**The anogenital ratio as an indicator of reproductive performance in dairy heifers**

Barbara Beci, Mieke Van Eetvelde, Louise Vanlommel, Geert Opsomer

Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Corresponding author: Barbara Beci: barbara.beci@ugent.be

Abstract

The anogenital distance (AGD) is considered a marker for prenatal androgen exposure and fertility in multiple species including humans. In dairy cattle, it is described as the length between the center of the anus and the clitoral base (AGDc). However, in other species, the distance from the center of the anus to the dorsal commissure of the vulva (AGDv) is also considered to be a predictor for fertility traits, as well as the anogenital ratio (AGR, defined as [AGDv/AGDc]\*100). The primary aim of the present study was to assess whether AGDv and AGR can be used as an indicator for reproductive performance in dairy heifers. Additionally, the relation between AGDv and AGDc and the correlation with other body measurements were explored. Data of 656 Holstein Friesian heifers at an age of 13.5 ± 1.08 months were analyzed. Respective means of 62.9 ± 8.20 mm (AGDv) and 107.6 ± 9.27 mm (AGDc) were recorded. The mean AGR ratio was calculated as 58.6 ± 6.75%, varying from 37.3 to 79.6%. The age of the heifers was not associated with any of the AGD measurements nor the ratio. Except for a very low correlation between heart girth and AGDc (r=0.09, P<0.05), both AGDs were largely uncorrelated with other body measurements. Linear regression models revealed that AGDc was not associated with any of the recorded fertility parameters. However, results revealed a negative association between AGDv and AGR and reproductive performance: heifers with a short AGDv and small AGR were younger at first AI (P≤0.003) and at conception (P=0.004). Based on ROC curve analyses, AGDv was the best indicator for pregnancy to first AI, with a threshold estimated at 65.3 mm. The pregnancy rate at first AI was 72.4% in heifers with a short AGDv (<65.3 mm, n=413) compared to 61.7% in heifers with a long AGDv (≥65.3mm, n=243). Hence, short-AGDv heifers had 63% higher odds to conceive at first AI compared to their long-AGDv counterparts (P=0.004). Additionally, an AGR threshold of 59,6% was determined: heifers with a small AGR (<59.6%) had 44% higher odds to be pregnant at first AI compared to heifers with an AGR ≥ 59.6%. Results of the present study suggest to consider AGDv and AGR as potential indicators for reproductive performance in dairy heifers. The latter implies that it is relevant to measure both AGDc and AGDv in future studies. The absence of correlation between body- and AGD-measurements furthermore suggests that AGD sizes are rather pre- than postnatally determined.

Keywords: anogenital distance, anogenital ratio, dairy heifer, reproductive performance

1. Introduction

Reproductive efficiency is crucial in dairy cattle management [1]. Good herd fertility is necessary to optimize productivity and longevity and to manage replacements, which is crucial to maintain herd size [2]. In heifers, optimal reproductive management is necessary to ensure first calving before the age of 24 months, thus reducing investment costs and shortening the generation interval [2,3]. Nowadays, it is generally accepted that only the optimal number of newborn females should be kept as replacements [4]. Therefore, there is an increasing need for tools to accurately select the heifers with the best (reproductive) potential.

Former selection among dairy cows was primarily focused on high milk yield and solids, resulting in an overall decrease in their reproductive performance [5,6]. It has been challenging to stop this negative trend and improve dairy cattle fertility through adaptations in the management, improved recording of fertility traits and a better knowledge of fertility related genes and genetic selection [5,7]. Nowadays, reproductive traits are routinely included in breeding goals [8], but many have been shown to have a low heritability, usually less than 5% [5]. This can be explained by the fact that traditional fertility traits are complex and therefore affected by more than just genetics [9]. In addition to the well-known effects of nutrition and disease on fertility, the prenatal environment has recently been shown to influence the expression of an animal’s genetic potential and therefore its phenotypic reproductive capacity [10]. Therefore, it would be beneficial to identify new, more detailed reproductive phenotypes that can preferably be determined early in life [5,11,12]. Such reproductive phenotypes might be good predictors for future reproductive performance as adult animals and thus provide helpful tools in the selection of youngstock [5,12].

