J. Dairy Sci. 105:8523–8534
https://doi.org/10.3168/jds.2022-21897

Anogenital distance is associated with postpartum estrous activity, intensity of estrous expression, ovulation, and progesterone concentrations in lactating Holstein cows

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ABSTRACT

The objectives of this retrospective observational study were to determine the associations of anogenital distance (AGD) with (a) postpartum estrous activity, (b) diameter of the preovulatory follicle, (c) intensity of estrous expression, (d) postestrus ovulation, (e) corpus luteum (CL) size, and (f) concentrations of progesterone at estrus and on d 7 after estrus. Lactating Holstein cows (n = 178; 55 primiparous, 123 multiparous) were enrolled into the study during the first postpartum week. All cows were continuously monitored by a pedometer-based automated activity monitoring (AAM) system for estrus. Postpartum estrous activity was assessed using the AAM estrus alerts, in which cows with at least one true estrus alert (i.e., a relative increase in steps from each cow's baseline detected by the AAM and the presence of at least one follicle >15 mm, a CL <20 mm, or no CL detected by ultrasound) by the first 50 d in milk (DIM) were considered to have commenced estrous activity. At the estrus alert >60 DIM, ovulation was determined by ultrasound at 24 h, 48 h, and 7 d after estrus, and blood samples were collected at estrus alert and on d 7 after estrus for progesterone analysis. The AGD was measured from the center of the anus to the base of the clitoris and classified as either short- or long-AGD using 2 cut-points of 148 mm (predictive of the probability of pregnancy to first insemination; short-AGD, n = 115; long-AGD, n = 63) and 142 mm (the median AGD; short-AGD, n = 90; long-AGD, n = 88). Regardless of the cut-point used, early postpartum estrous activity by 50 DIM (67 vs. 54%), duration of estrus (11.6 vs. 9.7 h), and preovulatory follicle diameter (20 vs. 19 mm) were greater in short-AGD than in long-AGD cows. Increased peak of activity at estrus in short-AGD cows (354 vs. 258% mean relative increase) was affected by an interaction between AGD and parity in which multiparous long-AGD cows had lesser relative increase in activity than primiparous cows (217 vs. 386%, respectively). Mean progesterone concentration at estrus was lesser in short-AGD (0.47 vs. 0.61 ng/mL) than in long-AGD cows. The ovulatory response at 24 h did not differ, but at 48 h (91 vs. 78%) and on d 7 after estrus (97 vs. 84%) it was greater in short-AGD cows. Although CL diameter on d 7 after estrus did not differ, short-AGD cows had greater progesterone concentration 7 d after estrus than long-AGD cows (4.1 vs. 3.2 ng/mL, respectively). In conclusion, greater proportions of short-AGD cows commenced estrous activity by 50 DIM, had larger preovulatory follicles, exhibited greater duration of estrus, had reduced progesterone concentration at estrus, had greater ovulation rates and progesterone concentration 7 d after estrus compared with long-AGD cows, with no difference in CL size between AGD groups. Because all the differences in physiological characteristics of short-AGD cows reported herein favor improved reproductive outcomes, we infer that these are factors contributing to improved fertility reported in short-AGD cows compared with long-AGD cows.

Key words: anogenital distance, estrous expression, ovulation, preovulatory follicle

INTRODUCTION

Reproductive performance is essential for efficient dairy production, but its decline has been noted over the decades and has been linked to a multitude of factors (Stevenson and Britt, 2017; Lucy, 2019). Accurate estrus detection is the essential first step in getting a cow pregnant in herds that do not adopt fixed-time AI programs. The occurrence of estrous behavior in lactating dairy cattle, however, has been reportedly reduced (Roelofs et al., 2010). Many factors affect expression of

Received January 30, 2022.
Accepted June 8, 2022.
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estrus, such as environmental temperature (Orihuela, 2000), parity (Tippenhauer et al., 2021), BCS (Madureira et al., 2015), and lactational status (Palmer et al., 2012; Madureira et al., 2015; Tippenhauer et al., 2021). Intensity and occurrence of estrous expression have been positively associated with fertility of lactating dairy cows that were artificially inseminated after either spontaneous estrus (Tippenhauer et al., 2021) or timed AI protocols (Pereira et al., 2016; Madureira et al., 2019). Literature regarding the physiological mechanisms behind the association of the intensity of estrous expression and reproductive outcomes is quite limited. In contrast, a recent publication linked hormonal factors such as estradiol and progesterone around the time of estrus with estrous expression (Madureira et al., 2021).

Anogenital distance (**AGD**), defined as the distance from the center of the anus to the clitoris in dairy cattle (Gobikrushanth et al., 2017), has been studied recently as a novel reproductive phenotype. Variability of AGD in dairy cows (Gobikrushanth et al., 2017) and heifers (Carrelli et al., 2021) and its heritability (Gobikrushanth et al., 2019) have been described. Moreover, an inverse relationship between AGD and fertility has been reported in both dairy (Gobikrushanth et al., 2017) and beef cattle (Battista, 2019). These reports in cattle are consistent with those of other animals such as rats (Zehr et al., 2001), rabbits (Bánszegi et al., 2012), and yellow-bellied marmots (Monclús and Blumstein, 2012). A similar inverse association between AGD and fertility in women has also been proposed (Mendiola et al., 2012; Mira-Escolano et al., 2014) based on indirect measures of fertility.

Variation in AGD is influenced by the level of androgen exposure during early fetal life when the dimorphic differentiation of the 2 sexes occur (Schwartz et al., 2019), with excess androgen exposure to female fetuses resulting in longer AGD in pigs (Drickamer et al., 1997), rats (Wolf et al., 2002), and sheep (Dean et al., 2012). When prenatal hyperandrogenism was experimentally induced in female rhesus monkeys, menarche was delayed, ovarian dysfunction and luteal deficiency were evident, and fecundity was greatly reduced (20 vs. 100%; Abbott et al., 1998). Wu et al. (2017) reported that women with longer AGD were more likely to fail to ovulate compared with women with shorter AGD. Furthermore, female sheep fetuses exposed to testosterone during early gestation failed to display overt female sexual behavior as adults, had perturbed estrous cycles, and reduced ovulatory response (Clarke et al., 1977).

