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

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ABSTRACT

Anogenital distance (AGD) has been defined in dairy cows as the distance from the center of the anus to the base of the clitoris. Initial reports on nulliparous Holstein heifers and first- and second-parity Holstein cows have found inverse relationships between AGD and measures of fertility. Our primary objective was to determine the relationship between AGD and measures of fertility in a larger population of North American Holstein cows to validate our previous finding that AGD is inversely related to fertility. Secondary objectives were to determine the associations between AGD and parity, and milk yield. Using digital calipers, we measured AGD in 4,709 Holstein cows [mean ± standard deviation (SD); parity 2.3 ± 1.4; days in milk (DIM) 154 ± 94; 305-d mature equivalent (ME) milk yield 13,759 ± 2,188 kg] from 18 herds in Western Canada and 1 herd in the USA. Anogenital distance (mm) was normally distributed with a mean (±SD) of 132 ± 12, ranging from 95 to 177, and a median of 133. Anogenital distance was linearly but inversely associated with pregnancy to first artificial insemination (P/AI1). For every 1-mm increase in AGD, the estimated probability of P/AI1 decreased by 0.8%. The optimum AGD cut-point that predicted probability of P/AI1 with sensitivity and specificity of 45 and 55%, respectively, was 129 mm. Consequently, data were categorized into either short (≤129) or long (>129) AGD groups across parities, and associations between AGD, parity (first, second, and third+), and fertility measures were determined. Rates of P/AI1 were greater (36 vs. 30%) in short- than in long-AGD cows; short-AGD cows required fewer AI per conception (2.3 vs. 2.4) and had fewer days open (137 vs. 142), and a greater proportion of short-AGD cows (67 vs. 64%) was pregnant by 150 DIM compared with long-AGD cows. The rates of pregnancy up to 150 (hazard ratio of 0.91) and 250 DIM (hazard ratio of 0.93) were smaller in long- than in short-AGD cows. Anogenital distance had a weak positive association with both parity (r = 0.22) and 305-d ME milk yield (r = 0.04). Results indicate an inverse relationship between AGD and measures of fertility in lactating cows, validating our earlier report. We infer that although selecting cows for short AGD is expected to have an adverse effect on milk yield, the anticipated gain in fertility will outweigh the small decline in milk yield, strengthening the potential of AGD as a novel reproductive phenotype for use in future breeding programs to improve fertility.

Key words: reproductive phenotype, genetic selection, fertility, milk yield

INTRODUCTION

Fertility is a multifaceted trait, and its decline in dairy cows has been attributed to a complex framework of genetic, environmental, and managerial factors and their interactions (Walsh et al., 2011). In addition, the genetic correlation between fertility and productive life indicates that fertility plays a significant role in cows' longevity (VanRaden et al., 2004; Sewalem et al., 2008). As a result, the dairy industry has shifted its focus primarily from milk production traits to incorporate more comprehensive breeding objectives, such as traits associated with improved health and fertility (Miglior et al., 2005), resulting in an upward trend in fertility in recent years (Ma et al., 2019; CDCB, 2021). The renewed interest in incorporation of fertility traits into dairy cattle improvement has led to recent investigations of novel reproductive phenotypes (Nyman et al., 2014; Young et al., 2017; Gobikrushanth et al., 2018a,b) to identify potential fertility traits for genomic selection (Fleming et al., 2019).

Anogenital distance (**AGD**), defined as the distance between the anus and external genitalia in both male

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and female mammals, is one such novel reproductive phenotype, which is sexually dimorphic (much longer in males than in females), and has been studied extensively in rodents (Drickamer, 1996; Wolf et al., 2002), rabbits (Bánszegi et al., 2012), and other species. It is known that AGD is determined by the level of exposure to androgens during fetal life in rats (Wolf et al., 2002) and sheep (Manikkam et al., 2004). In early research, Jainudeen and Hafez (1965) found that androgens injected between the allantochorion and the endometrium of pregnant cattle (37 to 80 d of gestation) caused masculinization of the external genitalia in female fetuses without affecting their gonads, but AGD was not reported by the authors. In another study (Jost et al., 1972) designed to investigate sexual organogenesis in experimentally induced freemartin cattle, AGD was longer (3.5 mm) in a freemartin fetus (gestational age of 60 d) that was positioned between 2 male fetuses, than in normal female fetuses (<2.0 mm). In cattle, the placenta is reportedly the main source of androgens in dams carrying a female fetus (Mongkonpunya et al., 1975). Serum testosterone concentrations in bovine fetuses of both sexes were highly variable and ranged from approximately 20 to 210 pg/mL in female fetuses. Serum testosterone concentrations of pregnant cows were also greater (330 ± 86 pg/mL) than those of nonpregnant cows (43 ± 3 pg/mL) and varied considerably, irrespective of fetal sex (Kim et al., 1972). Similarly, highly variable concentrations of maternal testosterone and androstenedione during gestation, even when cows are carrying female fetuses, have been reported by Gaiani et al. (1984). Collectively, these findings indicate that existing natural variation in fetal and maternal testosterone concentrations is likely the prenatal determinant of AGD in the bovine fetus.

In dairy cattle, AGD, defined as the distance from the center of the anus to the base of the clitoris (Gobikrushanth et al., 2017) was found to be normally distributed and highly variable, in a study using 921 lactating Canadian Holstein cows. Subsequent studies in Irish Holstein-Friesian (n = 1,180; Gobikrushanth et al., 2019) and Iranian Holstein cows (n = 86; Akbarinejad et al., 2019) confirmed the normal distribution and variation of AGD. In Canadian Holsteins, AGD was inversely associated with fertility in first- and second-parity cows; however, no apparent associations were found between AGD and fertility in cows of third and greater parity (Gobikrushanth et al., 2017). Short AGD tended to be associated with improvements in some measures of fertility in Iranian Holstein cows, including days to first AI, pregnancy to first AI, and proportion of repeat breeders (i.e., cows that failed to conceive after 3 AI; Akbarinejad et al., 2019). In Irish Holstein-Friesian cows, however, no significant associations were found between AGD and fertility measures of interest (Gobikrushanth et al., 2019). More recently, short AGD has been associated with improvements to measures of fertility in nulliparous Holstein heifers (n = 1,692) such as fewer numbers of AI (services) per conception, younger age at conception, and greater proportion of heifers pregnant to first AI (Carrelli et al., 2021), further demonstrating the inverse relationship between AGD and fertility in North American Holstein cattle. Validating previous findings of the distribution, variability, and inverse relationship of AGD with measures of fertility in a larger population of lactating Holstein cows is necessary to confirm the potential application of AGD as a novel reproductive phenotype. If AGD becomes 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, because antagonistic relationships between milk production and fertility have been described in the past (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 evident in a larger population of cows of the same breed. The primary objective, therefore, was to determine the relationship between AGD and measures of fertility in a larger population of North American Holstein cows, to validate our previous finding that AGD is inversely related to fertility. Secondary objectives were to determine the associations between AGD and parity, and AGD and 305-d mature equivalent (**ME**) milk yield.

