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

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ABSTRACT

The inverse association between anogenital distance (AGD; the distance from the center of the anus to the base of the clitoris) and fertility, its moderate heritability, and high variability reported in dairy cattle make AGD a promising candidate for further exploration as a reproductive phenotype. In addition to heritability, repeatability (i.e., consistency in measurements taken at different time points) is important for a reproductive phenotype to be considered useful in genetic selection. Therefore, our primary objective was to determine the repeatability of AGD from birth to breeding age (≈16 mo) in Holstein heifer calves, and during different stages of the estrous cycle, gestation, and lactation in Holstein cows. We also determined the associations among AGD, height (at the hip), and body weight (BW) at birth. In calves (n = 48), we recorded BW (kg) and height (cm) at birth and measured AGD (mm) at approximately 0, 2, 6, 9, 12, and 16 mo of age. In cows, AGD was measured at different stages of the estrous cycle (proestrus, estrus, metestrus and diestrus; n = 20), gestation (30, 90, 180, and 270 d; n = 78), and lactation (30–300 d in milk in 30-d increments; n = 30). Calf height and BW at birth had a weak positive association with AGD at birth. The AGD increased linearly from birth to breeding age, but there was no association between the AGD at birth and at breeding age in heifers. Although any 2 consecutive AGD measurements were correlated, 6 mo was the earliest age at which AGD was moderately correlated (r = 0.41) with that of breeding-age heifers. The AGD was neither influenced by the different stages of estrous cycle nor lactation and remained highly repeatable (r ≥ 0.95). Although AGD measurements at 30, 90, and 180 d of gestation (126.9, 126.7, and 127.7 mm, respectively) were strongly correlated (r ≥ 0.97) with each other, AGD at 270 d of gestation (142.8 mm) differed from AGD at all earlier stages of gestation. In summary, AGD measured at birth did not reflect AGD at breeding age in heifers, but AGD measurements in cows had high repeatability at all stages of the estrous cycle, gestation, and lactation, except at 270 d of gestation. Therefore, AGD could be measured reliably at any of the aforesaid physiological states in cows due to its high repeatability, except during late gestation. The earliest gestational stage when pregnancy-associated increase in AGD occurred, however, could not be definitively established in the present study.

Key words: reproductive phenotype, estrous cycle, gestation, lactation

INTRODUCTION

A novel reproductive phenotype that is strongly associated with fertility outcomes and has high variability, heritability, and repeatability could be considered as a fertility trait for genetic selection to improve fertility in dairy cattle (Berry et al., 2014; Miglior et al., 2017). In addition, it should be clearly defined, consistently recorded, easily obtained at a low cost, and measurable early in life (Shook, 1989).

Conventional fertility traits such as interval traits (e.g., days open), binary traits (e.g., pregnant vs. nonpregnant), and count traits (e.g., services per conception) all have low heritability estimates ranging from 0.02 to 0.04 and are affected by management decisions (e.g., voluntary waiting period) or recording errors (Berry et al., 2014), resulting in slow genetic progress. Emerging fertility traits such as commencement of luteal activity postpartum have greater heritability (0.16; Veerkamp et al., 2000) than that of conventional traits. Antral follicle count, an anatomically manifested trait, had a moderate heritability of 0.31 (Walsh et al., 2014) and high repeatability of 0.86 (Koyama et al., 2018) to 0.95 in dairy cattle (Burns et al., 2005). Antral follicle count, however, is difficult to obtain as it requires skilled personnel and specialized equipment, and its association with fertility differs between *Bos taurus* and *Bos indicus* cattle (Morotti et al. (2017). Other physiologic traits, such as hormone concentrations, not only require

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frequent monitoring but are also time-consuming, often invasive in nature, and expensive to quantify, making it difficult to implement in commercial dairy operations. The reproductive tract scoring system first reported in beef cattle has a heritability estimate of 0.32 (Anderson et al., 1991) and is positively associated with pregnancy per AI in beef (Anderson et al., 1991) and dairy cattle (Young et al., 2017; Madureira et al., 2020). The latter trait, however, is subjective, labor-intensive, and has low repeatability (Holm et al., 2009; Gutierrez et al., 2014). Therefore, research on novel reproductive phenotypes is considered important for selecting cows to improve fertility.

Anogenital distance (**AGD**; the distance from the center of the anus to the genitals) is a relatively new reproductive phenotype that has been of research and clinical interest in many mammalian species, and more recently, in dairy cows (Gobikrushanth et al., 2017). Excessive exposure of female fetuses to testosterone during prenatal development lengthens AGD in rats (Wolf et al., 2004). Ewes treated with i.m. injections of 100 mg of testosterone propionate twice weekly from 30 to 90 d of gestation gave birth to both male and female lambs with long AGD compared with lambs born to control ewes (Manikkam et al., 2004). The placenta is the major source of androgen to the bovine fetus (Mongkonpunya et al., 1975), and its concentrations are highly variable (Kim et al., 1972). Regardless of the sex of the fetus carried, mean (± SEM) serum testosterone concentrations were greater in pregnant cows (330 ± 86 pg/mL) than in nonpregnant cows (43 ± 3 pg/mL) with a great degree of variation (Kim et al., 1972). Studies that compared the reproductive outcomes between short- and long-AGD females reported that short-AGD females had indicators of better fertility than those with long AGD (i.e., earlier attainment of puberty in rats; Zehr et al., 2001), larger litter size, and heavier pups in rabbits (Bánszegi et al., 2012); additionally, studies show improved overall reproductive performance in gilts; that is, greater success to the first 4 breeding attempts (Drickamer et al., 1997).