Although the inverse association between AGD and fertility in dairy cows first reported by Gobikrushanth et al. (2017) has been confirmed by others in dairy cows (Akbarinejad et al., 2019; Grala et al., 2021; Carrelli et al., 2022), dairy heifers (Carrelli et al., 2021) and partially in beef cattle (Battista, 2019), the underlying physiological factors contributing to fertility differences between short- and long-AGD cattle have not been investigated. Determining the associations between AGD and estrous behavior characteristics, ovarian function and progesterone concentrations will help improve our understanding of the mechanisms at play. As short AGD is considered a female-leaning phenotype resulting from lesser exposure to testosterone (Hotchkiss et al., 2007) during prenatal development, we hypothesized that short-AGD cows may have an earlier resumption of postpartum estrous activity, develop larger preovulatory follicles, have a stronger intensity of estrus, have greater ovulatory response, develop a larger corpus luteum (**CL**), and have progesterone concentrations that favor improved reproductive outcomes compared with in cows with long AGD.

Thus, the objectives of this study were to determine the association of AGD in lactating dairy cows with (a) postpartum estrous activity, (b) size (diameter) of the preovulatory follicle, (c) intensity of estrous expression, (d) ovulatory response, (e) CL size, and (f) concentrations of progesterone at estrus and on d 7 after estrus.

MATERIALS AND METHODS

This retrospective observational study was conducted at the University of British Columbia's Dairy Education and Research Centre in Agassiz, Canada (49°13′59″N, 121°46′01″W) from January 2019 to January 2020. All animal procedures were approved by the Animal Care Committee of the University of British Columbia (protocol no. A18–0039) and by the Animal Care and Use Committee for Livestock of the University of Alberta (protocol no. 00002883).

Animals, Housing, and Management

Lactating Holstein cows (n = 208; 65 primiparous, 143 multiparous) were enrolled into the study during the first week after calving. The experimental herd of approximately 270 lactating cows had an average 305-d mature equivalent milk yield of 13,896 kg/cow.

Cows were housed in wood-framed barns with a freestall design, equipped with deep sand-bedded stalls, fans, and an automated manure scraper. Milking was performed twice daily (at approximately 0500 and 1500 h) in a conventional milking parlor. Fresh TMR was delivered twice daily (0700 and 1600 h). The TMR was formulated following the NRC (2001) guidelines to meet or exceed the requirements of a 620-kg Holstein cow producing 40 kg/d of 3.5% FCM. Water and TMR were available for ad libitum intake.

Cows were inseminated based on estrous activity alert (described below) after 60 DIM following the a.m./p.m. rule, at approximately 0700 or 1900 h. If no activity was recorded until 120 DIM, cows were enrolled into a standard Ovsynch protocol (Pursley et al., 1995) and subjected to timed AI.

Monitoring Postpartum Estrous Activity and Ovarian Ultrasonography

All cows were monitored for estrus by an automated activity monitoring (**AAM**) system. As a component of the AAM system, a leg-mounted pedometer (AfiPedometer Plus Tag; AfiMilk) was attached to the right hind limb of each cow before calving and it remained on the cow during her entire production life. For the present study, activity data (steps/h) recorded 24 h/d, in 1 h blocks by a wireless receiver box were analyzed, starting from the day of calving until first AI after the voluntary waiting period of 60 DIM. The threshold for an estrus alert relies on a relative increase in steps from each cow's baseline in addition to the number of steps within 1 h blocks, where blocks with lesser steps/h require a greater relative increase in steps from baseline, and vice versa (e.g., a cow with 0 to 49 steps/h would require an increase in steps of 180% to be alerted, whereas a cow with 700+ steps/h would require an increase of only 135%). Peak of activity was defined as the maximum activity reached above the threshold (Madureira et al., 2015). The AAM system used in this study has a sensitivity of 80.9% and specificity of 86.7% (Mayo et al., 2019). Duration of estrus was defined as the interval in hours from the onset to the end of an estrus alert. The AAM system was checked for alerts twice daily during milking times for cows in estrus.

At each estrus alert that occurred between 10 and 50 DIM, ovaries were examined by ultrasonography (Ibex Pro; E.I. Medical Imaging) using a 7.5 MHz linear-array transrectal transducer to determine if the alert was true or false. Cows were classified as having a true estrus alert if they had at least one follicle greater than 15 mm in diameter and either a CL less than 20 mm in diameter or no CL detectable at the time of the alert and an ovulation confirmed (presence of a new CL) 7 d after estrus. Cows were considered to have commenced postpartum estrous activity when at least one true estrus alert (≥1 alert; estrus) occurred during the first 50 DIM and considered anestrus if no true estrus alert occurred by 50 DIM.

In a subset of 117 cows (66% of total), ovarian ultrasonography was performed to determine ovulation at 24 h, 48 h, and 7 d after estrus alert when the alert occurred after the voluntary waiting period of 60 DIM. Ovulation was determined by the disappearance of the putative preovulatory follicle at 24 and 48 h and confirmed by the presence of a CL 7 d after estrus. Ovulation failure was declared when ovulation at 48 h postestrus was not detected, and a new CL was absent 7 d after estrus.