The anogenital distance (AGD) has been proposed as a novel indicator of reproductive performance in cattle [13,14]. It is a general term that describes the distance between the anus and genitals in both sexes in different species [15,16]. In human females, AGD specifically refers to the distance between the center of the anus and either the clitoral base (AGDc) or posterior fourchette of the vulva (AGDv) [17,18]. Studies in laboratory animals and humans have repeatedly shown that in females, a longer AGD is associated with lower reproductive performance [19–21]. Moreover, in human studies, the anogenital ratio (AGR, describing the mutual relationship between both) has been proposed as a more consistent parameter amongst different races and unrelated to other body morphometrics [22]. In addition, the AGR has been shown to be a better indicator for fertility problems than the individual AGD measurements [23]. However, so far, bovine studies are rather scarce. In dairy cattle, AGD has been studied as the distance between the anus and the base of the clitoris (AGDc) and shows promising results as a moderately heritable indicator for reproductive success [14,24,25]. To the best of our knowledge, the anogenital distance to the dorsal commissure of the vulva (AGDv) or the ratio between both AGD measurements have not yet been studied in cattle.

The principal aim of the present study was to assess whether AGDv and the ratio between AGDv and AGDc could be used as indicators for reproductive performance in dairy heifers. Therefore, the association between these measurements and the fertility results of 656 animals was analyzed. In addition, the correlation between AGDv and AGDc and the correlation between these and other body measurements were also explored.

**2. Materials and methods**

***2.1 Selection of herds and animals and data collection***

The present study was part of a larger study on different aspects of anogenital distances in dairy heifers. As there was no literature available on AGDv in cattle, the initial sample size calculation was based on results of a previous study on AGDc, reporting an average AGDc of 107 ± 10.5 mm in nulliparous heifers [25,26]. After measuring 141 animals on 9 herds for the current study, we observed a monthly increase of 3.8 mm per month of age. Using a power calculation in R (pwr.anova.test), we concluded that 153 animals per age group (12-13, 13-14, and 14-15 months) were necessary to make inferences about AGDc growth in the population. Therefore, the sample size was set at 459 animals.

The present cross-sectional study was conducted on 27 commercial dairy herds located in Flanders and The Netherlands. Farms were selected based on their willingness to collaborate and the availability of accurate fertility data. An informed consent was obtained from all herd managers and all national animal handling regulations and guidelines were strictly followed. All herds had a year-round calving pattern without outdoor access for heifers. Heifers were housed in freestall barns and fed according to their requirements for maintenance and growth. Rations included mainly grass silage, hay, straw, corn silage and minerals. Heifers were artificially inseminated by the farmer or an AI technician after visual estrus detection, no synchronization protocols were applied. Pregnancy diagnosis was performed on a regular base by experienced veterinarians using transrectal ultrasonography.

The study focused on heifers around the age of first insemination. Therefore, during regular farm visits, all heifers between 11 and 16 months old were included in the study. AGDs were always measured by the same individual using 15-cm stainless-steel digital calipers with a 0.01 mm accuracy (Kreator KRT705004®, VARO, Belgium). The tail was lifted and the vulvar lips were gently spread to make the dorsal commissure of the vulva and the clitoris clearly visible. Anogenital distance to the vulva (AGDv) was defined as the distance from the center of the anus to the dorsal commissure of the vulva (Fig. 1). Anogenital distance to the clitoris (AGDc) was defined as the distance between the center of the anus and the base of the clitoris (Fig. 2). The anogenital ratio (AGR) was calculated as the ratio between AGDv and AGDc (AGR = AGDv/AGDc) and expressed as a percentage.

To assess the overall body size of each heifer, additional measurements of hip height (HH, in cm) and heart girth (HG, in cm) were performed. Hip height was measured from the ground to the spine parallel to the hook bones, using a livestock measuring stick (Agradi, The Netherlands). Heart girth was measured with a plastic-coated fiber measuring tape (Animeter®, Albert Kerbl GmbH, Germany), as the circumference around the thorax immediately behind the elbows. To determine the body condition of the animals, back fat thickness (BFT, in mm) was measured ultrasonographically (Tringa Linear, Esaote/Pie Medical, Maastricht, The Netherlands) as described by Schröder and Staufenbiel (2006) [27].

Breed, birth date, insemination dates and results of pregnancy diagnoses were extracted from the herd database or provided by the farmer. Only heifers with at least 75% of Holstein Friesian blood proportion that were between 335 and 480 days old at the time of the measurements, were included for further analyses. In addition, animals with unknown or incomplete fertility information (missing insemination dates or pregnancy diagnosis, n=29) were excluded. Heifers bred by natural service (n=11) were removed because an exact date of conception was not available.

***2.2 Statistical Analyses***

All data were loaded into the statistical program R version 3.4.1.

In a previous bovine study, Gobikrushanth et al. (2017) stated that, because of the high repeatability and low variation between measurements, a single AGDc determination was sufficient [25]. However, to the best of our knowledge, no literature on the repeatability of AGDv measurements in cattle is available. Hence, in a preliminary trial, the AGDv was determined three times in 50 animals by the same individual. Similar to the results reported by Gobikrushanth et al. (2017) about AGDc, a high correlation (r=0.92, P<0.001) and a low coefficient of variation (CV=4%) between the repeated AGDv measurements were observed [25]. Hence, for the rest of the study, AGDv was only determined once by the same examiner.