MATERIALS AND METHODS

Animals and Management

All animal use protocols were approved by the University of Alberta (Edmonton, Canada) Animal Care and Use Committee for Livestock (AUP no. 00002883) and by the University of Idaho (Moscow) Animal Care and Use Committee (protocol no. IACUC-2019-61). The study was conducted using lactating Holstein cows from 3 institutional and 15 commercial dairy farms across Western Canada (Alberta and British Columbia), and 1 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. The average number of cows measured on a per-farm basis was 248 (minimum 10, maximum 1,823), with herd sizes ranging from approximately 40 to 6,100 cows. Cows were housed in either freestall (15 herds) or tiestall barns (4 herds), offered a TMR (primarily composed of barley or corn silage, alfalfa silage, alfalfa hay, and concentrates) formulated according to National Research Council (2001) guidelines and had unrestricted access to water. Fresh feed was delivered either once or twice daily, and milking was performed 2 to 3 times daily. Cows were artificially inseminated based on electronic activity monitoring systems, ovulation synchronization protocols, or a combination of both. Assuming a modest difference of 5% in pregnancy to first AI between cows of short- and long-AGD groups, a priori power analysis ($\alpha = 0.05$, $\beta = 0.20$; MedCalc version 20.006, MedCalc Software Ltd.) determined the need for a minimum sample size of 2,754 cows for the population (i.e., both groups combined).

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 20.3-cm stainless steel digital calipers (Pro.Point, Princess Auto Ltd.) as described by Gobikrushanth et al. (2017). A single AGD measurement was obtained for each cow, measured by 1 of 2 experienced individuals. Some farms were visited more than once if only a portion of the herd could be accessed in a single visit. For the sake of convenience and time efficiency, AGD was measured in all cows (n = 5,545) that were accessible and had no apparent perineal abnormalities such as inflamed or lacerated vulva, as indicators of trauma at calving. Later, based on records, cows that were (1) within the 14-d period before or after calving (n = 145; to avoid periparturient changes in AGD, as per Gobikrushanth et al., 2017), (2) >180 d of gestation at the time of AGD measurement (n = 461; to avoid potential increase in AGD associated with gestational stage as per Rajesh et al., 2022), (3) designated as "do not breed" before their first insemination (n = 111), (4) sold or dead with no fertility data (n = 71), (5) either >500 DIM at the time of AGD measurement (n = 34) or >8 lactations (n = 3) (considered outliers), or (6) more than one of these criteria (n = 11), were excluded from analyses, resulting in a final population of 4,709 cows.

Data pertaining to 305-d ME milk yield and fertility measures [pregnancy to first AI (**P/AI1**), pregnancy to second AI (**P/AI2**), pregnancy to third AI (**P/AI3**), times bred, number of AI per conception, days open (interval from calving until subsequent conception), and pregnancy information up to 250 DIM] were retrieved for all cows through DairyComp305 herd management software (Valley Agricultural Software Inc.). Data retrieval occurred approximately 9 to 10 mo after AGD measurement, allowing sufficient time for all cows to have either completed or be over 250 DIM of the lactation in which AGD was measured. Only data from the first diagnosis of pregnancy (i.e., pregnancy per AI at 5 to 6 wk after each AI) were considered in this study.

Statistical Analyses

All data were analyzed using SAS version 9.4 (SAS Institute Inc.). Descriptive statistics such as mean, standard error of the mean (SEM), standard deviation (SD), median, minimum, and maximum for AGD, as well as normality of the data were determined by the UNIVARIATE procedure. The least squares means (LSM, ±SEM) for lactation number, AGD, DIM when AGD was measured, 305-d ME milk yield, times bred, AI per conception, P/AI1, P/AI2, and P/AI3, proportions of cows pregnant at 150 and 250 DIM, days open, and days of gestation when AGD was measured in pregnant cows across dairy farms were determined using the generalized linear mixed models (GLIMMIX) procedure. The model was specified as Poisson distribution ("link = log s dist = poisson") for times bred and AI per conception and as binary distribution ("dist = binary link logit") for binary variables (P/AI1, P/AI2, P/AI3, and proportions of cows pregnant at 150 and 250 DIM).

Potential difference in AGD between the Canadian (n = 2,886) and US (n = 1,823) dairy cow populations was tested by ANOVA using the MIXED procedure, and both data sets were eventually combined for all further analyses because the AGD means did not differ (132.3 vs. 132.2 mm, respectively; $P = 0.98$).

The linear and quadratic associations between AGD (predictor continuous variable) and binomial outcomes of interest (i.e., P/AI1, P/AI2, P/AI3, and cumulative pregnancy at 150 and 250 DIM), respectively, were determined by simple and polynomial regression models using the LOGISTIC procedure of SAS for 4,655 cows. Later, the optimum AGD threshold (cut-point) predictive of P/AI1, including sensitivity and specificity, was determined using the receiver operating characteristic (**ROC**) curve analysis. The ROC curves analyze sensitivity and 1 − specificity. Sensitivity is the proportion of cows above the optimum AGD cut-point diagnosed as pregnant to first AI, and specificity is the proportion of cows below the optimum AGD cut-point diagnosed as not pregnant to first AI. The optimum AGD cut-point was chosen based on the highest Youden's J statistic index. The significance of the optimum AGD cut-point was determined based on the area under the curve (**AUC**), where the AUC ranged from 0.50 to

1.00, with AUC of 0.50 considered noninformative and AUC of 1.00 considered perfect, as previously described (Swets, 1988).

Based on the AGD cut-point established by ROC curve analysis, cows of all parity groups combined were categorized as either short (≤129 mm) or long (>129 mm) AGD, to examine the influence of AGD on measures of fertility. The differences in reproductive outcomes such as P/AI1, P/AI2, P/AI3, and cumulative pregnancy at 150 and 250 DIM between short- and long-AGD cows were tested by the GLIMMIX procedure, specifying a binary distribution ("dist = binary link logit") in the model statement. The differences in times bred as well as AI (services) per conception between short- and long-AGD cows were tested by the GLIMMIX procedure specifying a Poisson distribution ("link = log s dist = poisson"), whereas days open data were analyzed, by default, as Gaussian distribution. The model included the fixed effects of AGD group (short or long), parity group (first, second, third+), and their interactions, whereas the effect of farm was treated as random. Because none of the interactions between AGD and parity groups on reproductive outcomes were significant, the interaction term was removed from the final model. The differences in LSM were tested using the Tukey-adjusted multiple means comparison test with an ilink option (for binomial variables, times bred, and AI per conception) or using a PDIFF test (for days open).

In addition to using the AGD cut-point of 129 mm established by the ROC curve analysis, the influence of AGD on fertility measures was further examined by the GLIMMIX procedure and the models described above, using 3 other AGD cut-point criteria. In the first approach, the "median AGD" of 133 mm was used as the cut-point to separate short- and long-AGD groups. In the second, "extreme ends" approach, the bottom 20%, middle 60%, and top 20% of AGD distribution were used to separate the short-, medium-, and long-AGD groups. Third, we used a "quartiles" approach to separate the AGD data into 4 groups. In all 3 approaches, cows were classified into different AGD groups across all parities combined.

Differences in the rate of pregnancy from calving up to 150 and 250 DIM between AGD categories [based on AGD 129 mm cut-point; short (≤129 mm) or long (>129 mm) AGD] were evaluated by the Kaplan-Meier survival analysis using the LIFETEST procedure. The results from the Kaplan-Meier survival analysis were tested by a Cox proportional hazard model using the PHREG procedure.