Anogenital Distance Measurement

Anogenital distance was measured once during the lactation from the center of the anus to the base of the clitoris, as described by Carrelli et al. (2021) using 20.3-cm stainless-steel digital calipers (Pro.Point, Princess Auto Ltd.) by the same technician trained in AGD measurement (mean DIM ± SE at AGD measurement was 159 ± 7). Anogenital distance data from cows that were up to 14 d after calving and pregnant cows that were >180 d of gestation at the time of AGD measurement were excluded from all analyses. The early postpartum cows were excluded because of the possible influence of parturient trauma on AGD (Gobikrushanth et al., 2017) and cows >180 d of gestation at AGD measurement were excluded to avoid potential increase in AGD associated with gestational age (Rajesh et al., 2022). After applying these filters, data from 178 cows (55 primiparous, 123 multiparous) were used in the final analyses. Because AGD is a phenotype that is consistent through most physiological states and not affected by the stage of lactation in Holstein cows (Rajesh et al., 2022), one measurement was considered sufficient. All AGD measurements and estrus alerts (AAM data) were collected during the same lactation and the associations were evaluated retrospectively.

Blood Sampling and Quantification of Progesterone

Blood samples (approximately 10 mL) were collected by puncturing a coccygeal vessel into Vacutainer tubes (Becton Dickinson) with K_2EDTA, at the time of estrus and on d 7 after estrus (first estrus alert after the voluntary waiting period of 60 DIM when AI occurred). Samples were placed immediately on ice, centrifuged within 3 h after collection (2,700 × g at 4°C for 15 min), and plasma harvested and stored at −20°C. Plasma progesterone concentrations were quantified using a commercial ELISA (Ovucheck Plasma; Biovet) previously validated for use in cattle by Broes and LeBlanc (2014). The intra- and inter-assay coefficients of variation were 6.2 and 6.6%, respectively.

Statistical Analyses

All statistical analyses were carried out with SAS Studio version 5.1 (SAS Institute Inc.). Parity was categorized as primiparous (first-lactation cows) and multiparous (≥second-lactation cows). Data on pregnancy at first AI (**PAI1**; diagnosed by transrectal ultrasonography at 32 ± 3 d post-AI) were retrieved using Dairy Comp 305 (Lactanet; Valley Agricultural Software Inc.) to determine the association between PAI1 and AGD (predictor continuous variable) by logistic regression analysis and the estimated probabilities of PAI1 were plotted against AGD (Figure 1). The receiver operating characteristic curve analysis was then performed to compute the optimum AGD cut-point that could predict the greatest probability of PAI1 (Figure 2). Details of the receiver operating characteristic curve analysis were described previously (Gobikrushanth et al., 2017; Carrelli et al., 2021). Based on the latter analysis, the optimum AGD cut-point to separate the cows into short- and long-AGD groups was computed to be 148 mm with a sensitivity of 0.85, specificity of 0.41, and the Youden index value of 0.26. Accordingly, AGD was then classified into short AGD (≤148 mm; n = 115) and long AGD (>148 mm; n = 63).

To remove any potential bias from using the AGD cut-point established based on the predicted probability of pregnancy per artificial insemination (**P/AI**) to make associations with reproductive parameters of the same population of cows, AGD groups were also classified using the median AGD of 142 mm into short- (≤142 mm; n = 85) and long-AGD (>142 mm; n = 93). Dependent outcomes assessed were activity from the AAM (i.e., peak of activity and duration), follicle diameter at estrus, ovulation, diameter of CL on d 7 after estrus, and progesterone concentration at estrus and on d 7 after estrus. Generalized linear mixed regression procedure (GLIMMIX) in SAS was applied with the model consisting of the fixed effects of AGD, milk production, parity, and the interaction between AGD and parity, with cow as the experimental unit. For all models, only variables with $P < 0.15$ were retained in final models. Differences with $P \leq 0.05$ were considered significant and those between $P > 0.05$ and ≤0.10 were considered as a tendency.

RESULTS AND DISCUSSION

The central objective of the present study was to investigate some of the underlying physiological factors that may be contributing to the greater fertility reported in dairy cows with short AGD (Gobikrushanth et al., 2017; Akbarinejad et al., 2019; Grala et al., 2021) than in those with long AGD.

Descriptive Statistics

Of the 178 cows used in the study, after exclusions based on criteria described in the previous section, 112