Pearson correlation coefficients were calculated to determine the correlation between the different AGDs and between the AGDs and the overall body measurements. To assess the association between the AGDs and the fertility parameters, linear mixed models were built using the lmer() function of the lme4 package [28], including herd as random effect. Outcome variables were age at first insemination, age at conception and number of inseminations until conception; explaining variables were both AGD measurements and the AGR. To determine the AGDc, AGDv and AGR thresholds significantly associated with the pregnancy rate after first AI, receiver operating characteristic (ROC) curves were constructed using the pRoc package [29]. Based on these ROC curves, thresholds were calculated above which a lower pregnancy rate at first AI could be expected and models were compared using the area under the curve (AUC). Based on the different thresholds, heifers were subsequently categorized as having a small (< threshold) or large (≥ threshold) AGDc, AGDv and AGR. Thereafter, associations between all groups (AGDc, AGDv and AGR) and fertility parameters were analyzed with logistic regression models using the lmer() function of the lme4 package [28]. Herd was included as a random effect in all models to account for variation in management between herds. Statistical significance and tendency were declared at P<0.05 and 0.05<P<0.1, respectively.

**3. Results**

Initially, 741 heifers from 27 herds were measured. After selection based on breed, age and the availability of fertility data, 679 animals remained in the dataset. By the time of analysis, 23 animals were not yet inseminated or examined for pregnancy, so present results are based on data of 656 heifers.

***3.1 Descriptive Analysis***

Heifers were measured at the age of 13.5 ± 1.08 months. Overall body measurements are depicted in Table 1.

*Table 1. Descriptive statistics for age, body measurements, anogenital distance to the vulva (AGDv) and clitoris (AGDc), and anogenital ratio (AGR=AGDv/AGDc) in Holstein Friesian heifers (n=656).*

|  |  |  |
| --- | --- | --- |
|  | Mean ± SD | Min – Max |
| Age (days) | 414 ± 32.9 | 335 – 480 |
| Heart girth (cm) | 176 ± 7.4 | 156.0 – 195.0 |
| Hip height (cm) | 138 ± 4.1 | 126.5 – 150.0 |
| Back Fat Thickness (mm) | 14 ± 3.7 | 4.0 – 31.0 |
| AGDv (mm) | 62.9 ± 8.20 | 37.2 – 94.0 |
| AGDc (mm) | 107.6 ± 9.27 | 76.4 – 140.0 |
| AGR (%) | 58.6 ± 6.75 | 37.3 – 79.6 |

A positive correlation was found between age of the heifer and HG (r=0.52, P<0.001), HH (r=0.47, P<0.001) and BFT (r=0.19, P<0.001). However, AGDs were not influenced by the age of the heifer and were largely uncorrelated with other body measurements: only a low correlation was found between HG and AGDc (r=0.09, P<0.05), while other correlations were not significant. The correlation between AGDv and AGDc was 0.48 (P<0.001).

Ten heifers were culled because of their inability to conceive after 1 to 7 AI’s (average of 3.5). The other 646 heifers were all diagnosed pregnant after 1 to 6 AI’s. Age at first AI and conception is shown in table 2.

*Table 2. Descriptive statistics for fertility parameters of included heifers.*

|  |  |  |  |
| --- | --- | --- | --- |
|  | n | Mean ± SD | Min – Max |
| Age at first AI (days) | 656 | 434 ± 40.0 | 344 – 634 |
| Age at conception (days) | 646 | 451 ± 51.8 | 344 – 683 |
| Number of AIs per pregnancy | 646 | 1.5 ± 0.85 | 1 – 6 |

***3.2 Linear Regression Models***

Results of the linear regression models showed that AGDc was not associated with fertility results. However, heifers with a longer AGDv were older at first AI (P<0.001) and at conception (P=0.004). In addition, the AGR showed similar results: for every 1% increase in AGR, heifers were 0.56 days older at first AI (P=0.003) and 0.79 days older at conception (P=0.004) (Table 3).