Associations between AGD and parity, and AGD and 305-d ME milk yield, were examined for all cows by both Pearson correlation coefficient using the CORR procedure and coefficient of determination using the REG procedure of SAS. For all comparisons, differences were considered significant if $P \leq 0.05$, and considered a tendency if $P > 0.05$ and ≤ 0.10.

RESULTS

Descriptive Statistics

Anogenital distance was normally distributed (mean ± SD, 132 ± 12) within a wide range of AGD estimates. The distributions of AGD in the first-, second-, and third+-parity groups are presented in Figure 1. Descriptive statistics, such as the number of cows used per farm, their mean lactation number, AGD, DIM when AGD measurement occurred, 305-d ME milk yield, times bred, number of AI per conception, P/AI1, P/AI2, P/AI3, cumulative pregnancy at 150 and 250 DIM, days open, and mean days of gestation for cows that were pregnant at the time of AGD measurement, are presented in Table 1. Mean (±SEM), minimum, and maximum AGD of cows belonging to the first-, second-, and third+-parity groups within short and long AGD categories were as presented in Table 2.

Optimum AGD Cut-Point to Predict P/AI1

Although both linear and quadratic associations between AGD and P/AI1 were significant, the scatter plot of estimated probability of P/AI1 on the predictor variable AGD revealed that the relationship was linear rather than polynomial (Figure 2). According to the results of the regression model, for every unit (1 mm) increase in AGD, the estimated probability of P/AI1 decreased by 0.8% ($P < 0.01$). The ROC curve analysis determined 129 mm AGD as the optimum cut-point to predict P/AI1, with a sensitivity of 45.5% and specificity of 59.5% ($P < 0.01$; Figure 3). Based on the AGD cut-point established by ROC curve analysis, cows of all parity groups combined were categorized as either short (≤129 mm) or long (>129 mm) AGD to examine the influence of AGD on measures of fertility.

Relationship Between AGD and Measures of Fertility

In addition to the aforementioned inverse linear association between AGD and P/AI1, cumulative pregnancy at 150 DIM had a similar inverse linear association with AGD, with the estimated probability of cumulative pregnancy at 150 DIM decreased by 0.6% ($P = 0.03$) for every 1-mm increase in AGD. However, the logistic regression models that tested the association between AGD and P/AI2, P/AI3, or P250 were not significant ($P = 0.29$, $P = 0.70$, and $P = 0.39$, respectively).

In the analyses comparing the differences in reproductive outcomes between short- and long-AGD groups, short-AGD cows had greater ($P < 0.01$) P/AI1 than long-AGD cows, but P/AI2 and P/AI3 did not differ between AGD categories. Moreover, cows with short AGD were bred fewer ($P < 0.01$) times, required fewer ($P = 0.01$) AI per conception, and had fewer ($P = 0.03$) days open than cows with long AGD (Table 3). The pregnancy risk up to 150 (hazard ratio of 0.91; $P = 0.01$) and 250 DIM (hazard ratio of 0.93; $P = 0.03$), determined by survival analysis, differed between AGD categories (Figures 4a and 4b). Although the cumulative proportion of cows pregnant at 150 DIM was greater ($P = 0.04$) in the short-AGD group than in the long-AGD group, the proportion of cows pregnant at 250 DIM did not differ between AGD groups (Table 3). When alternative approaches—that is, median AGD, extremes (top and bottom 20%) of AGD distribution, and quartiles—were used to create short- and long-AGD categories, short-AGD cows had consistently greater ($P \leq 0.05$) P/AI1 than long-AGD cows in all statistical scenarios; however, most other fertility measures did not differ (Table 4).

Associations Between AGD, Parity, and 305-d ME Milk Yield

Anogenital distance was positively correlated with parity group (Figure 5a); however, the relationship was not strong (r = 0.214; $P < 0.01$). The AGD was also positively, albeit poorly, correlated with 305-d ME milk yield (Figure 5b; r = 0.041; $P < 0.01$). The phenotypic variations in AGD explained by these 2 variables were small (coefficients of determination $R^2 = 0.0458$ and 0.0017, respectively).

DISCUSSION

The current study was conducted primarily to validate previous findings from our laboratory that lactating Holstein cows with short AGD were more fertile than those with long AGD. Associations between AGD and parity, as well as milk production, were also examined—the latter for the first time. Present results confirm our previous findings (Gobikrushanth et al., 2017) that an inverse relationship exists between AGD and fertility in lactating North American Holstein cows.

Anogenital distance was normally distributed and highly variable in the present study, comparable to what was previously reported by Gobikrushanth et al. (2017). Mean AGD across all parities in the current study was 1 mm greater than the mean AGD of Canadian Holsteins (Gobikrushanth et al., 2017), 13 mm greater than the AGD of Irish Holstein-Friesians (Gobikrushanth et al., 2019), and 18 mm greater than that of Iranian Holstein cows (Akbarinejad et al., 2019). The AGD was normally distributed and highly variable in all described populations, indicating that the mean AGD is much smaller among the population of cows in Ireland and Iran than those in North America, likely due to the smaller size of Holsteins in those countries. Moreover, mean AGD for first-, second-, and third+-parity cows in the current study were only 2 mm, 1 mm, and 1 mm greater, respectively, than those observed

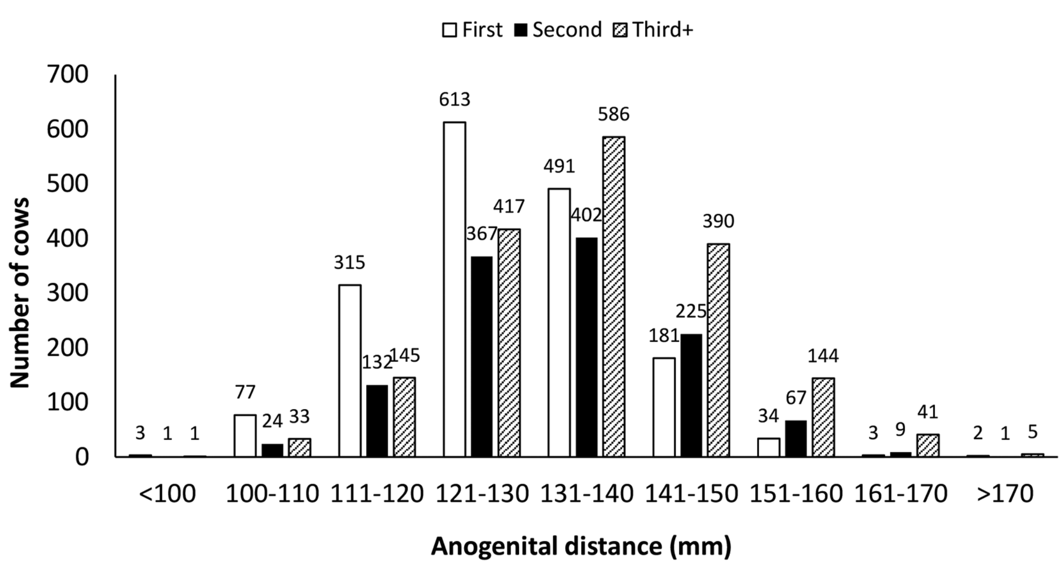


Figure 1. Distribution of anogenital distance in first-parity (unfilled bars: n = 1,719), second-parity (filled bars: n = 1,228), and third+-parity cows (hatched bars: n = 1,762).