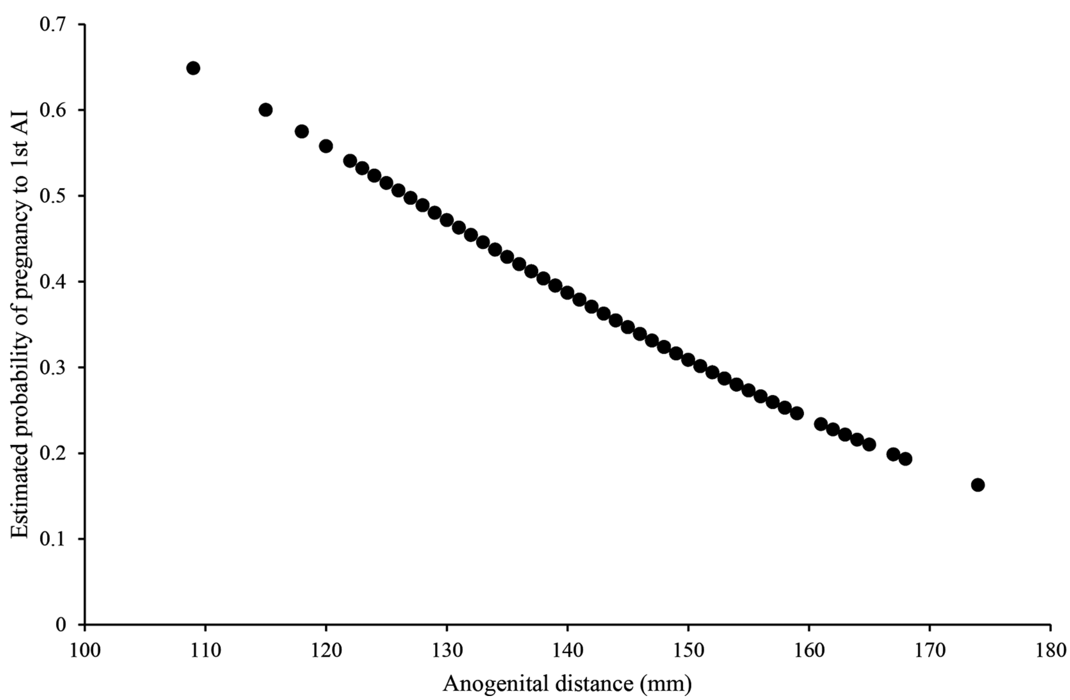

Figure 1. Scatter plot depicting the estimated probability of pregnancy at first AI plotted against anogenital distance in 178 dairy cows. An inverse relationship was found, with the odds of pregnancy at first AI declining by 3.5% for every 1-mm unit increase in anogenital distance ($P < 0.01$). The data presented in this figure are a subset (<4%) of a larger data set reported elsewhere (Carrelli et al., 2022).

Table 1. Distribution of cows with short and long anogenital distance (AGD) within parity when AGD was categorized based on 2 different cut-points[1]

Item	Short AGD (≤148 mm)		Long AGD (>148 mm)		Short AGD (≤142 mm)		Long AGD (>142 mm)	
	Primi (42)	Multi (73)	Primi (13)	Multi (50)	Primi (36)	Multi (49)	Primi (19)	Multi (74)
Mean ± SEM (mm)	133 ± 1.2	136 ± 0.9	152 ± 2.2	156 ± 1.1	131 ± 1.5	133 ± 1.0	150 ± 1.0	153 ± 0.9
Minimum, maximum (mm)	109, 148	118, 148	149, 157	149, 174	109, 141	118, 142	143, 157	143, 174

[1]The AGD was measured from the center of the anus to the clitoris and categorized into short- and long-AGD groups based on either the optimum cut-point predictive of pregnancy at first AI computed using the receiver operating characteristic curve analysis (short AGD: ≤148 mm vs. long AGD: >148 mm) or based on the median AGD (short AGD: ≤142 mm vs. long AGD: >142 mm); Primi = primiparous; Multi = multiparous; the numbers of cows appear in parentheses.

(63%) were pregnant at the time of AGD measurement with a mean (± SEM) gestational age of 86 ± 5 d (minimum, maximum; 0, 177). The distribution of short- and long-AGD cows within parity groups based on the 2 different AGD cut-points of 148 and 142 mm, and their corresponding mean, minimum, and maximum AGD are presented in Table 1. The mean (± SD) AGD in the present study was 142 ± 12.5 for all parities combined, which is 10 to 11 mm larger than previously reported for North American Holstein cows of mixed parities (Gobikrushanth et al., 2017; Carrelli et al., 2022). The reasons attributable to the increased AGD of cows in this herd may be the larger-than-average frame size of cows in this herd (personal communication from the herd manager) and lower-than-average herd replacement rate (27% vs. the provincial average of 47%) resulting in a greater proportion of third+ parity cows (49% vs. the provincial average of 35%). The proportions of multiparous and primiparous cows in the study were 69 and 31%, respectively.

AGD and Early Postpartum Estrous Activity

As hypothesized, AGD was associated with early postpartum estrous activity. Whereas a greater proportion of short-AGD cows had resumed estrous activity (≥1 estrus alert; $P \leq 0.03$) by 50 DIM, among the cows that were considered anestrous (no estrus alerts) during the same 50-d early postpartum period, a greater proportion ($P \leq 0.05$) was from the long-AGD group, regardless of the AGD cut-point used (Table 2). In addition, female rats of the short-AGD phenotype that were not exposed to testosterone propionate (0 mg/kg) during fetal life in utero had more estrous cycles and were 100% cyclic compared with females of the long-AGD phenotype that were exposed to 2 levels of testosterone propionate (1.5 and 2.5 mg/kg) treatments in utero, which manifested fewer estrous cycles and reduced cyclicity of 75 and 66%, respectively (Hotchkiss et al., 2007). A recent study by Borchardt et al. (2021) reported that cows with no estrous expression (determined by AAM) from 7 to 40 DIM had inferior reproductive performance compared with cows that displayed estrous activity during the corresponding time. Resuming ovarian cyclicity before the first AI, especially early in the lactation, has been linked to improved reproductive performance (Thatcher and Wilcox, 1973; Galvão and Santos, 2010) with one study (Bruinjé et al., 2017) reporting much greater probabilities of P/AI in primiparous and multiparous cows (odds ratios of 3.85 and 3.45, respectively) that had one or more estrous cycles before first AI compared with cows that had none.

AGD and Intensity of Estrous Expression

The intensity of estrous expression in the present study was determined using 2 indicators: first, a relative

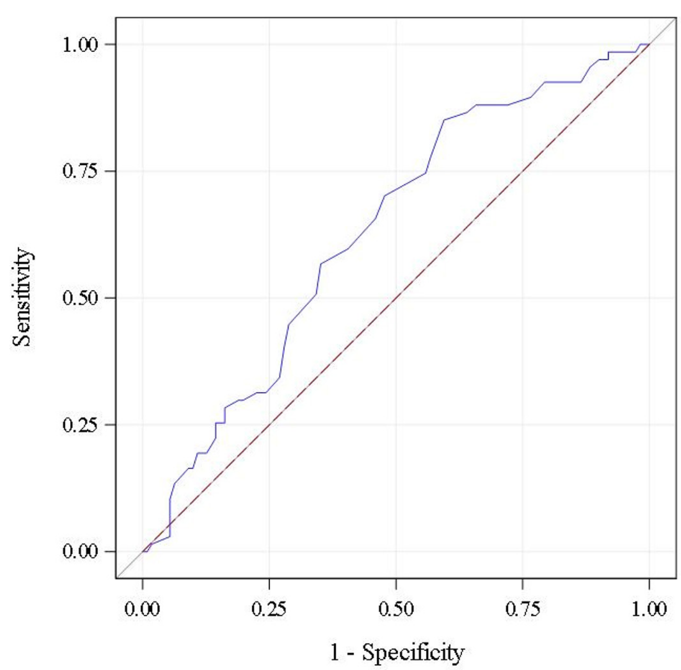

Figure 2. The receiver operating characteristic curve that determined the anogenital distance cut-point of 148 mm predictive of probability of pregnancy at first AI in 178 cows (area under the curve: 0.63; sensitivity: 85.1%; specificity: 40.5%; $P < 0.01$).