Gobikrushanth et al. (2017) recently characterized AGD in Canadian Holstein cows and reported a greater pregnancy per AI and cumulative pregnancy risk by 250 DIM in first- and second-parity cows with short AGD (≤127 mm) than in those with long AGD (>127 mm). Other studies have also reported such inverse associations between AGD and indices of fertility in dairy cattle (Akbarinejad et al., 2019; Carrelli et al., 2021, 2022; Grala et al., 2021). A heritability estimate of 0.37 was later reported for AGD in Irish Holstein-Friesian cows (Gobikrushanth et al., 2019).

Repeatability is an indication of within-individual consistency of measurements. Only traits that are consistent within the same individual but have variability among individuals in a population can respond well to genetic selection (Boake, 1989). Therefore, repeatability, often determined as Pearson correlation of coefficient (Wolak et al., 2012), is an important criterion for a reproductive phenotype before it could be considered as a fertility trait for genetic selection. In addition, it should have strong association with fertility and high heritability.

We hypothesized that (1) a positive correlation exists between AGD of a newborn calf and that of breeding-age heifers; thus, AGD at birth could be used to predict the AGD of breeding-age heifers; and (2) AGD is not affected by physiological states of life, that is, stages of the estrous cycle, gestation, and lactation in adult cattle. Therefore, the main objectives were to determine if AGD measured at an early age could predict AGD at breeding age (~16 mo) in heifers, and if AGD is affected by different physiological states such as the stages of the estrous cycle (proestrus, estrus, metestrus, diestrus), gestation, and lactation in cows. A secondary objective was to determine the associations among AGD, height, and BW at birth in the newborn calf.

MATERIALS AND METHODS

Animals and Experimental Design

The studies were conducted using Holstein cattle at the University of Alberta's Dairy Research and Technology Center, a 146-cow tiestall barn located in Edmonton, Alberta, Canada. Calves were reared indoors in individual pens from 0 to 21 d of age, and in group pens equipped with automatic milk feeders from 22 to 60 d of age, when weaning occurred. After weaning, calves were moved to outdoor dry-lot pens with homogeneous age groups housed together. Calves were raised on milk, with ad libitum access to concentrates and chopped hay, until weaning. For postweaning diets, calves were offered free-choice alfalfa hay and a mash supplement containing millrun, soybean hulls, malt sprouts, and ground wheat grain as the main ingredients. At approximately 13 mo of age, heifers were moved into a breeding pen and placed on a diet containing barley and oat silage and free-choice alfalfa hay. Mineral supplements were given from 7 mo of age onward, and unrestricted access to clean drinking water was provided at all stages. From weaning until approximately 8 wk before the expected date of first calving, heifers were housed at the Roy Berg Research Ranch in Kinsella, Alberta. Cows were housed individually in tiestalls provided with rubber mattresses covered with a layer of wood shavings (changed daily) and were let out for exercise for up to 3 h on weekdays. Cows were ad libitum fed a TMR of alfalfa silage, barley silage, chopped hay, and concentrates once daily (0800 h), formulated according to the guidelines of the National Research Council (2001) to meet the requirement of a 650-kg lactating cow producing 45 kg of milk per day. Cows always had free access to drinking water and were milked twice daily in tiestalls. Calves and cows were cared for according to the guidelines of the Canadian Council on Animal Care (2009), and all animal use protocols were approved by the Animal Care and Use Committee for Livestock, University of Alberta (protocol no. 00002883).

All the studies were prospective longitudinal studies with repeated measures over time. Within each objective, we measured the same set of animals at different time points, and the change in AGD in the same animal over time was the variable of interest. Unless specified otherwise, means (± SD) are presented. Sample size requirements for each study were determined by a priori power analysis (version 20.006; MedCalc Software Ltd.).

AGD from Birth to Breeding Age

We defined AGD as the distance from the center of the anus to the base of the clitoris and measured it using 15.2-cm stainless steel digital calipers (Procise, The Innovak Group) as previously described by Gobikrushanth et al. (2017). Anogenital distance was measured 3 consecutive times for each animal by resetting the calipers to zero between each measurement, and the mean of the 3 measurements was used. All AGD measurements were taken by the same individual to minimize errors. The height at the hip (from the ground to the calf's rump, above the hip bones) was measured using an aluminum livestock measuring stick (Jeffers Livestock) immediately after the first AGD measurement. Both height and AGD were measured by the same individual in all animals, with a second individual assisting to restrain calves. The date of birth and BW of calves at birth were obtained from the herd records.

Forty-eight calves were enrolled in the study with AGD measurements obtained in the first week (0 mo) of birth (3.3 ± 2.1 d), then at 2 mo (62.5 ± 4.3 d), 6 mo (184.6 ± 12.4 d), 9 mo (270.9 ± 9.3 d), 12 mo (359.9 ± 10.4 d), and at breeding age or approximately 16 mo (489.0 ± 31.9 d).