Table 1. Distribution of cows (n) by farm and LSM ± SEM for lactation number (Lact no.), anogenital distance (AGD), DIM when AGD was measured, 305-d mature equivalent milk yield (305MY), times bred (TBRED), AI per conception (AIPC), percentage pregnancy per first (P/AI1), second (P/AI2), and third AI (P/AI3), proportion of cows pregnant at 150 (P150) and 250 (P250) DIM, days open (DOPEN), and days of gestation (DGEST) when AGD was measured (SEM not shown for percentages)

Farm[1]	n/farm	Lact no.	AGD (mm)	DIM	305MY (kg)	TBRED	AIPC	P/AI1 (%)	P/AI2 (%)	P/AI3 (%)	P150 (%)	P250 (%)	DOPEN	DGEST
CAN1	211	1.8 ± 0.09	136 ± 0.8	157 ± 6.4	15,086 ± 142.5	2.5 ± 0.13	2.2 ± 0.13	28	32	42	65	97	139 ± 5.3	84 ± 5.3
CAN2	81	2.5 ± 0.15	136 ± 1.2	165 ± 10.3	13,653 ± 230.0	2.2 ± 0.21	2.1 ± 0.19	36	39	63	63	95	130 ± 7.8	97 ± 7.0
CAN3	321	1.8 ± 0.08	123 ± 0.6	154 ± 5.2	13,419 ± 115.5	3.0 ± 0.11	3.0 ± 0.10	21	27	32	63	93	144 ± 4.1	84 ± 4.0
CAN4	10	3.9 ± 0.42	126 ± 3.5	202 ± 41.4	10,087 ± 654.6	3.0 ± 0.61	3.0 ± 0.98	11	0	20	0	67	254 ± 39.2	94 ± 49.2
CAN5	178	2.3 ± 0.10	138 ± 0.8	159 ± 7.0	14,265 ± 155.6	2.7 ± 0.14	2.5 ± 0.13	33	35	33	64	94	137 ± 5.4	86 ± 5.0
CAN6	74	2.5 ± 0.16	134 ± 1.3	182 ± 10.8	13,127 ± 240.7	2.2 ± 0.22	1.9 ± 0.21	40	43	48	44	83	177 ± 8.4	70 ± 7.9
CAN7	136	2.1 ± 0.11	129 ± 0.9	155 ± 7.9	13,809 ± 177.5	2.5 ± 0.17	2.4 ± 0.16	39	27	38	66	95	126 ± 6.2	91 ± 5.6
CAN8	80	2.2 ± 0.15	123 ± 1.2	150 ± 10.3	12,947 ± 231.5	2.3 ± 0.22	2.0 ± 0.20	41	32	54	73	94	134 ± 8.2	83 ± 7.6
CAN9	391	2.3 ± 0.07	132 ± 0.6	146 ± 4.7	12,452 ± 104.7	2.7 ± 0.10	2.5 ± 0.09	29	35	46	66	94	135 ± 3.6	90 ± 3.6
CAN10	144	2.3 ± 0.11	142 ± 0.9	154 ± 7.7	13,172 ± 172.5	2.2 ± 0.16	2.1 ± 0.15	35	44	52	72	97	133 ± 6.0	84 ± 5.9
CAN11	143	1.9 ± 0.11	138 ± 0.9	169 ± 7.7	13,280 ± 173.1	2.1 ± 0.16	1.9 ± 0.15	44	47	45	75	98	135 ± 6.1	97 ± 5.6
CAN12	43	2.4 ± 0.20	130 ± 1.7	195 ± 14.1	15,223 ± 315.7	2.7 ± 0.29	2.5 ± 0.27	28	30	45	42	76	191 ± 11.0	81 ± 11.0
CAN13	51	2.0 ± 0.19	131 ± 1.5	226 ± 13.0	13,747 ± 289.9	2.6 ± 0.27	2.5 ± 0.26	17	43	40	59	93	144 ± 10.6	103 ± 8.3
CAN14	127	2.2 ± 0.12	131 ± 1.0	161 ± 8.2	11,983 ± 183.7	2.5 ± 0.17	2.4 ± 0.16	38	28	38	64	92	134 ± 6.4	89 ± 5.7
CAN15	224	2.5 ± 0.09	128 ± 0.7	148 ± 6.2	14,599 ± 138.3	2.4 ± 0.13	2.2 ± 0.12	44	31	45	80	96	114 ± 4.7	94 ± 4.3
CAN16	182	2.1 ± 0.10	129 ± 0.8	202 ± 6.9	12,832 ± 153.5	2.1 ± 0.14	1.8 ± 0.14	40	51	47	63	96	148 ± 5.5	97 ± 5.0
CAN17	178	2.4 ± 0.10	142 ± 0.8	153 ± 6.9	13,880 ± 155.2	2.6 ± 0.15	2.6 ± 0.13	38	31	37	72	97	124 ± 5.2	84 ± 4.7
CAN18	312	2.0 ± 0.10	133 ± 0.6	164 ± 5.3	14,113 ± 117.2	3.2 ± 0.11	2.7 ± 0.11	28	21	31	66	93	135 ± 4.3	82 ± 4.0
USA1	1,823	2.5 ± 0.03	132 ± 0.3	143 ± 2.2	14,069 ± 48.5	3.2 ± 0.05	3.0 ± 0.04	23	32	34	65	91	141 ± 1.7	52 ± 1.7
Overall	4,709	2.3 ± 0.02	132 ± 0.2	154 ± 1.4	13,759 ± 31.9	2.8 ± 0.03	2.6 ± 0.03	29	32	37	66	93	138 ± 1.1	75 ± 1.1
Minimum, maximum	10, 1,823	1, 8	95, 177	15, 498	3,778, 21,260	1, 16	1, 13	11, 44	0, 51	20, 63	0, 80	67, 98	32, 573	0, 180
n/variable		4,709	4,709	4,677	4,708	4,708	4,174	4,655	3,221	2,100	4,175	4,173	4,175	2,357

[1]Farm identification codes reflect farm locations in Canada (CAN) or the United States (USA).