Table 2. Reproductive indices hypothesized to be associated with anogenital distance (AGD) of lactating Holstein cows after estrus alerts by an automated activity monitoring system; AGD was categorized based on 2 different cut-points[1]

Item	AGD cut-point of 148 mm				AGD cut-point of 142 mm			
	Short AGD	Long AGD	*P*-value	Int,[2] *P*-value	Short AGD	Long AGD	*P*-value	Int, *P*-value
Estrous activity up to 50 DIM[3]								
% Estrus (≥1 alert; n/n)	65.2 (75/115)	54.0 (34/63)	0.002	0.72	69.4 (59/85)	53.7 (50/93)	0.03	0.67
% Anestrus (no alert; n/n)	34.8 (40/115)	46.0 (29/63)	0.05		30.6 (26/85)	46.2 (43/93)	0.01	
Reproductive index after 60 DIM								
Follicle diameter[4] (mm)	19.8 ± 0.4	18.7 ± 0.5	0.08	0.44	20.2 ± 0.4	18.6 ± 0.5	0.01	0.20
% Ovulation by 24 h[5] (n/n)	33.8 (26/77)	35.0 (14/40)	0.31	0.23	36.1 (22/61)	32.1 (18/56)	0.77	0.31
% Ovulation by 48 h[5] (n/n)	90.9 (70/77)	75.0 (30/40)	0.03	0.35	90.2 (55/61)	80.3 (45/56)	0.07	0.19
% Ovulation by 7 d[5] (n/n)	96.1 (74/77)	82.5 (33/40)	0.01	0.60	97.0 (59/61)	85.7 (48/56)	0.04	0.71
CL diameter[6] (mm)	28.7 ± 0.5	28.0 ± 0.7	0.41	0.17	28.5 ± 0.6	28.2 ± 0.7	0.74	0.34
Progesterone at estrus[7] (ng/mL)	0.45 ± 0.03	0.60 ± 0.05	0.03	0.44	0.48 ± 0.07	0.62 ± 0.07	0.05	0.38
Progesterone 7 d after estrus[8] (ng/mL)	4.0 ± 0.2	3.1 ± 0.2	0.002	0.38	4.2 ± 0.2	3.3 ± 0.2	0.001	0.30

[1]The AGD was measured from the center of the anus to the clitoris and categorized into short- and long-AGD groups based on either the optimum cut-point predictive of pregnancy at first AI computed using the receiver operating characteristic curve analysis (short AGD: ≤148 mm vs. long AGD: >148 mm) or based on the median AGD (short AGD: ≤142 mm vs. long AGD: >142 mm).

[2]Int = AGD × parity interaction.

[3]Cows were considered to have been in estrus when at least one true estrus alert occurred from 10 to 50 DIM and considered anestrus when no estrus alert occurred during the same period.

[4]Diameter (mean ± SEM) of the putative preovulatory follicle at estrus in a subset of 117 cows. All cows of this subset were included in the analysis for the preovulatory follicle diameter, independent of the ovulatory response.

[5]Ovulation was determined (n = 117) by the disappearance of the preovulatory follicle at 24 and 48 h and on d 7 after estrus by the presence of a corpus luteum (CL) on the same ovary as the preovulatory follicle. Four cows ovulated after 48 h.

[6]The CL diameter (mean ± SEM) measured on d 7 after estrus.

[7]Mean (± SEM) concentration of progesterone at estrus.

[8]Mean (± SEM) concentration of progesterone on d 7 after estrus. Only cows that ovulated were included in the analysis for progesterone concentration on d 7 after estrus.

increase in activity at estrus, and second, the duration of estrus. Irrespective of the 2 AGD cut-points (148 and 142 mm) used, short-AGD cows had greater increase in activity than in long-AGD cows. When the 148 mm AGD cut-point was applied, the increased peak of activity at estrus in short- versus long-AGD cows (354 ± 19.6 vs. 258 ± 27.2% mean relative increase), however, was affected by an interaction ($P = 0.001$) between AGD and parity (Figure 3) in which multiparous long-AGD cows had lesser relative increase in activity than primiparous cows (217 vs. 386%, respectively). When cows were classified as short- and long-AGD using the median AGD of 142 mm as the cut-point, the above interaction was less evident ($P = 0.10$). The increased peak of activity at estrus in short- versus long-AGD cows were 345 ± 20.1 versus 280 ± 22.9%, respectively. Multiparous cows with long-AGD tended to have a lesser relative increase in activity at estrus than multiparous cows with short-AGD and a lesser increase in activity than primiparous cows of both long- and short-AGD groups. Thus, although the cows that were in the short AGD group presented an overall greater increase in activity at estrus than cows in the long-AGD group, the differences were due to the lesser relative increase in activity among multiparous cows of the long-AGD group.

Regardless of the AGD cut-point, cows in the short-AGD group had longer ($P < 0.001$) duration of estrous expression than cows in the long-AGD group. The differences in the duration of estrus in short- and long-AGD groups separated using the 148 mm cut-point are shown in Figure 4. The mean duration of estrus for short- and long-AGD cows separated using the median AGD (142 mm) were 11.8 and 9.8 h, respectively.