*Table 3. Results of the linear regression models for the association between anogenital distance measurements and age at first AI (n=656), age at conception and number of AI’s until conception (n=646), with herd included as a random factor.*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Age at first AI |  | Age at conc |  | Number of AIs per pregnancy |
|  | Estimate | P |  | Estimate | P |  | Estimate | P |
| AGDv | 0.53 | <0.001 |  | 0.66 | 0.004 |  | 0.002 | 0.60 |
| AGDc | 0.19 | 0.19 |  | 0.14 | 0.49 |  | 0.003 | 0.49 |
| AGR (%) | 0.56 | 0.003 |  | 0.79 | 0.004 |  | 0.001 | 0.9 |

The optimal thresholds of AGDc and AGDv to predict pregnancy at first AI, as determined by the ROC curve analyses, were 102.5 mm and 65.3 mm, with a respective AUC of 52.6% and 54% (Fig.3 A and B). The optimal threshold of the AGR to predict pregnancy at first AI was 59.6% (AUC of 53.2%; Fig. 3 C). Based on the thresholds, heifers were categorized into groups for having a small (< threshold) or large (≥threshold) AGDc, AGDv and AGR.

None of the fertility indices were significantly different between heifers with a small and large AGDc (Table 4 and 5). Heifers with a small AGDv had a pregnancy rate at first AI of 72.4%, compared to 61.7% in large-AGDv heifers. Logistic regression models revealed that heifers with a small AGDv had 63% higher odds to become pregnant at first AI compared to their large-AGDv counterparts (P=0.004; Table 4). In addition, heifers in the small AGDv group were 7.8 ± 2.68 days younger at first AI (P=0.004) and 11.6 ± 3.81 days younger at conception (P=0.002) compared to heifers in the large AGDv group (Table 5). Results of AGR were similar to those of the AGDv. Heifers with a small AGR had 44% higher odds to become pregnant at first AI compared to their large-AGR counterparts (P=0.030; Table 4). As a result, heifers in the small AGR group were 7.7 ± 3.72 days younger at conception compared to heifers in the large AGR group (P=0.04), despite their similar age at first AI (P=0.20, Table 5).

**

*Fig.3 Receiver operating characteristic (ROC) curve analyses for AGDc (A), AGDv (B) and AGR (C) predicting the probability of pregnancy at first AI in dairy heifers.*

*Table 4. Results of the logistic regression models for the association between AGDc, AGDv and AGR groups (based on ROC curve analyses) and pregnancy rate to first AI.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Group | n | Pregnancy to first AI (%) | Odds ratio estimate | 95% CI | P-value |
| AGDv  | <65.3 mm | 413 | 72.4% | 1.63 | 1.16-2.28 | 0.004 |
|  | ≥65.3 mm | 243 | 61.7% | ref. |  |  |
| AGDc | <102.5 mm | 202 | 73.3% | 1.39 | 0.96-20.3 | 0.08 |
|  | ≥102.5 mm | 454 | 66.3% | ref. |  |  |
| AGR | <59.6% | 364 | 72.9% | 1.44 | 1.04-2.01 | 0.03 |
|  | ≥59.6% | 292 | 64.0% | ref. |  |  |

*Table 5. Results of the logistic regression models for the association between AGDc, AGDv and AGR groups (based on ROC curve analyses) and age at first AI (n=656), age at conception and number of AIs per pregnancy (n=646).*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Age at first AI |  | Age at conc |  | Number of AIs per pregnancy |
|  | Group | Estimate | P |  | Estimate | P |  | Estimate | P |
| AGDv | <65.3 mm | ref. | 0.004 |  | ref. | 0.002 |  | ref. | 0.14 |
|  | ≥65.3 mm | 7.8 |  |  | 11.6 |  |  | 0.10 |  |
| AGDc | <102.5 mm | ref. | 0.38 |  | ref. | 0.22 |  | ref. | 0.38 |
|  | ≥102.5 mm | 2.5 |  |  | 4.9 |  |  | 0.06 |  |
| AGR | <59.6% | ref. | 0.20 |  | ref. | 0.04 |  | ref. | 0.13 |
|  | ≥59.6% | 3.3 |  |  | 7.7 |  |  | 0.10 |  |