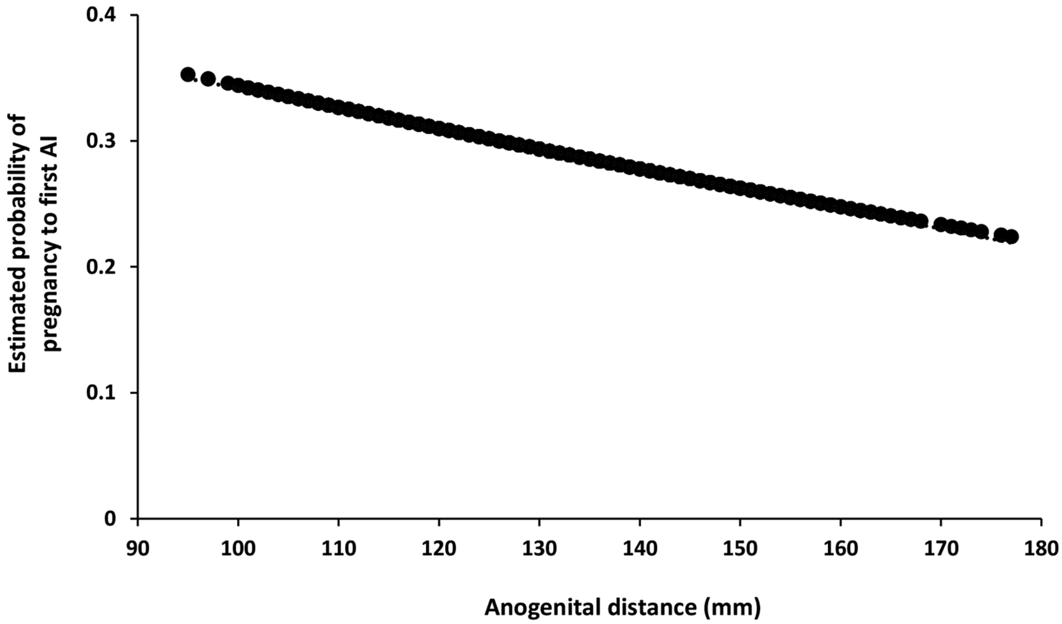


Figure 2. Estimated probability of pregnancy to first AI plotted against anogenital distance (AGD) in 4,655 lactating dairy cows. For every 1-unit (millimeter) increase in AGD, the odds of conceiving to first AI decreased by 0.8% ($P < 0.01$).

previously in Canadian Holsteins (Gobikrushanth et al., 2017). Despite AGD being highly variable, similarities in mean AGD and its range across these 2 North American populations (Gobikrushanth et al., 2017 and the present study) of Holstein cows suggest that measurement of AGD is highly repeatable within the North American Holstein population.

Anogenital distance measurements are highly repeatable in lactating Holstein cows during different physiological states, including all phases of the estrous cycle, lactation, and gestation, except during the last 2 wk of gestation (Rajesh et al., 2022). Consequently, measuring AGD in the present study at random stages of the estrous cycle, lactation, and most stages of gestation should not have affected AGD. The study of Rajesh et al. (2022) was limited in that AGD was not measured monthly through the entire gestation, but only 4 times at approximately 1, 3, 6, and 9 mo of gestation. Although a significant increase in AGD was evident only at 9 mo (270.5 ± 4.4 d) of gestation, the authors of the study acknowledged that pregnancy-associated AGD increase likely occurred earlier than 9 mo; that is, between 6 and 9 mo of gestation, although they could not definitively establish the earliest gestational stage when the AGD increase occurred. For that reason, to eliminate the potential influence of gestational stage on AGD in the present study, we excluded data from all cows that were >180 d of gestation at the time of AGD measurement. Furthermore, AGD data from periparturient cows (that is, 14 d pre- and postcalving) were excluded from all analyses, minimizing the possible variation in AGD due to edema and inflammation

Table 2. Mean anogenital distance (AGD, distance from the center of the anus to the base of the clitoris) in lactating dairy cows of all parities combined, and first-, second-, and third+-parity groups after categorizing them into short- and long-AGD groups using the 129-mm cut-point, and both AGD groups combined, from all 19 herds

	AGD group, mean ± SEM				
Parity group	Short, ≤129 mm (n)	Long, >129 mm (n)	AGD groups combined, mean ± SEM (n)	Minimum (mm)	Maximum (mm)
All parities	121 ± 0.1 (1,975)	140 ± 0.2 (2,734)	132 ± 0.2 (4,709)	95	177
First parity	121 ± 0.6 (947)	138 ± 0.6 (772)	128 ± 0.3 (1,719)	95	174
Second parity	123 ± 0.6 (484)	140 ± 0.6 (744)	133 ± 0.3 (1,228)	99	177
Third+ parity	123 ± 0.8 (544)	142 ± 0.8 (1,218)	135 ± 0.3 (1,762)	97	176

Table 3. Measures of fertility in lactating cows of short- and long-anogenital distance groups (AGD, distance from the center of the anus to the base of the clitoris), expressed as LSM ± SEM (SEM not shown for percentages)

Fertility measure[1]	n	Short AGD[2]	Long AGD[3]	P-value
P/AI1, %	4,655	36 (n = 1,953)	30 (n = 2,702)	0.001
P/AI2, %	3,221	35 (n = 1,308)	33 (n = 1,913)	0.14
P/AI3, %	2,100	39 (n = 846)	41 (n = 1,254)	0.40
Times bred	4,708	2.5 ± 0.08 (n = 1,974)	2.6 ± 0.09 (n = 2,734)	0.002
AI per conception[4]	4,174	2.3 ± 0.09 (n = 1,780)	2.4 ± 0.09 (n = 2,394)	0.009
Days open[4]	4,175	137 ± 4.1 (n = 1,781)	142 ± 4.0 (n = 2,394)	0.03
Pregnant by 150 DIM, %	4,175	67 (n = 1,781)	64 (n = 2,394)	0.04
Pregnant by 250 DIM, %	4,173	94 (n = 1,781)	94 (n = 2,392)	0.77

[1]Cows that were inseminated once but whose pregnancy information was unavailable (n = 54) were excluded from this analysis; P/AI1, P/AI2, and P/AI3 denote pregnancy per first, second, and third AI.

[2]Cows with AGD less than or equal to the optimum AGD cut-point (≤129 mm) were considered short AGD.

[3]Cows with AGD greater than the optimum AGD cut-point (>129 mm) were considered long AGD.

[4]Could not be determined in 534 cows that had not conceived by the end of the study.

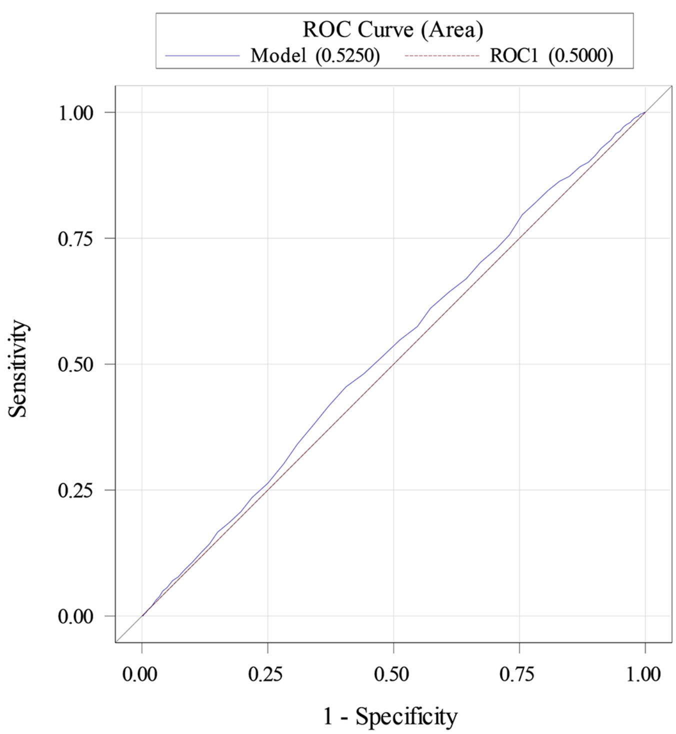


Figure 3. Receiver operating characteristic (ROC) curve analysis to determine the optimum anogenital distance (AGD) predictive of probability of pregnancy to the first AI in 4,655 lactating dairy cows. Optimum AGD cut-off was 129 mm, with a sensitivity of 45.5% and a specificity of 59.5%; $P < 0.01$.

of the external genitalia in the days leading up to and after calving. Other exclusion criteria applied were described under Methodology.