AGD at Different Stages of the Estrous Cycle

Twenty cows in their early lactation (33.3 ± 10.6 DIM) were enrolled and subjected to transrectal ultrasonography using a scanner (Aloka 500, Aloka Co Ltd.) equipped with a 7.5-MHz linear array transducer to determine ovarian cyclicity indicated by the presence of at least 1 corpus luteum (**CL**). Upon confirming cyclicity, cows were subjected to estrous synchronization using an intravaginal insert (controlled internal drug release device; **CIDR**) containing 1.38 g of progesterone (EAZI-BREED CIDR, Zoetis) and 100 μg (i.m.) of GnRH (gonadorelin acetate; Fertiline, Vetoquinol N. A. Inc.) concurrently. Upon CIDR removal 7 d later, cows received 500 μg (i.m.) of cloprostenol (Estrumate, Merck Intervet Corp.) followed by a second dose 24 h later. Ovaries were scanned by ultrasonography concurrent with each dose of cloprostenol, and thereafter at 48-h intervals, through one complete estrous cycle spanning 2 consecutive ovulations, and major ovarian structures were documented. Anogenital distance was measured in all cows at the same frequency as ovarian ultrasonography throughout the estrous cycle. Blood samples were collected from a coccygeal vessel into evacuated tubes containing sodium heparin (Vacutainer, BD Life Sciences) on alternate days, immediately before ovarian ultrasonography, throughout the cycle to quantify progesterone. Samples were centrifuged at 1,500 × g for 20 min at 4°C no later than 30 min after collection, and then plasma was harvested and kept frozen at −20°C until assayed.

Plasma concentrations of progesterone were quantified at the Endocrine Services Laboratory (Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, SK, Canada) using a commercial radioimmunoassay kit (ImmuChem Progesterone^{125} kit; ICN Pharmaceuticals, Inc.). All samples were quantified in a single assay with intra-assay coefficients of variations of 7.1% for low- (mean, 0.9 ng/mL), 12.6% for medium- (mean, 5.2 ng/mL), and 6.7% for high-reference samples (mean, 12.5 ng/mL), respectively, and assay sensitivity was 0.15 ng/mL.

The average estrous cycle length in the present study was 23.4 ± 2.7 d. The proestrus, estrus, metestrus, and diestrus stages were determined retrospectively based on established norms for the length of each stage in a normal estrous cycle (Senger, 2005), CL dynamics, and plasma concentrations of progesterone (Henricks et al., 1971; Kastelic et al., 1990; Sartori et al., 2004). As the start of estrus could not be precisely determined in all cases, the day an ovulation was confirmed was considered metestrus, and the preceding sampling day was considered estrus. Based on these criteria, the mean concentrations of progesterone and CL sizes for proestrus, estrus, metestrus, and diestrus stages were 0.4 ± 0.8, 0.0 ± 0.0, 0.1 ± 0.2, and 3.5 ± 1.4 ng/mL, and 18.2 ± 3.6, 9.0 ± 6.5, 11.2 ± 7.1, and 24.1 ± 4.1 mm, respectively.

AGD at Different Stages of Gestation and Lactation

Seventy-eight cows confirmed pregnant at 30 to 32 d post-AI were enrolled in the gestational stages of this study. The AGD measurements were obtained at approximately 30 (36.6 ± 3.2), 90 (98.6 ± 5.0), 180 (187.9 ± 5.1), and 270 (270.5 ± 4.4) d of gestation.

Thirty lactating cows were enrolled in the lactational stages of this study; AGD measurements were obtained at approximately 30 (33.8 ± 2.3), 60 (64.9 ± 3.2), 90 (94.2 ± 3.2), 120 (124.7 ± 3.0), 150 (154.9 ± 4.2), 180 (184.1 ± 3.6), 210 (212.8 ± 3.7), 240 (242.4 ± 3.6), 270 (272.9 ± 5.7), and 300 (304.2 ± 6.7) DIM.

Statistical Analysis

Data were analyzed using SAS version 9.4 (SAS Institute Inc.). The descriptive statistics such as mean, standard deviation, minimum, and maximum for the days of AGD measurements, AGD, and calf height and BW at birth, as well as normality for AGD in each of the 4 objectives, were determined using the UNIVARIATE procedure of SAS. The relationships among AGD, BW, and height at hip at birth were tested by linear regression model using the REG procedure of SAS. The repeatability of AGD between different stages of the estrous cycle, gestation, and lactation was determined by Pearson correlation coefficient (r; −1 to +1) using the CORR procedure of SAS. The difference in AGD among different ages and stages of estrous cycle, gestation, and lactation were determined by MIXED procedure of SAS (results presented as LSM ± SEM). Anogenital distance was modeled against the fixed effects of age (calf or heifer study) and parity (in the parous cow studies); stages of the estrous cycle, gestation, or lactation; and their interactions. The interaction term was excluded from the model because no interactions ($P >$ 0.10) were evident. The experimental unit (i.e., heifer or cow) nested within age or stage of estrous cycle, gestation, or lactation was included as a repeated statement because AGD was measured repeatedly on the same animal at different time points. The differences in least squares means were tested using the PDIFF multiple comparison test and declared significant if $P \leq$ 0.05 and considered a tendency if $P > 0.05$ but ≤ 0.10.