**4. Discussion**

***4.1 Anogenital measurements and overall body measurements***

In this study, we introduced the anogenital distance to the dorsal commissure of the vulva (AGDv) and the anogenital ratio (AGR) as potential fertility markers in dairy cattle, besides the already described AGDc. Nulliparous heifers around the age of first insemination (13.5 ± 1.08 months of age) were the focus of this study. In this age group, farmers can still benefit from selecting the animals with the best expected reproductive performance. Additionally, in nulliparous heifers, the anatomy of the vulva and perineal region is not influenced by a previous calving allowing for accurate anogenital measurements. We found an average AGDc of 107.6 ± 9.27 mm, which is similar to previously reported results in nulliparous heifers [14]. The average AGDv was 62.9 ± 8.20 mm, demonstrating that the variation in AGDv is relatively larger amongst individuals compared to the AGDc. This is similar to what has been described in several human studies [17,30–32]. In addition to the AGD measurements, the anogenital ratio (AGR) was calculated as AGDv divided by AGDc and expressed as a percentage, as first introduced by Callegari et al. in 1987. The AGR has been more frequently determined in human newborns [22,33] and is believed to be a reliable indicator of prenatal androgen exposure. Furthermore, while AGDc and AGDv varied relatively strongly between studies, AGR was found to be rather consistent between races and sexes [22], and was not correlated with body size [22]. The latter suggests that the AGR is a more stable marker in comparison to both singular AGD measurements [17,22]. In the current research, a quite large variation in AGR was recorded among the studied heifers (min 37.3% - max 79.6%), suggesting a relatively large difference in prenatal androgen exposure. Lack of androgens (testosterone) during the masculinization programming window in females is the reason that there is no cranial migration of the genital tubercule resulting in a shorter AGD compared to males [34–36]. Studies in female rats have shown that intrauterine exposure to endocrine disruptors such as azole fungicide prochloraz and ethinyl estradiol resulted in longer AGD at birth [37]. To the best of our knowledge, the effect of specific endocrine disruptors on the AGD in cattle has not been studied yet.

Besides the AGD measurements, other body measurements such as HG, HH and BFT were determined to investigate their mutual correlations. As one would expect, older heifers were heavier, taller and deposited more back fat. Even though a moderate correlation (r=0.48) was found between AGDv and AGDc, both AGDs were largely uncorrelated with overall body measurements. This is in accordance with previous Holstein Friesian studies where only weak correlations between AGDc and HH [25], body weight and BCS [26] were found. Furthermore, Rajesh et al. (2022) demonstrated that AGDc is highly repeatable throughout different physiological states in cows. AGDc measurements were neither influenced by stage of the estrous cycle, nor stage of gestation up to 180 days [13].

Within the studied age group, there was considerable variation in AGDc and AGDv, although no correlation with age was found. The latter suggests that these AGD measures are highly variable but minimally influenced by postnatal factors, as has also been demonstrated in previous bovine AGDc studies [14,25,26].

***4.2 Anogenital measurements and fertility***

Results from a limited number of bovine studies report an inverse relationship between AGDc and reproductive performance in lactating Holstein cows and nulliparous heifers [14,25,38]. Cows with a long AGDc (>127.1 mm) had lower conception rates to first AI compared to cows with a shorter AGDc and also demonstrated a decreased likelihood of pregnancy by 250 days in lactation [25]. Longer AGDc also tended to be associated with higher serum anti-Müllerian hormone concentrations, more days to first service, lower first service to conception rates and a higher proportion of repeat breeders [39]. An observational study by Madureira et al. (2022) reported that greater proportions of short AGDc cows commenced estrous activity by 50 days in lactation, had larger preovulatory follicles, exhibited a longer estrus and had higher ovulation rates compared to long-AGDc cows [40]. Moreover, first parity cows with a short AGDc were more likely to be pregnant within the first six weeks of mating, resulting in a 20-day shorter calving to conception interval compared to long AGDc cows [24]. Carrelli et al. (2021) found that every 1 mm increase in AGDc in dairy heifers, reduced the predicted probability of pregnancy by 1.9% [14]. They also defined the AGDc threshold for heifers to be 110 mm and demonstrated that heifers defined as having a short AGDc (≤110 mm) needed fewer services per conception (1.5 vs. 1.7), conceived earlier (448.4 vs. 454.3 d) and had higher pregnancy rates to first AI (58.3 vs. 49.6%) compared to the heifers with a long AGDc (>110 mm) [14]. In addition, a recent study on superovulation in cows and heifers reported a greater number of preovulatory follicles, as well as higher odds of yielding fertilized ova and viable embryos, in short- compared to long-AGDc cows. However, embryo yield and quality did not differ between short and long-AGDc heifers [41].

In the current study in Belgian and Dutch Holstein Friesian heifers, no linear association was found between AGDc and age at first AI, age at conception, or number of AIs per pregnancy. The optimum AGDc threshold to predict the pregnancy rate at first AI was estimated at 102.5 mm. This is much smaller than the threshold of 110 mm published by Carrelli et al. (2021), despite a similar range in AGDc values [14]. In addition, none of the fertility indices were significantly different between small AGDc (<102.5 mm) and large AGDc (≥102.5) heifers. This is in contrast with the study of Carrelli et al. (2021), who reported a negative association between AGDc and fertility parameters in nulliparous Canadian Holstein heifers. Although this lack of association could be attributed to the smaller sample size in the current study (656 versus 1,692 heifers, respectively), another possible explanation could be the difference in fertility between the studied populations. Gobikrushanth et al. (2019) were also not able to demonstrate an inverse relationship between reproductive variables and AGDc in 1,180 Irish cows. The authors stated that the lack of association between AGDc and fertility could be attributed to the fact that their study was done in a more fertile population of cows in Ireland [26]. In our findings, the overall pregnancy rate to first AI was 68.4%, which is much higher than the reported 58.3% (short AGD heifers) and 49.6% (long AGD heifers) in the Canadian study by Carrelli et al. (2021) [14]. Hence, we suggest that associations between AGDc and fertility outcomes vary between studies and may be less evident in a highly fertile population.