In the first report by Gobikrushanth et al. (2017), P/AI1 was greater in cows with short AGD than in cows with long AGD, among first- (54 vs. 31%) and second-parity cows (44 vs. 28%). However, AGD was not associated with P/AI1 in third+-parity cows in that study. Parity × AGD interactions were not evident in the present study, and the differences in pregnancy to AI between short- and long-AGD cows were seen only during first service (P/AI1) but not during subsequent services (P/AI2 and P/AI3). In Iranian Holstein cows, the risk of P/AI1 tended to be 19 percentage points greater in cows with short AGD (49%) than with long AGD (30%; Akbarinejad et al., 2019). Our present results corroborate those of the latter study, albeit less pronounced, with P/AI1 being only 5 percentage points greater in short-AGD cows than in long-AGD cows. New evidence shows that dairy cows classified as short-AGD resume postpartum estrous activity sooner, have greater intensity and duration of estrus, are more likely to ovulate, and have greater circulating concentrations of progesterone 7 d post-AI compared with long-AGD cows (A. Madureira, University of British Columbia, Vancouver, Canada; T. Burnett, University of Guelph, Ridgetown, Canada; J. Carelli, M. Gobikrushanth, R. Cerri, University of British Columbia, Vancouver, Canada, and D. Ambrose, unpublished data). Therefore, it is plausible that short-AGD cows in the present study, irrespective of parity, had an early onset of cyclicity, developed dominant follicles that were more estrogenic (Sartori et al., 2004), or had other beneficial alterations in the systemic (Lucy et al., 2014) or local (Leroy et al., 2008) metabolic and endocrine milieus, conducive for greater conception and pregnancy sustenance. Although these are speculative deliberations, they are testable hypotheses worth pursuing in future research.

The number of repeat breeders in Iranian Holsteins differed significantly between AGD groups, where the proportion of repeat breeders among long-AGD cows was twice that of short-AGD cows (33% vs. 16%; Akbarinejad et al., 2019). In the present study, short-AGD cows were bred fewer times (2.5 vs. 2.7) and required fewer AI per conception (2.3 vs. 2.4) than long-AGD cows, although the differences were small. These results differ from those of Akbarinejad et al. (2019), who found that the mean number of AI per conception did not differ between AGD groups. Moreover, the interval from calving to conception (days open) was 38 d greater in cows with long AGD than in those with short AGD in that study (Akbarinejad et al., 2019). Although the interval from calving to conception was not as long, a similar positive relationship between AGD and days open was evident in the present study, wherein long-AGD cows remained open, on average, 5 d longer than short-AGD cows. Although the relationships between AGD and days open are comparable between the present study and that of Akbarinejad et al. (2019), the large (33 d) difference in the intervals from calving to conception between the 2 studies may be attributable to the larger population size and greater variability of factors in the present study (n = 4,709; 19 herds) compared with that (n = 86; 1 herd) of Akbarinejad et al. (2019). The method used to categorize cows into short- and long-AGD groups differed between the 2 studies, as the median AGD was used as the cut-point in the latter study. However, the method used to categorize cows into short and long AGD in the present study was similar to those of Gobikrushanth et al. (2017), Carrelli et al. (2021), and Grala et al. (2021); that is, a cut-point established based on ROC curve analysis. When the extreme ends (bottom 20% vs. top 20%) and quartile methods were used as cut-points to create AGD groups, P/AI1 between short- and long-AGD groups differed significantly, and when the median AGD was used as the cut-point they tended to differ (Table 4), indicating that any one of these methods could be used to define

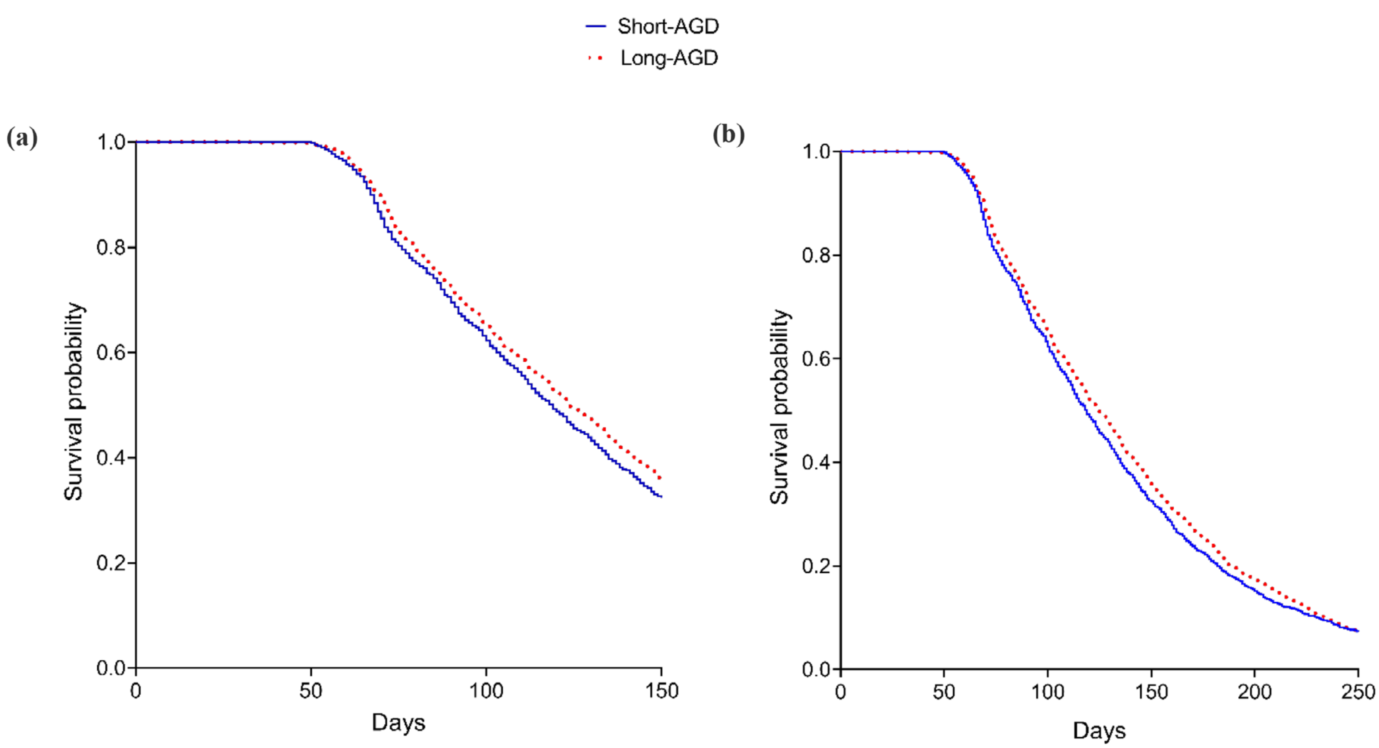


Figure 4. Kaplan-Meier survival curves illustrating the survival probability of pregnancy up to (a) 150 DIM by anogenital distance (AGD) groups in 4,175 lactating dairy cows and (b) 250 DIM by AGD groups in 4,173 lactating dairy cows. The rate of pregnancy up to 150 DIM was significantly lower [hazard ratio of 0.91 (95% CI: 0.84–0.98); $P = 0.01$] for long-AGD cows (≥129 mm; n = 2,394) than for short-AGD cows (<129 mm; n = 1,781). The rate of pregnancy up to 250 DIM was also significantly lower [hazard ratio of 0.93 (95% CI: 0.88–0.99); $P = 0.03$] in long-AGD cows (≥129 mm; n = 2,392) than in short-AGD cows (<129 mm; n = 1,781).