RESULTS AND DISCUSSION

Anogenital distance measurements differed ($P <$ 0.01) by age from birth until approximately 16 mo, and were normally distributed in heifer calves and within the stages of the estrous cycle, gestation, and lactation in mature cows. Similarly, AGD was normally distributed in studies with larger numbers of cattle (Gobikrushanth et al., 2017; Akbarinejad et al., 2019; Carrelli et al., 2021).

Anogenital distance was positively and moderately associated with calf BW at birth; however, only 8% of the variation in AGD was explainable by changes in BW at birth (Figure 1a). The AGD tended ($P = 0.06$) to be positively associated with height within a week of birth, but only 7% of the variation in AGD could be attributed to changes in height (Figure 1b). In addition, AGD increased ($P < 0.05$) as the calf grew and reached breeding age (Figure 2), following an isometric growth pattern. In other words, the increase in AGD was in proportion to body size, and the relative growth was presumably at a constant rate. Weak associations were previously reported between AGD and hip height (r = 0.20; $P < 0.01$) and age (r = 0.30; $P < 0.01$) in Canadian Holstein cows (Gobikrushanth et al., 2017) and between AGD and BW (r = 0.32; $P < 0.01$) and BCS (r = 0.14; $P < 0.01$) in Irish Holstein cows (Gobikrushanth et al., 2019). However, in infants, birth weight and length (cm) were strongly correlated with AGD (Sathyanarayana et al., 2010). Similarly, in another human study (Thankamony et al., 2009), a positive association with AGD and birth weight was observed in a linear regression model.

Anogenital distance at birth was not indicative of the adult AGD in the present study. The youngest age at which the AGD was associated with the AGD at breeding age was 6 mo (r = 0.41, $P = 0.05$), but the measurements of AGD between adjacent age intervals were moderately correlated (r = 0.33–0.62; Table 1). In a human study (Priskorn et al., 2018), a significant but low correlation (r = 0.19, $P = 0.02$) was found between AGD at 3 and 18 mo of age in female infant. In another study, AGD increased rapidly from birth to 12 mo of age in both male and female infants with only a minor increase after the first year until 24 mo of age (Papadopoulou et al., 2013). Similarly, although the increase in AGD was linear and relatively fast in heifers from birth to 16 mo of age in the current study, any further increase in AGD as an influence of parity was much less, albeit significant, based on data available from the parous cows used in the present study and as reported in previous studies (Gobikrushanth et al., 2017, 2019; Carrelli et al. 2022).

Our results suggested that AGD at 6 mo could have some predictive value for adult AGD. Although the sample size of 48 heifer calves was determined by a priori power analyses, only 22 of the 48 heifers completed the study for reasons beyond our control (i.e., study disruption due to the COVID-19 pandemic situation), and AGD measurements at all ages from birth to 16 mo were available only in 20 heifers. Therefore, these findings must be confirmed in the future with

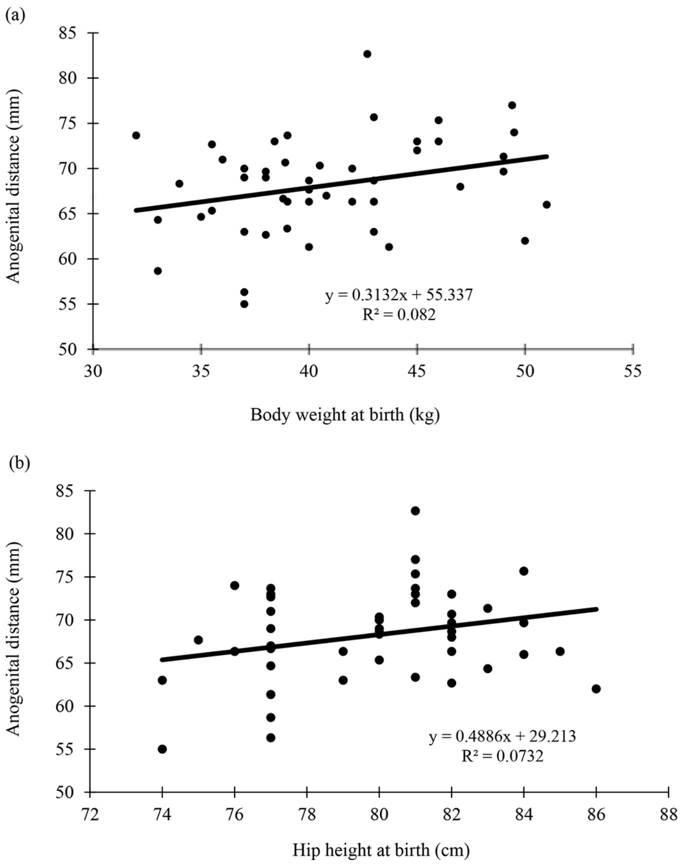

Figure 1. Associations between anogenital distance and (a) calf BW at birth ($R^2 = 0.08$; $P = 0.04$), and (b) hip height at birth ($R^2 = 0.07$; $P = 0.06$) in 48 heifer calves.