In contrast to the findings on AGDc, results of the present study did reveal an inverse linear relationship between AGDv and reproductive success in Holstein Friesian heifers. Animals with a longer AGDv were older at first AI and at conception. The AUC of the ROC curves also revealed AGDv to be the best predictor of pregnancy to first AI: short-AGDv heifers (AGDv < 65.3 mm) had 63% higher odds to conceive at first AI compared to their long-AGDv counterparts (AGDv ≥ 65.3 mm). Hence, we suggest that future studies should include AGDv besides AGDc, as currently no other data on AGDv in cattle is available. However, based on current findings and literature in other species, we can presume that -similar to AGDc - the variation in AGDv between populations is substantial. This might result in very population-dependent thresholds for AGDv, making it difficult to compare findings and to extrapolate results to other cattle populations.

Finally, our current findings show a negative association between AGR and fertility parameters, similar to AGDv. A larger AGR was associated with in an older age at first AI and at conception. Moreover, heifers with a greater AGR – meaning a relatively long AGDv compared to AGDc, and thus indicating a shorter vulvar length – had a reduced pregnancy rate after the first AI. Hence, we suggest that AGR should be considered as a predictor for reproductive success in cattle. More so, following the results of human studies, AGR could be a more consistent marker than single AGD measurements, usable across populations [22]. Based on these findings, it seems relevant to measure both the AGDv and AGDc in cattle, in order to determine AGR and to further assess which measurement is the better indicator of reproductive success. Moreover, a recent study showed that the ratio between anterior and posterior AGD was longer in women suffering from polycystic ovary syndrome (PCOS). These authors therefore suggested using the AGR instead of single AGD measurements as a biomarker of PCOS [23]. The link between the AGR and PCOS could be very interesting for further research in dairy cattle, where a similar disease (Cystic Ovarian Disease) is quite common [42].

**5. Conclusions**

The AGDc, AGDv and AGR were determined in Belgian and Dutch Holstein heifers around the age of first insemination. To the best of our knowledge, this is the first study reporting AGDv and AGR measurements in dairy cattle. The AGDs were not correlated with other body measurements, nor with the age within this specific age group, suggesting that the AGDs are prenatally determined rather than postnatally. Compared to AGDc, AGDv and AGR were shown to be better indicators for future reproductive performance. Further research is warranted to assess whether these findings can be reproduced in even younger heifers as well as to identify the prenatal factors determining the length of the anogenital distances.

**Credit autorship Contribution Statement**

**Barbara Beci**: Methodology, data collection and analysis, writing - original draft. **Mieke Van Eetvelde**: Methodology, data analysis, writing - review & editing. **Louise Vanlommel**: Data collection. **Geert Opsomer**: Conceptualization, methodology, supervision, review & editing.

**Funding**

This research did not receive any funding from agencies in the public, commercial, or

not-for-profit sectors.

**Declaration of competing interest**

None of the authors has any conflict of interest to declare.

**Acknowledgements**

All farm personnel involved in the study are cordially thanked.

**Software and data repository resources**

None of the data were deposited in an official repository.

**References**

[1] Dillon P, Berry DP, Evans RD, Buckley F, Horan B. Consequences of genetic selection for increased milk production in European seasonal pasture based systems of milk production. Livest Sci 2006;99:141–58. https://doi.org/10.1016/J.LIVPRODSCI.2005.06.011.

[2] Boulton AC, Rushton J, Wathes DC. An empirical analysis of the cost of rearing dairy heifers from birth to first calving and the time taken to repay these costs. Animal 2017;11:1372–80. https://doi.org/10.1017/S1751731117000064.

[3] Tozer PR, Heinrichs AJ. What Affects the Costs of Raising Replacement Dairy Heifers: A Multiple-Component Analysis. J Dairy Sci 2001;84:1836–44. https://doi.org/10.3168/JDS.S0022-0302(01)74623-1.

[4] Mohd Nor N, Steeneveld W, Mourits MCM, Hogeveen H. The optimal number of heifer calves to be reared as dairy replacements. J Dairy Sci 2015;98:861–71. https://doi.org/10.3168/JDS.2014-8329.