Table 4. Comparing the effects of anogenital distance (AGD, distance from the center of the anus to the base of the clitoris) on measures of fertility when different AGD cut-off criteria were applied; results presented as LSM ± SEM

	AGD cut-off criterion applied											
	≤ vs. > Median, 133 mm[2]			Bot 20%, Mid 60%, Top 20%[3]				Quartiles[4]				
Variable[1]	Short	Long	*P*-value	Short	Medium	Long	*P*-value	Short	Low-Int	High-Int	Long	*P*-value
AGD	126 ± 0.5	145 ± 0.5	<0.001	117 ± 0.4	132 ± 0.3	148 ± 0.4	<0.001	119 ± 0.3	129 ± 0.3	136 ± 0.3	147 ± 0.3	<0.001
TBRD	2.5 ± 0.09	2.6 ± 0.09	0.31	2.5 ± 0.09	2.6 ± 0.08	2.6 ± 0.09	0.14	2.5 ± 0.09	2.5 ± 0.09	2.7 ± 0.10	2.6 ± 0.09	0.009
AIPC	2.3 ± 0.09	2.4 ± 0.09	0.36	2.3 ± 0.09	2.3 ± 0.09	2.4 ± 0.09	0.50	2.3 ± 0.09	2.3 ± 0.09	2.4 ± 0.10	2.4 ± 0.10	0.05
P/AI1	34 ± 0.02	31 ± 0.02	0.10	35 ± 0.02	33 ± 0.02	29 ± 0.02	0.02	35 ± 0.02	35 ± 0.02	31 ± 0.02	30 ± 0.02	0.01
P/AI2	34 ± 0.02	33 ± 0.02	0.44	37 ± 0.03	33 ± 0.02	33 ± 0.03	0.20	35 ± 0.02	36 ± 0.02	31 ± 0.02	33 ± 0.02	0.13
P/AI3	40 ± 0.02	41 ± 0.02	0.59	40 ± 0.03	40 ± 0.02	42 ± 0.03	0.85	41 ± 0.03	40 ± 0.03	38 ± 0.03	43 ± 0.03	0.40
P150	66 ± 0.02	63 ± 0.02	0.08	66 ± 0.02	66 ± 0.02	62 ± 0.02	0.24	67 ± 0.02	67 ± 0.02	63 ± 0.02	63 ± 0.02	0.15
P250	93 ± 0.009	94 ± 0.01	0.37	93 ± 0.01	93 ± 0.009	95 ± 0.01	0.25	93 ± 0.01	94 ± 0.01	93 ± 0.01	94 ± 0.01	0.49
DOPEN	139 ± 3.9	143 ± 4.2	0.10	139 ± 4.4	140 ± 4.0	142 ± 4.4	0.54	138 ± 4.3	137 ± 4.3	143 ± 4.3	143 ± 4.4	0.07

[1]TBRD = times bred; AIPC = AI (services) per conception; P/AI1 = pregnancy per first AI; P/AI2 = pregnancy per second AI; P/AI3 = pregnancy per third AI; P150 = cumulative proportion of cows pregnant at 150 DIM; P250 = cumulative proportion of cows pregnant at 250 DIM; DOPEN = days open.

[2]Applying the median AGD of 133 mm as the cut-off to separate short- and long-AGD groups.

[3]Applying the bottom 20%, middle 60%, and top 20% of AGD distribution to separate short-, medium-, and long-AGD groups.

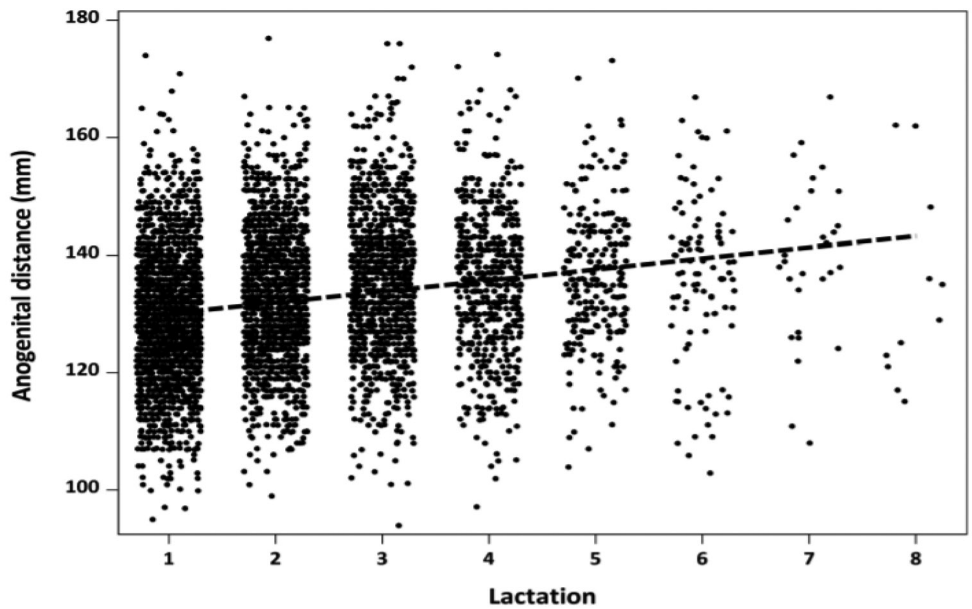
[4]Applying the quartile method to separate groups with short, low-intermediate, high-intermediate, and long AGD.

a threshold AGD value to determine associations between AGD and fertility measures of interest. Thus, if ROC curve analysis fails to provide an AGD cut-point predictable of P/AI1, the aforementioned methods could be alternatives. It is recommended, however, to use ROC curve analysis to find the optimal cut-point when possible, because 5 fertility measures differed when the cut-point of 129 mm, as determined via ROC curve analysis, was used, compared with only 1 fertility measure (P/AI1) that consistently differed or tended to differ when other cut-points (median, extreme ends, and quartiles) were used.

The survival analysis approach determined that the rates of pregnancy up to 150 and 250 DIM were smaller for long-AGD than short-AGD cows in the present study. Similarly, the rate of pregnancy up to 250 DIM was smaller in long-AGD than in short-AGD cows of first (hazard ratio of 0.68) and second parities (hazard ratio of 0.76) in a previous report from our research group (Gobikrushanth et al., 2017). In another report (Carrelli et al., 2021), nulliparous heifers with long AGD had reduced pregnancy risk up to 15 mo of age compared with short-AGD heifers (hazard ratio of 0.59). Despite significant differences in the rate of attaining pregnancy evident from the Kaplan-Meier survival curves up to both 150 and 250 DIM between short- and long-AGD cows, the proportion of cows pregnant differed only at 150 DIM, as determined by ANOVA. This indicates that, although the smaller rate of pregnancy attainment in long-AGD cows continued up to or beyond 200 DIM, this difference was no longer evident at 250 DIM. We infer that despite delayed rate of pregnancy in long-AGD cows, they do get pregnant given sufficient time.