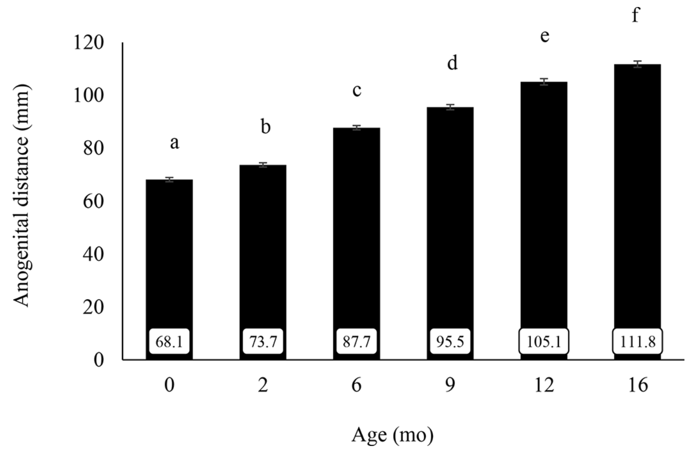

Figure 2. Bar chart showing the mean (± SEM) anogenital distance (AGD) measurements of heifers at different ages from 0 to 16 mo. Means with different letters (a–f) differ ($P < 0.05$). The numbers at the base of each bar indicate mean AGD in millimeters.

a larger number of heifers. We found no association between AGD at 0 mo and at 6 mo of age or later, except a tendency ($P = 0.07$) for an association at 9 mo (Table 1). The most rapid rate of growth in reproductive organs occurs between 7 and 10 mo of age in Holstein heifers, in preparation for puberty (Desjardins and Hafs, 1969). Because the age at onset of puberty varies greatly among heifers, with puberty occurring as early as 6 mo of age in some instances (Kinder et al., 1995; Bruinjé et al., 2021), the possible differences in puberty-associated changes in the external genitalia might have contributed to a greater variation in AGD at 6 mo compared with that at 0 mo of age.

Table 1. Pearson correlation coefficients denoting the associations of anogenital distance (AGD) among the different stages (0–16 mo of age) of development in Holstein heifers (no. of heifers given in parentheses)[1]

Age in mo	0	2	6	9	12	16
0	1.00 (48)					
2	0.57 <0.01 (48)	1.00 (48)				
6	−0.09 0.95 (46)	0.33 0.02 (46)	1.00 (46)			
9	0.32 0.07 (32)	0.3 0.08 (32)	0.54 <0.01 (32)	1.00 (32)		
12	0.24 0.28 (22)	0.35 0.09 (22)	0.36 0.09 (22)	0.62 <0.01 (21)	1.00 (22)	
16	0.27 0.21 (22)	0.32 0.14 (22)	0.41 0.05 (22)	0.31 0.16 (21)	0.62 <0.01 (20)	1.00 (22)

[1]Correlation coefficients (r) of AGD by age (mo) are shown in the top row for each age and associated P-values are given below the r values.

Anogenital distance was not influenced by the different stages of the estrous cycle (Figure 3; $P = 0.99$) but was strongly correlated (Table 2; r > 0.98) and thus considered highly repeatable within the same animal. Similarly, Barrett et al. (2015) measured AGD in women once each during early follicular phase, mid-cycle, and the luteal phase, repeated over 3 menstrual cycles, and found that AGD did not differ across stages of the cycle. In contrast, Dušek and Bartoš (2012) reported that AGD in female mice was influenced by the stages of the estrous cycle (LSM ± SE for proestrus, metestrus, and diestrus were 7.2 ± 0.1, 7.2 ± 0.1, 7.3 ± 0.1 mm, respectively; $P < 0.05$), although the mean AGD differed by only 0.1 mm. Nevertheless, the repeatability of AGD measurements in the latter study was relatively high (r > 0.66). Estrogen is the major hormone found at a greater concentration during the follicular phase of the estrous cycle in cattle, which gradually increases from 3 to 10 pg/mL during proestrus and exceeds 10 pg/mL at the onset of estrus with a nondetectable concentration of progesterone (Henricks et al., 1971). Mild swelling of the vulva and reddening of the vulvar mucosa are commonly observed in cows in estrus (Diskin and Sreenan, 2000); therefore, we hypothesized that swelling of the vulva would alter the AGD measurement taken at estrus, as observed by Dušek and Bartoš (2012) in estrous mice, compared with other stages of estrous cycle. Our hypothesis was not supported as we found no evidence of any significant alteration in AGD during estrus compared with other stages of the estrous cycle.

The AGD was affected by the stages of gestation, with AGD measured at late gestation (270 d) being greater than those measured at 30, 90, and 180 d of gestation (Figure 4, Table 3). The tissue around the perineal region becomes edematous with the progression of mild swelling of the vulva several days before calving to a pronounced swelling of the vulva at impending parturition, which aids in ease of parturition (Berglund et al., 1987). Relaxin and estrogen in the placenta of cows have a major role in the relaxation of pubic ligaments and preparation of the maternal birth canal for parturition (Schuler et al., 2018). The correlations of AGD among 30, 90, and 180 d of gestation were all high (r > 0.97), but were only moderate (r > 0.63) with AGD at 270 d of gestation. Although the vulvar enlargement during impending parturition likely altered the AGD at 270 d of gestation, it remained moderately correlated with the AGD at previous stages of gestation. Because results indicated a significant increase in AGD at 270 d of gestation, we recommend that AGD measurement be avoided during late gestation. In this regard, AGD during the early postpartum period, say the first 2 wk, is also likely to be affected by edema and inflammation resulting from parturient trauma. Given this, AGD measurement must be avoided in the 14-d periods immediately before and after calving, as reported by Gobikrushanth et al. (2017). Moreover, despite the strong evidence presented in this study that AGD was not altered up to 180 d of gestation, our study was not designed to record AGD measurements between 180 and 270 d of gestation. Therefore, we cannot be certain that AGD was not affected during that (180–270 d) window. It is possible that gradual increments in AGD began during this period culminating in the significant increase documented at 270 d of gestation. Future studies must document any AGD changes that may occur from 180 to 270 d of gestation to conclusively determine the earliest gestational stage when pregnancy-associated increase in AGD becomes evident in cattle.