Many factors affect the intensity of estrous expression in lactating dairy cattle that include cow factors (e.g., body condition, milk production, lameness, hormone concentrations at estrus) and environmental factors (e.g., ambient temperature, cow comfort, barn design, footing). In a study using wild house mice, Drickamer (1996) reported that the occurrence of estrus was greater in female mice with short AGD (≤median AGD) than in those with long AGD (>median AGD) and females with short AGD also had a greater percentage of pregnancy (86 vs. 56%) than females with long AGD. In rabbits (Feknous et al., 2021), long-AGD females presented a greater rate of sexual receptivity, greater plasma testosterone concentrations, and displayed

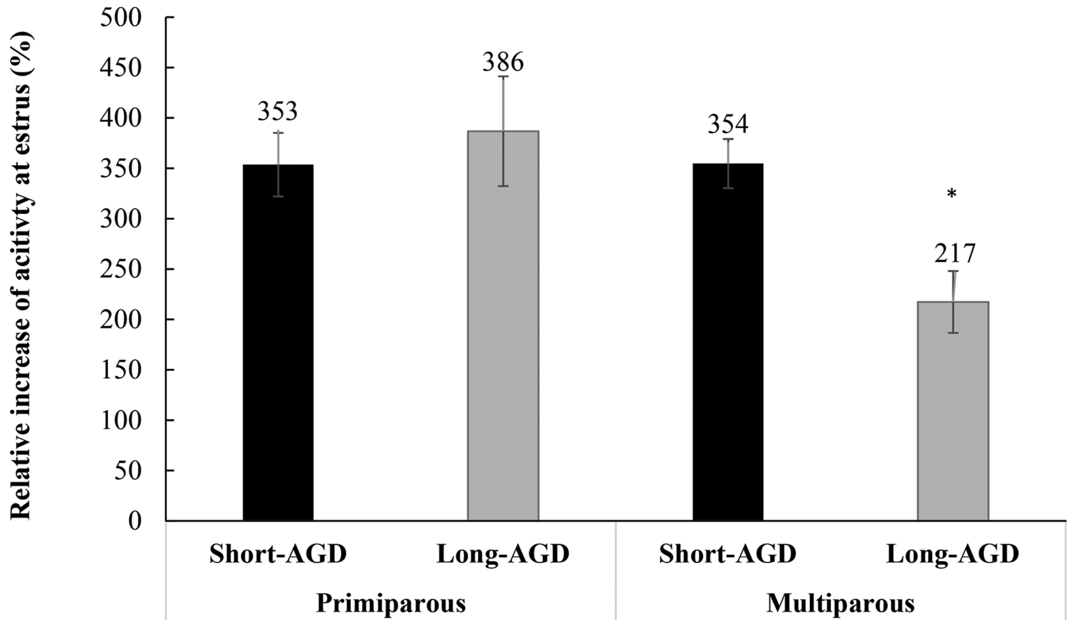

Figure 3. Interaction of anogenital distance (AGD) with parity (primiparous = first-lactation cows and multiparous = ≥second-lactation cows) and relative increase of activity at estrus alert. Anogenital distance was categorized as short (≤148 mm) and long (>148 mm) using the cut-point determined by the receiver operating characteristic curve analysis. Multiparous cows with long-AGD had a lesser relative increase in activity at estrus than multiparous cows with short AGD and a lesser increase in activity than primiparous cows of both long- and short-AGD groups ($P = 0.001$). Error bars denote SD.

more aggressive (male-like) sexual behavior than short-AGD females. Nevertheless, the number of implanted embryos was greater in female rabbits of short AGD (9.1 vs. 8.7) and the embryonic and fetal survival rates were also greater in short-AGD females resulting in a greater litter size at birth (9.0 vs. 7.8) when compared with long-AGD females.

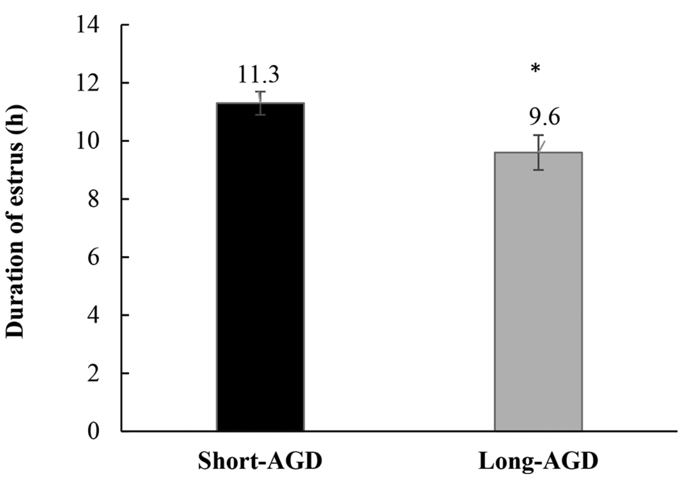

Figure 4. Duration of estrus (hours from onset to the end of each estrus alert) presented by anogenital distance (AGD) group. Anogenital distance was categorized as short (≤148 mm) and long (>148 mm) using the cut-point determined by the receiver operating characteristic curve analysis. Short-AGD cows had longer duration of estrus than long-AGD cows ($P < 0.01$). Error bars denote SD.

Detection of estrus is an essential component of a successful reproductive program in dairy herds that do not rely on programs that synchronize ovulation, because monitoring estrous behavior is important to determine the ideal time for AI and to forecast the approximate time of ovulation in dairy cattle. In response to increasing difficulties in detecting estrus, various technological advances have been made to improve the ability to detect estrus (Firk et al., 2002). Among these technologies are AAM systems that rely on a secondary characteristic of estrus, that is, an increase in physical activity observed in cows during estrus (Jónsson et al., 2011). Use of a variety of technologies that measure different cow traits (steps per hour, resting time, lying time, and so on) during estrus gives us the potential to use these data for phenotypic characterization and genetic selection.