Despite the strengths of including several dairy farms and thousands of cows in the study, it also had limitations, in that we had no control over herd management practices and as to when AGD was measured relative to calving or AI. Measuring AGD at approximately the same stages of lactation and gestation in all cows would have been the preferable approach, but it was not practical within this study. Moreover, the variations in herd size, parity, rations fed, level of milk production, reproductive management practices, AI technician effects, length of dry period, and other factors could not be controlled, making the study population more heterogeneous than desired. For example, differences in reproductive management practices, particularly in herds using ovulation synchronization protocols versus activity monitoring technologies, may mask the response, especially in terms of outcomes such as days open. Due to inconsistencies in on-farm data recording of whether cows were subjected to AI after detected estrus or timed AI, we were unable to incorporate this

(a)



(b)

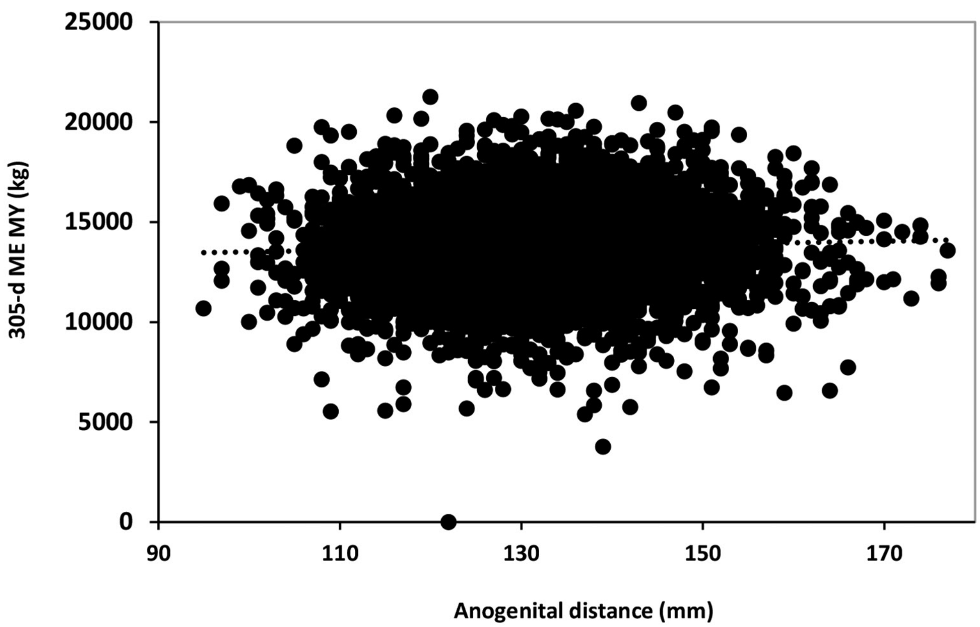


Figure 5. Association between (a) parity and anogenital distance (y = 1.927x + 127.79; R^2 = 0.05; $P < 0.01$) and (b) 305-d mature equivalent milk yield (ME MY) in kilograms and anogenital distance (y = 7.5947x + 12,752; R^2 = 0.0017; $P < 0.01$) in lactating dairy cows (n = 4,709). Data points jittered in (a) to visualize the distribution.

component into our analysis. Despite the lack of homogeneity and tighter control of the aforementioned factors, the findings of an inverse association between AGD and fertility in this large population provide further evidence that AGD influences fertility in North American Holstein cows.

The 5-percentage point difference in P/AI1 (36 vs. 31%) between short- and long-AGD cows in the present study was lower than anticipated. Nevertheless, our findings corroborate previous reports (Gobikrushanth et al., 2017; Akbarinejad et al., 2019; Carrelli et al., 2021; Grala et al., 2021) that dairy cattle with short

AGD are more fertile than those with long AGD. Using only primiparous New Zealand Holstein-Friesian grazing cows that were bred to have either positive (+5%) or negative (−5%) genetic merit for fertility traits, Grala et al. (2021) recently reported that AGD was shorter in cows with a positive genetic merit for fertility than in those with a negative genetic merit for fertility. Cows with a short AGD were more likely to be pregnant earlier in the mating season than those with a long AGD. Moreover, the time from calving to conception was 20 d earlier in short-AGD cows than in long-AGD cows. That study (Grala et al., 2021) not only supports our present findings but also corroborates previous reports, with the only exception being the Irish study conducted with grazing Holstein-Friesian cows (Gobikrushanth et al., 2019).

The correlation between measures of fertility and milk production has received considerable attention in the past, where an antagonistic relationship between dairy cattle fertility and milk production has been reported (Oltenacu et al., 1991; Dematawewa and Berger, 1998; VanRaden et al., 2004). If AGD is successfully established as a fertility trait for consideration in future genetic selection programs, it becomes important to know whether selecting for short AGD (indicative of improved fertility) would have a negative effect on milk yield in dairy cows. Therefore, the association between AGD and 305-d ME milk yield was also investigated in the present study. Only 0.15% of the variation in AGD was explainable by changes in 305-d ME milk yield, indicating that the phenotypic selection for AGD is unlikely to cause any significant decline in milk yield. The simultaneous improvements in genetic merit for both milk production and fertility are possible despite the antagonistic relationship between fertility and milk yield (Shanks et al., 1978; Berry et al., 2014, 2016).

Traditional fertility traits, such as number of AI, calving interval, and days open, tend to have low heritability estimates (0.01 to 0.08; Berry et al., 2014; Fleming et al., 2019) and limitations associated with these metrics, suggesting that indicator traits could be very useful for increasing accuracy of estimated breeding values for fertility (Miglior et al., 2017). 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 is that those detailed phenotypes could have noticeably greater heritability than traditional measures of fertility (Miglior et al., 2017). A heritability estimate of 0.37 has been reported for AGD in Irish Holstein-Friesians (Gobikrushanth et al., 2019). Accordingly, if we attribute approximately 40% of the variation in AGD to the genetic effect, much higher than that of traditional fertility traits, measuring AGD and genotyping animals in a sufficiently large reference population will allow estimation of marker effects, which can then be used to determine genomic breeding values. In addition, combining genomic predictions for both novel and traditional fertility traits is likely to provide a more accurate and faster means of improving dairy cow fertility.

In conclusion, even though the magnitude of difference in P/AI1 between short- and long-AGD cows was smaller than previously reported, the present study conducted on a large population of North American Holstein cows confirms earlier findings that AGD is inversely associated with measures of fertility. The rate of attaining pregnancy up to 150 and 250 DIM was smaller in cows with long AGD than in those with short AGD. Both parity and 305-d ME milk yield had weak positive associations with AGD, the latter indicating that milk yield may be adversely affected by selecting for short AGD. Nonetheless, the anticipated fertility gains from selecting cows for short AGD is greater than the probable decline in milk yield (5% vs. 0.17%), strengthening the potential of AGD as a novel reproductive phenotype for use in future breeding programs to improve fertility. Before AGD can be recommended for use in selection for fertility, however, the phenotypic and genotypic associations between AGD and existing fertility indices such as daughter pregnancy rate, cow conception rate, and heifer conception rate should be explored.

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