The stages of lactation at 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 DIM did not affect AGD (Figure 5). The correlation among AGD measured at different stages of lactation was very high (Table 4; r > 0.95). As

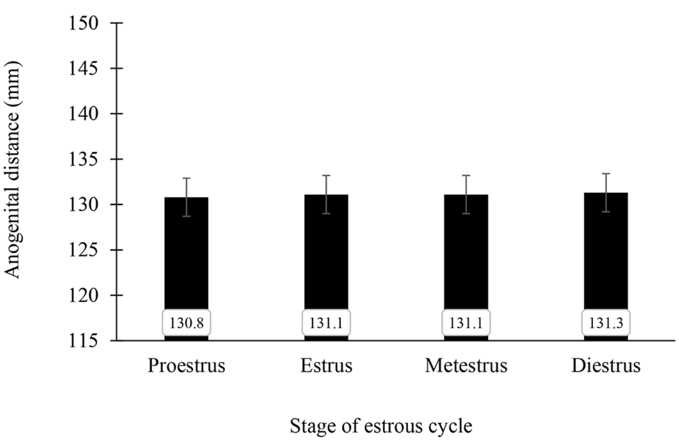

Figure 3. Bar chart showing the LSM (± SEM) of anogenital distance (AGD) measurements at different stages of the estrous cycle. Means did not differ ($P = 0.99$). The numbers at the base of each bar indicate mean AGD in millimeters.

Table 2. Pearson correlation coefficients denoting the associations of anogenital distance (AGD) among the different stages of the estrous cycle (n = 20 at each stage) in Holstein cows[1]

Stage of cycle	Metestrus	Diestrus	Proestrus	Estrus
Metestrus	1.00			
Diestrus	0.99	1.00		
	<0.01			
Proestrus	0.98	0.98	1.00	
	<0.01	<0.01		
Estrus	0.98	0.98	0.98	1.00
	<0.01	<0.01	<0.01	

[1]Correlation coefficients (r) of AGD by stage of estrous cycle are shown in the top row for each stage and associated P-values are given below the r values.

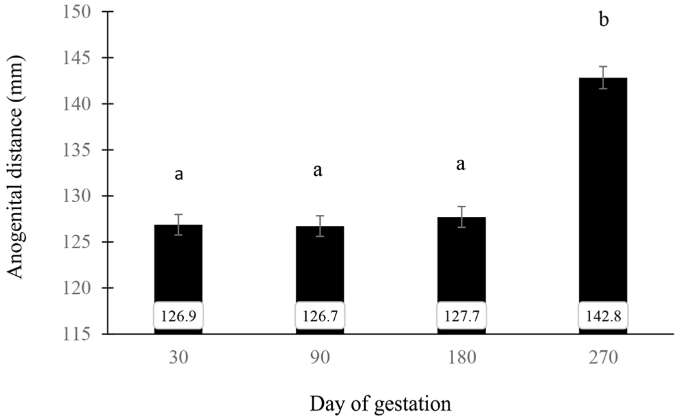

Figure 4. Bar chart showing LSM (±SEM) anogenital distance (AGD) measurements in mm at different stages of gestation. Means with different letters (a, b) differ ($P < 0.05$). The numbers at the base of each bar indicate mean AGD in millimeters for the corresponding stage of gestation.

Table 3. Pearson correlation coefficients denoting the associations of anogenital distance (AGD) measurements among the different stages (30, 90, 180, and 270 d) of gestation in Holstein cows (no. of cows appear in parentheses)

Day of gestation	30	90	180	270
30	1.00 (79)			
90	0.97 <0.01 (79)	1.00 (79)		
180	0.96 <0.01 (78)	0.97 <0.01 (78)	1.00 (78)	
270	0.62 <0.01 (69)	0.62 <0.01 (69)	0.63 <0.01 (68)	1.00 (69)

[1]Correlation coefficients (r) of AGD by day of gestation are shown in the top row for each stage of gestation and associated P-values are given below the r values.

hypothesized, AGD did not change during the different stages of lactation. These results implied that AGD could be measured at any phase of lactation.

The possible confounding effects of the different physiological states on each other (for example, stages of lactation confounded with stages of estrous cycle or with stages of gestation) were not considered in this study because AGD was not affected by any of the physiological states, with the only exception being late (270 d) gestation. The mean (± SD) stage of gestation when cows assigned to the lactational stages study reached 300 DIM (stage of last AGD measurement) was 164 ± 63 d, which indicated that the stages of gestation could not have been a confounder in the lactation study.