AGD and Diameter of the Preovulatory Follicle, Ovulation, and CL Size

The mean (± SD) diameter of the preovulatory follicle at estrus, for both AGD groups combined, was 19.5 ± 3.7 mm. All cows subjected to ovarian ultrasonography (n = 117) were included in the analysis for the preovulatory follicle diameter, independent of their ovulatory response. Depending on the AGD cut-point applied, the diameter of the putative preovulatory follicle either differed ($P = 0.01$) or tended ($P = 0.08$) to differ between short- and long-AGD cows (Table 2) with the follicles being larger in short-AGD cows.

Larger preovulatory follicles in dairy cows have been associated with greater circulating estrogen concentrations (Bello et al., 2006; Lopes et al., 2007; Madureira et al., 2021) and more overt estrous behavior (Lyimo et al., 2000; Cummins et al., 2012; Madureira et al., 2021). In a study that used data from 130 cows (Bello et al., 2006), larger preovulatory follicles were associated with greater P/AI with evidence of a quadratic rather than a linear effect, and the maximum probability of pregnancy was associated with preovulatory follicles of ~16 mm in diameter. In a larger retrospective study (n = 1,048) with similar objectives, Colazo et al. (2015) did not find a significant association between follicle size (diameter) and P/AI. However, the association between follicle size measured at timed AI and the predicted probability of pregnancy loss between 32 and 60 d of gestation was significant with a greater probability of pregnancy loss in cows that ovulated follicles >20 mm. Based on the results of the latter studies, although larger preovulatory follicles may not necessarily equate to greater P/AI, the increased fertility in short-AGD cows described in the literature may be an indirect benefit derived through increased estrogen production in larger follicles resulting in greater intensity and duration of estrus and increased ovulatory responses.

We did not quantify estradiol concentrations in the present study; nonetheless, because of the greater intensity of estrous expression observed in short-AGD compared with long-AGD cows, it is plausible that short-AGD cows had greater circulating estrogen concentrations at estrus. Quantifying estrogen concentrations in the peripheral circulation during estrus or in the follicular fluid of preovulatory follicles to compare estrogen profiles of short- and long-AGD cows is warranted in future studies to further understand the differences in the ovarian physiology between AGD groups.

Overall, 34% (40/117), 86% (100/117), and 92% (107/117) of cows ovulated by 24 h, 48 h, and after 48 h (confirmed on d 7 after estrus), respectively. Whereas the proportions of cows that ovulated by 24 h did not differ between short- and long-AGD groups, a greater proportion of short-AGD cows ovulated by 48 h than long-AGD cows (Table 2). The proportion of total ovulation including cows that ovulated beyond 48 h after estrus based on confirmation of a CL by ultrasonography on d 7 after a true estrus alert was also greater in the short-AGD than in the long-AGD group (Table 2).

Notably, the occurrence of cumulative proportions of ovulation at 48 h and 7 d after estrus was greater by up to 16 and 13 percentage points, respectively, in short-AGD cows than in long-AGD cows. Moreover, depending on the AGD cut-point, the proportion of cows that failed to ovulate in the present study was 3 to 4% in the short AGD group versus 14 to 18% in the long-AGD group. In an earlier report, using cows from the same herd as the present study, an ovulation failure rate of 6.7% was reported in cows that had been in true estrus based on AAM alerts (Burnett et al., 2018). Whereas the 3 to 4% ovulation failure in short-AGD cows found in the present study falls well within the norms of this herd, the 4- to 5-fold greater ovulation failure in long-AGD cows suggests compromised ovulatory mechanisms in long-AGD cows. Whether reduced estradiol concentrations at estrus weaken the LH surge (Reames et al., 2011), leading to a greater proportion of failed ovulations in cows of the long-AGD group, remains to be explored and could form the basis for future research.

The CL diameter measured on d 7 after estrus did not differ between short- and long-AGD groups in the present study (Table 2) negating our hypothesis that short-AGD cows will develop larger CL. Although larger preovulatory follicles in dairy cows have been associated with the development of larger CL after ovulation (Cummins et al., 2012), this was not evident in the present study.

AGD and Progesterone Concentrations at Estrus and on d 7 After Estrus

Cows with short AGD had a lesser mean concentration of progesterone at estrus than in cows with long AGD, but mean progesterone concentration on d 7 after estrus was greater in short-AGD cows than in long-AGD cows. Only cows that had an ovulatory response (presence of CL on d 7 after estrus) were included in the analysis for progesterone concentration. Data for AGD and progesterone concentration at estrus and on d 7 after estrus are summarized in Table 2.

The importance and the dynamics of progesterone concentrations during critical time points of the estrous cycle, early embryonic development and pregnancy in dairy cattle, have been reviewed (Wiltbank et al., 2014). For instance, low progesterone concentrations at estrus and AI are associated with greater probability of pregnancy in dairy cows (Colazo et al., 2017; Bruinjé et al., 2019) with the chances of pregnancy greatly reduced ($P < 0.01$) when progesterone concentrations at AI exceeded 0.5 ng/mL (Colazo et al., 2017). The optimal progesterone concentration threshold at timed AI for P/AI at 32 d was ≤0.5 ng/mL, indicating the significance of having very low progesterone concentrations at AI for greater P/AI. The authors of the previous study found that none (0%) of the 58 cows that had >0.8 ng/mL progesterone concentration at AI conceived in their study, whereas the P/AI was in the range of 48 to 56% when progesterone concentrations were ≤0.5 ng/mL (Colazo et al., 2017). Similar results were found by Ambrose et al. (2015) and Madureira et al. (2021) associating low progesterone concentrations at estrus with greater P/AI. Madureira et al. (2021) also reported a strong association between low progesterone at AI and estrous expression as determined by the relative increase in activity by AAM. In this context, administering a low-dose natural prostaglandin (dinoprost, 10 mg) in conjunction with timed AI increased P/AI in lactating dairy cows (Ambrose et al., 2015), although such an effect was not demonstrable in a larger study of multiple herds (Sauls et al., 2018). Our present finding that short-AGD cows had lesser mean progesterone concentrations at estrus than in long-AGD cows is intriguing. This indicates that one of the physiological factors contributing to the enhanced fertility in short-AGD cows may be a superior luteolytic mechanism that ensures rapid and complete luteolysis, reducing progesterone concentrations below the optimum level (e.g., 0.5 ng/mL; Colazo et al., 2017) required for the highest probability of P/AI.