[5] Berglund B. Genetic improvement of dairy cow reproductive performance. Reprod Domest Anim 2008;43 Suppl 2:89–95. https://doi.org/10.1111/J.1439-0531.2008.01147.X.

[6] Walsh SW, Williams EJ, Evans ACO. A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 2011;123:127–38. https://doi.org/10.1016/J.ANIREPROSCI.2010.12.001.

[7] Overton MW, Dhuyvetter KC. Symposium review: An abundance of replacement heifers: What is the economic impact of raising more than are needed? J Dairy Sci 2020;103:3828–37. https://doi.org/10.3168/JDS.2019-17143.

[8] Miglior F, Muir BL, van Doormaal BJ. Selection indices in Holstein cattle of various countries. J Dairy Sci 2005;88:1255–63. https://doi.org/10.3168/JDS.S0022-0302(05)72792-2.

[9] Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001;84:1277–93. https://doi.org/10.3168/JDS.S0022-0302(01)70158-0.

[10] Wathes DC. Developmental Programming of Fertility in Cattle—Is It a Cause for Concern? Animals 2022;12:2654. https://doi.org/10.3390/ani12192654.

[11] Carthy TR, Berry DP, Fitzgerald A, McParland S, Williams EJ, Butler ST, et al. Risk factors associated with detailed reproductive phenotypes in dairy and beef cows. Animal 2014;8:695–703. https://doi.org/10.1017/S1751731114000354.

[12] Meier S, McNaughton LR, Handcock R, Amer PR, Beatson PR, Bryant JR, et al. Heifers with positive genetic merit for fertility traits reach puberty earlier and have a greater pregnancy rate than heifers with negative genetic merit for fertility traits. J Dairy Sci 2021;104:3707–21. https://doi.org/10.3168/JDS.2020-19155.

[13] Rajesh I, Gobikrushanth M, Carrelli JE, Oba M, Ambrose DJ. Repeatability of anogenital distance measurements from birth to maturity and at different physiological states in female Holstein cattle. J Dairy Sci 2022;105:2699–707. https://doi.org/10.3168/JDS.2021-21419.

[14] Carrelli JE, Gobikrushanth M, Corpron M, Rajesh I, Sandberg W, Colazo MG, et al. Relationship of anogenital distance with fertility in nulliparous Holstein heifers. J Dairy Sci 2021;104:8256–64. https://doi.org/10.3168/jds.2020-19940.

[15] Swan SH, Kristensen DM. Anogenital Distance: A Marker of Steroidal Endocrine Disruption. Encyclopedia of Reproduction 2018:588–93. https://doi.org/10.1016/B978-0-12-801238-3.64379-9.

[16] Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: Relationship to male reproductive development and its disorders. Journal of Clinical Endocrinology and Metabolism 2013;98:2230–8. https://doi.org/10.1210/JC.2012-4057.

[17] Callegari C, Everett S, Ross M, Brasel JA. Anogenital ratio: Measure of fetal virilization in premature and full-term newborn infants. J Pediatr 1987;111:240–3. https://doi.org/10.1016/S0022-3476(87)80075-6.

[18] Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. Environ Health 2004;3. https://doi.org/10.1186/1476-069X-3-8.

[19] Wu XY, Li ZL, Wu CY, Liu YM, Lin H, Wang SH, et al. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocr J 2010;57:201–9. https://doi.org/10.1507/ENDOCRJ.K09E-205.

[20] Bánszegi O, Szenczi P, Dombay K, Bilkó Á, Altbäcker V. Anogenital distance as a predictor of attractiveness, litter size and sex ratio of rabbit does. Physiol Behav 2012;105:1226–30. https://doi.org/10.1016/J.PHYSBEH.2012.01.002.

[21] Mira-Escolano MP, Mendiola J, Mínguez-Alarcõn L, Melgarejo M, Cutillas-Tolín A, Roca M, et al. Longer anogenital distance is associated with higher testosterone levels in women: a cross-sectional study. BJOG 2014;121:1359–64. https://doi.org/10.1111/1471-0528.12627.

[22] Numsriskulrat N, Srilanchakon K, Pronprechatham C, Pornkunwilai S, Supornsilchai V. Sex-specific ranges and ratios for anogenital distance among Thai full-term newborns. BMC Pediatr 2022;22:1–6. https://doi.org/10.1186/S12887-022-03325-Y/TABLES/4.

[23] Simsir C, Pekcan MK, Aksoy RT, Ecemis T, Coskun B, Kilic SH, et al. The ratio of anterior anogenital distance to posterior anogenital distance: A novel-biomarker for polycystic ovary syndrome. J Chin Med Assoc 2019;82:782–6. https://doi.org/10.1097/JCMA.0000000000000150.