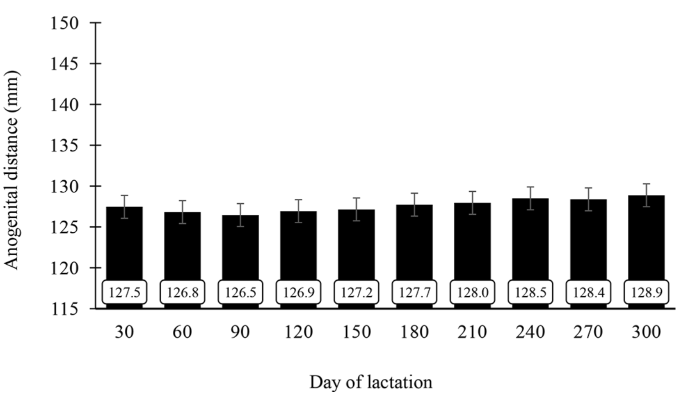

Figure 5. Bar chart showing LSM (± SEM) of anogenital distance (AGD) measurements at different phases of lactation. Means did not differ ($P = 0.96$). The numbers at the base of each bar indicates mean AGD.

In summary, to our knowledge, this was the first study to establish the repeatability of AGD at various ages from birth to breeding age in Holstein heifers, and at important physiological states in Holstein cows. Although AGD measured at birth did not reflect the AGD of breeding-age heifers, AGD measured at 6 mo of age did, to a certain extent. Moreover, AGD was neither affected by any stage of the estrous cycle or lactation nor by most stages of gestation. Our results indicated that AGD is highly repeatable and remains largely unaffected by postnatal physiological states in Holstein cows. We concluded that AGD at 6 mo is a reliable indicator of AGD at 9, 12, and 16 mo of age in nulliparous heifers, and AGD could be measured consistently through most physiological states in parous cows, except during the last 2 wk of gestation. The earliest gestational stage when pregnancy-associated increase in AGD occurred, however, could not be definitively established in the present study.

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Table 4. Pearson correlation coefficients denoting the associations of anogenital distance (AGD) measurements among the different stages (30–300 d, DIM) of lactation in Holstein cows (n = 70 at each stage)[1]

DIM	30	60	90	120	150	180	210	240	270	300
30	1.00									
60	0.95	1.00								
	<0.01									
90	0.94	0.96	1.00							
	<0.01	<0.01								
120	0.94	0.95	0.96	1.00						
	<0.01	<0.01	<0.01							
150	0.94	0.97	0.96	0.97	1.00					
	<0.01	<0.01	<0.01	<0.01						
180	0.90	0.94	0.96	0.94	0.96	1.00				
	<0.01	<0.01	<0.01	<0.01	<0.01					
210	0.90	0.94	0.96	0.95	0.96	0.97	1.00			
	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01				
240	0.90	0.93	0.95	0.95	0.95	0.96	0.98	1.00		
	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01			
270	0.90	0.94	0.96	0.97	0.95	0.94	0.98	0.97	1.00	
	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		
300	0.90	0.92	0.94	0.95	0.93	0.94	0.96	0.96	0.97	1.00
	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	

[1]Correlation coefficients (r) of AGD by DIM are shown in the top row for each stage and associated *P*-values are given below the r values.

REFERENCES

Akbarinejad, V., F. Gharagozlou, M. Vojgani, E. Shourabi, and M. J. M. Makiabadi. 2019. Inferior fertility and higher concentrations of anti-Müllerian hormone in dairy cows with longer anogenital distance. Domest. Anim. Endocrinol. 68:47–53. https://doi.org/10.1016/j.domaniend.2019.01.011.

Anderson, K. J., D. G. LeFever, J. S. Brinks, and K. G. Odde. 1991. The use of reproductive tract scoring in beef heifers. Agri-Practice. 12:19–26.

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

Barrett, E. S., L. E. Parlett, and S. H. Swan. 2015. Stability of proposed biomarkers of prenatal androgen exposure over the menstrual cycle. J. Dev. Orig. Health Dis. 6:149–157. https://doi.org/10.1017/S2040174414000646.

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

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

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

Bruinjé, T. C., J. P. Rosadiuk, F. Moslemipur, H. Sauerwein, M. A. Steele, and D. J. Ambrose. 2021. Differing planes of pre- and post-weaning phase nutrition in Holstein heifers: II. Effects on circulating leptin, luteinizing hormone, and age at puberty. J. Dairy Sci. 104:1153–1163. https://doi.org/10.3168/jds.2020-18810.

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/10.1095/biolreprod.104.036277.

Canadian Council on Animal Care. 2009. Guidelines on the care and use of farm animals in research, teaching, and testing. Accessed Aug. 27, 2021. https://ccac.ca/Documents/Standards/Guidelines/Farm_Animals.pdf.

Carrelli, J. E., M. Gobikrushanth, M. Corpron, I. Rajesh, W. Sandberg, M. G. Colazo, A. Ahmadzadeh, M. Oba, and D. J. Ambrose. 2021. Relationship of anogenital distance with fertility in nulliparous Holstein heifers. J. Dairy Sci. 104:8256–8264. https://doi.org/10.3168/jds.2020-19940.

Carrelli, J. E., M. Gobikrushanth, M. Corpron, W. Sandberg, I. Rajesh, A. Ahmadzadeh, M. Oba, and D. J. Ambrose. 2022. Associations between anogenital distance and measures of fertility in lactating North American Holstein cows: A validation study. J. Dairy Sci. (JDS 20827; under second review)

Desjardins, C., and H. D. Hafs. 1969. Maturation of bovine female genitalia from birth through puberty. J. Anim. Sci. 28:502–507. https://doi.org/10.2527/jas1969.284502x.