Contrary to the requirement for extremely low progesterone concentrations at estrus and AI, elevated progesterone concentrations 7 d after AI favor early embryonic development and pregnancy establishment. Supplemental progesterone treatment given to cows from d 5 to 9 (d 1 = AI) resulted in significant increases in trophoblast length and interferon-tau concentrations in the uterus (Mann et al., 2006) and treatments that increased progesterone concentrations after AI have increased conception rates but not consistently (Stevenson et al., 2007). Many studies have demonstrated a strong association between progesterone concentrations measured on d 5 to 7 after AI and P/AI (Stronge et al., 2005; Bruinjé et al., 2019; Madureira et al., 2021). Moreover, cows with high genetic merit for fertility had 34% greater progesterone concentrations in circulation than in cows with lesser genetic merit for fertility from 5 to 13 d of the estrous cycle (Cummins et al., 2012). Therefore, our finding that mean progesterone concentration on d 7 after estrus was greater ($P < 0.01$) in short-AGD cows than in long-AGD cows is of importance, which may be another factor contributing to the improved fertility in short-AGD cows. The present study was not designed to probe deeper into the underlying processes, but the findings that progesterone concentrations on d 7 after estrus were greater in the peripheral circulation despite the lack of an increase in CL size imply that short-AGD cows may have better steroidogenic mechanisms in place for increased progesterone production or reduced progesterone clearance. Again, this is an area for future research.

Anogenital distance is the result of increased exposure to androgens during prenatal development of the fetus in rodents (Hotchkiss et al., 2007), and Gobikrushanth et al. (2017) reported that AGD is independent of postnatal factors such as age and height in dairy cows. High exposure to androgens in utero results in androgenization of the female reproductive system, manifested by increased AGD, which has been associated with reduced fertility in rodents (Bánszegi et al., 2012), humans (Mendiola et al., 2012), and cattle (Gobikrushanth et al., 2017; Grala et al., 2021; Carrelli et al., 2021). The primary source of androgens in pregnant cows is the placenta (Mongkonpunya et al., 1975) and concentrations of androgens in maternal circulation can be highly variable among individual animals, even in cows carrying female fetuses (Gaiani et al., 1984).

Understanding the physiological reasons behind the variance in AGD in dairy cattle will require more research into the relationship between maternal androgen concentrations and AGD. That being said, AGD is a low-cost morphometric measure with an inverse relationship with fertility in cattle reported from diverse populations of cattle (Gobikrushanth et al., 2017; Akbarinejad et al., 2019; Battista, 2019; Carrelli et al., 2021; Grala et al., 2021; Carrelli et al., 2022) that can be measured with reliability early (6 mo of age) in life (Rajesh et al., 2022), and is moderately heritable ($h^2 = 0.37$; Gobikrushanth et al., 2019). On the contrary, Roxström et al. (2010) evaluated the heritability of the frequency and intensity of estrous expression in cattle and reported a low heritability ($h^2 = 0.01$ to 0.03) showing the limited efficacy of the latter phenotype for genetic selection. In addition, days from calving to first "high activity alert," a characteristic that can be associated with days from calving to first estrus, also has been shown to have relatively low heritability ($h^2 = 0.12$ to 0.18) and repeatability (Løvendahl and Chagunda, 2009), making AGD a new phenotype with potential for use in genetic improvement of fertility. Genomic regions of interest for AGD have been identified in some studies (Gobikrushanth et al., 2019; Grala et al., 2021) although none of the genetic markers or the associated genes are known to be related to fertility in cattle.

In summary, significant differences were found in the interval from calving to resumption of postpartum estrous activity, duration of estrus, ovulatory response, and circulating progesterone concentrations (at estrus and on d 7 after estrus) between short- and long-AGD cows, all favoring short-AGD cows. The size of the preovulatory follicle was larger in short-AGD cows, but the CL size did not differ between the 2 AGD groups.

The characteristics of short-AGD cows in this study are all in line with properties associated with greater fertility, and thus further support the inverse relationship between AGD and fertility. This study has shed light on several physiological differences between short- and long-AGD cows and has identified factors that contribute to improved fertility in short-AGD cows. To our knowledge, this is the first study to describe the relationship between AGD, estrus characteristics, and ovarian function in dairy cattle and we expect the present findings to pave the way for future studies.

ACKNOWLEDGMENTS

This study was supported by contributions from the Dairy Research Cluster Initiative II (Dairy Farmers of Canada, Agriculture and Agri-Food Canada, the Canadian Dairy Network and the Canadian Dairy Commission; Ottawa, ON, Canada), Strategic Research and Development Program of Alberta Agriculture and Forestry (Edmonton, AB, Canada), Alberta Milk (Edmonton, AB, Canada), and WestGen Endowment Fund (Abbotsford, BC, Canada). The authors acknowledge LactaNet (Guelph, ON, Canada) and Holstein Canada (Brantford, ON, Canada) for their in-kind support. J. E. Carrelli received a Canadian Dairy Commission Graduate Scholarship. Partial funding was also provided by the Natural Sciences and Engineering Research Council, Discovery Grant (Ottawa, ON, Canada). The authors are thankful to the staff, students, and interns of the UBC Dairy Education and Research Center (Agassiz, BC, Canada) for their assistance during this research project. The authors have not stated any conflicts of interest.

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