[24] Grala TM, Price MD, Kuhn-Sherlock B, Burke CR, Meier S. 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 2021;104:12939–52. https://doi.org/10.3168/jds.2020-19948.

[25] Gobikrushanth M, Bruinjé TC, Colazo MG, Butler ST, Ambrose DJ. Characterization of anogenital distance and its relationship to fertility in lactating Holstein cows. J Dairy Sci 2017;100:9815–23. https://doi.org/10.3168/jds.2017-13033.

[26] Gobikrushanth M, Purfield DC, Kenneally J, Doyle RC, Holden SA, Martinez PM, et al. The relationship between anogenital distance and fertility, and genome-wide associations for anogenital distance in Irish Holstein-Friesian cows. J Dairy Sci 2019;102:1702–11. https://doi.org/10.3168/jds.2018-15552.

[27] Schröder UJ, Staufenbiel R. Invited review: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. J Dairy Sci 2006;89:1–14. https://doi.org/10.3168/JDS.S0022-0302(06)72064-1.

[28] Bates D, Mächler M, Bolker BM, Walker SC. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 2015;67:1–48. https://doi.org/10.18637/JSS.V067.I01.

[29] Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: An open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:1–8. https://doi.org/10.1186/1471-2105-12-77/TABLES/3.

[30] Shah R, Alshaikh B, Schall JI, Kelly A, Ford E, Zemel BS, et al. Endocrine-sensitive physical endpoints in newborns: ranges and predictors. Pediatr Res 2021;89:660–6. https://doi.org/10.1038/S41390-020-0950-2.

[31] Özkan B, Konak B, Çayir A, Konak M. Anogenital distance in Turkish newborns. J Clin Res Pediatr Endocrinol 2011;3:122–5. https://doi.org/10.4274/JCRPE.V3I3.24.

[32] Vieiralves RR, Ribeiro GS, Alves EF, Sampaio FJ, Favorito LA. Are anogenital distance and external female genitalia development changed in neural tube defects? Study in human fetuses. J Pediatr Urol 2020;16:654.e1-654.e8. https://doi.org/10.1016/j.jpurol.2020.07.015.

[33] van der Straaten S, Springer A, Zecic A, Hebenstreit D, Tonnhofer U, Gawlik A, et al. The External Genitalia Score (EGS): A European Multicenter Validation Study. J Clin Endocrinol Metab 2020;105. https://doi.org/10.1210/CLINEM/DGZ142.

[34] Dean A, Smith LB, Macpherson S, Sharpe RM. The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. Int J Androl 2012;35:330–9. https://doi.org/10.1111/J.1365-2605.2011.01236.X.

[35] Welsh M, Saunders PTK, Fisken M, Scott HM, Hutchison GR, Smith LB, et al. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest 2008;118:1479–90. https://doi.org/10.1172/JCI34241.

[36] Jain VG, Goyal V, Chowdhary V, Swarup N, Singh RJ, Singal A, et al. Anogenital distance is determined during early gestation in humans. Hum Reprod 2018;33:1619–27. https://doi.org/10.1093/HUMREP/DEY265.

[37] Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, Svingen T. Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Arch Toxicol 2019;93:253–72. https://doi.org/10.1007/S00204-018-2350-5.

[38] Carrelli JE, Gobikrushanth M, Corpron M, Sandberg W, Rajesh I, Ahmadzadeh A, et al. Associations between anogenital distance and measures of fertility in lactating North American Holstein cows: A validation study. J Dairy Sci 2022;0. https://doi.org/10.3168/JDS.2021-20827.

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

[40] Madureira AML, Burnett TA, Carrelli JE, Gobikrushanth M, Cerri RLA, Ambrose DJ. Anogenital distance is associated with postpartum estrous activity, intensity of estrous expression, ovulation, and progesterone concentrations in lactating Holstein cows. J Dairy Sci 2022;105. https://doi.org/10.3168/JDS.2022-21897.

[41] Rajesh I, Colazo MG, Gobikrushanth M, Carrelli JE, Oba M, Ambrose DJ. Superovulatory response, anti-Müllerian hormone concentration and antral follicle count in Holstein cattle with short or long anogenital distance. Theriogenology 2023;195:249–56. https://doi.org/10.1016/J.THERIOGENOLOGY.2022.10.036.

[42] Vanholder T, Opsomer G, de Kruif A. Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. Reprod Nutr Dev 2006;46:105–19. https://doi.org/10.1051/RND:2006003.

**Figure captions**

**Figure 1.** Measurement of the anogenital distance between the center of the anus and the dorsal commissure of the vulva (AGDv) using digital calipers.

 

**Figure 2.** Measurement of the anogenital distance between the center of the anus and the base of the clitoris (AGDc) using digital calipers.