Diskin, M. G., and J. M. Sreenan. 2000. Expression and detection of oestrus in cattle. Reprod. Nutr. Dev. 40:481–491. https://doi.org/10.1051/rnd:2000112.

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

Dušek, A., and L. Bartoš. 2012. Variation in ano-genital distance in spontaneously cycling female mice. Reprod. Domest. Anim. 47:984–987. https://doi.org/10.1111/j.1439-0531.2012.02003.x.

Gobikrushanth, M., T. C. Bruinje, M. G. Colazo, S. T. Butler, and D. J. Ambrose. 2017. 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, J. Kenneally, R. C. Doyle, S. A. Holden, P. M. Martinez, E. R. Canadas, T. C. Bruinjé, M. G. Colazo, D. J. Ambrose, and S. T. Butler. 2019. The relationship between anogenital distance and fertility, and genome-wide associations for anogenital distance in Irish Holstein-Friesian cows. J. Dairy Sci. 102:1702–1711. https://doi.org/10.3168/jds.2018-15552.

Grala, T. M., M. D. Price, B. Kuhn-Sherlock, C. R. Burke, and S. Meier. 2021. Investigating anogenital distance and antral follicle count as novel markers of fertility within a herd of cows with positive or negative genetic merit for fertility traits. J. Dairy Sci. 104:12939–12952. https://doi.org/10.3168/jds.2020-19948.

Gutierrez, K., R. Kasimanickam, A. Tibary, J. M. Gay, J. P. Kastelic, J. B. Hall, and W. D. Whittier. 2014. Effect of reproductive tract scoring on reproductive efficiency in beef heifers bred by timed insemination and natural service versus only natural service. Theriogenology 81:918–924. https://doi.org/10.1016/j.theriogenology.2014.01.008.

Henricks, D. M., J. F. Dickey, and J. R. Hill. 1971. Plasma estrogen and progesterone levels in cows prior to and during estrus. Endocrinology 89:1350–1355. https://doi.org/10.1210/endo-89-6-1350.

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

Kastelic, J. P., D. R. Bergfelt, and O. J. Ginther. 1990. Relationship between ultrasonic assessment of the corpus luteum and plasma progesterone concentration in heifers. Theriogenology 33:1269–1278. https://doi.org/10.1016/0093-691X(90)90045-U.

Kim, C. K., S. S. C. Yen, and K. Benirschke. 1972. Serum testosterone in fetal cattle. Gen. Comp. Endocrinol. 18:404–407. https://doi.org/10.1016/0016-6480(72)90230-4.

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

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

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.

Manikkam, M., E. J. Crespi, D. D. Doop, C. Herkimer, J. S. Lee, S. Yu, M. B. Brown, D. L. Foster, and V. Padmanabhan. 2004. Fetal programming: Prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. Endocrinology 145:790–798. https://doi.org/10.1210/en.2003-0478.

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.

Mongkonpunya, K., Y. C. Lin, P. A. Noden, W. D. Oxender, and H. D. Hafs. 1975. Androgens in the bovine fetus and dam. Exp. Biol. Med. (Maywood) 148:489–493. https://doi.org/10.3181/00379727-148-38567.

Morotti, F., A. F. Zangirolamo, N. C. da Silva, C. B. da Silva, C. O. Rosa, and M. M. Seneda. 2017. Antral follicle count in cattle: Advantages, challenges, and controversy. Anim. Reprod. 14:514–520. https://doi.org/10.21451/1984-3143-AR994.

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

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

Priskorn, L., J. H. Petersen, N. Jørgensen, H. B. Kyhl, M. S. Andersen, K. M. Main, A. M. Andersson, N. E. Skakkebaek, and T. K. Jensen. 2018. Anogenital distance as a phenotypic signature through infancy. Pediatr. Res. 83:573–579. https://doi.org/10.1038/pr.2017.287.

Sartori, R., J. M. Haughian, R. D. Shaver, G. J. M. Rosa, and M. C. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J. Dairy Sci. 87:905–920. https://doi.org/10.3168/jds.S0022-0302(04)73235-X.

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

Schuler, G., R. Fürbass, and K. Klisch. 2018. Placental contribution to the endocrinology of gestation and parturition. Anim. Reprod. 15(Suppl. 1):822–842. https://doi.org/10.21451/1984-3143-AR2018-0015.

Senger, P. L. 2005. Reproductive cyclicity – terminology and basic concepts. Pages 144–163 in Pathways to Pregnancy and Parturition. 2nd rev. ed. Current Conceptions Inc.

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.

Thankamony, A., K. K. Ong, D. B. Dunger, C. L. Acerini, and I. 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.

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.

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.

Wolak, M. E., D. J. Fairbairn, and Y. R. Paulsen. 2012. Guidelines for estimating repeatability. Methods Ecol. Evol. 3:129–137. https://doi.org/10.1111/j.2041-210X.2011.00125.x.

Wolf, C. J., G. A. LeBlanc, and L. E. Gray Jr.. 2004. Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. Toxicol. Sci. 78:135–143. https://doi.org/10.1093/toxsci/kfh018.

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. 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.

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