ANDRÉIA FERREIRA MACHADO

ASSOCIATION OF PHENOTYPIC AND GENETIC TRAITS WITH FERTILITY IN DAIRY CATTLE

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Simone Eliza Facioni Guimarães

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ABSTRACT

MACHADO, Andréia Ferreira Machado, D.Sc. Universidade Federal de Viçosa, February, 2024. **Association of phenotypic and genetic traits with fertility in dairy cattle.** Adviser: Simone Elisa Facioni Guimarães.

In this thesis, the three chapters explore the association of phenotypic traits with fertility in dairy cattle. The objective of the first chapter was to characterize anogenital distance (AGD) as a proxy for Anti-Mullerian hormone (AMH) and *in vitro* embryo production (IVEP) in *Bos indicus* Gyr cattle. A single AGD measurement was collected from 552 donors from six herds in Brazil. Each donor had a raw average calculated for the number of recovered oocytes, viable oocytes, and produced embryos. It was observed that AGD had a normal distribution and was highly variable among the Gyr population. A phenotypic association of a greater number of produced embryos as AGD decreased was identified, while a positive and low genetic correlation was observed between AGD and viable oocytes and AGD and embryo rate. In summary, for the Gyr breed, AGD was phenotypically inversely associated with a quantity-related parameter, such as the total number of produced embryos. In contrast, AGD showed a low genetic correlation with qualitative-related outcomes such as viable oocytes and embryo rate. For the second chapter, the objective was to characterize phenotypic traits such as body condition score (BCS), weight, Anti-Mullerian hormone, antral follicle count (AFC), and anogenital distance in nulliparous Holstein heifers and their association with fertility. All measures were collected on 698 Holstein heifers from a single heifer yard in the State of Kansas (USA). Data from weight and fertility outcomes were retrieved from the on-farm herd management system on weekly backups. A tendency of association between AMH and AGD and a correlation between AFC and AGD was identified. Greater pregnancy at first AI and all services were observed for nulliparous heifers on the High BCS, as for Moderate AMH groups. To conclude, for the nulliparous Holstein heifers an association between the phenotypes AMH, AFC, and AGD was identified, whereas just BCS and AMH were associated with fertility. For the third chapter, the objective was to estimate the genetic and phenotypic association of oocytes and embryo production with linear type traits in Gyr dairy cattle. A repeatability model was applied to 14,251 ovum pick-up events from 1,916 Gyr donors, while 604 donors from the same group had their body measurements taken, donors were from six different herds in Brazil. Moderate heritability was observed for IVEP (0.20 to 0.38) and type traits (0.22 to 0.40). A high genetic correlation (0.83 to 0.99) was observed between the IVEP traits, and low to high correlations (-0.07 to 0.90) were demonstrated for the type traits. Low phenotypic correlations

(0.01 to 0.13) between IVPE and type traits were observed, in contrast to a moderate genetic correlation indicated between IVEP and type traits, especially for ilium width (0.29 to 0.41), rump area (0.31 to 0.38), and hip height (0.23 to 0.33). Therefore, our study indicates the possibility of indirectly affecting IVEP selection based on type traits.

Keywords: Reproduction. Gyr cattle. Holstein cattle. Genetics.

RESUMO

MACHADO, Andréia Ferreira Machado, D.Sc. Universidade Federal de Viçosa, Fevereiro, 2024. **Associação de características fenotípica de genética com fertilidade em bovinos leiteiros.** Orientadora: Simone Eliza Facioni Guimarães.

Nesta tese, os três capítulos exploram a associação de características fenotípicas com a fertilidade de bovinos leiteiros. O objetivo do primeiro capítulo foi caracterizar a distância anogenital (AGD) como um representante do hormônio Anti-Mulleriano (AMH) e da produção *in vitro* de embriões (IVEP) em bovinos *Bos indicus* da raça Gir. Uma única medida de AGD foi coletada de 552 doadoras de seis rebanhos no Brasil. Cada doadora teve uma média calculada para número de oócitos recuperados, oócitos viáveis e embriões produzidos. Observou-se que AGD tinha a distribuição normal e era altamente variável na população Gir. Foi identificada uma associação fenotípica de maior número de embriões produzidos à medida que AGD diminuía, enquanto uma correlação genética positiva e baixa foi observada entre AGD e oócitos viáveis e entre AGD e taxa de embriões. Em resumo, para a raça Gir, AGD foi fenotipicamente associada de forma inversa a um parâmetro quantitativo, o número total de embriões produzidos. Em contraste, AGD apresentou uma baixa correlação genética com resultados qualitativos, como oócitos viáveis e taxa de embriões. No segundo capítulo, objetivou-se caracterizar fenótipos como o escore de condição corporal (BCS), peso, hormônio Anti-Mulleriano, contagem de folículos antrais (AFC) e distância anogenital em novilhas holandesas nulíparas e sua associação com a fertilidade. Todas as medidas foram coletadas em 698 novilhas Holandesas de um centro de recria de novilhas no Estado do Kansas (EUA). Os dados de peso e fertilidade foram recuperados do sistema de gestão do rebanho na fazenda por meio de backups semanais. Identificou-se uma tendência de associação entre AMH e AGD e uma correlação entre AFC e AGD. Maior taxa de prenhez ao primeiro serviço e em todos serviços foi observado para novilhas nulíparas no High BCS, da mesma forma que no grupo High AMH. Concluindo, para novilhas Holandesas nulíparas foi identificada uma associação entre os fenótipos AMH, AFC e AGD, enquanto apenas BCS e AMH foram associados à fertilidade. No terceiro capítulo, o objetivo foi estimar a associação genética e fenotípica da produção oocitária e de embriões com as características lineares de tipo em bovinos leiteiros da raça Gir. Um modelo de repetibilidade foi aplicado a 14.251 eventos de coleta de oócitos de 1.916 doadoras Gir, enquanto 604 doadoras do mesmo grupo tiveram suas medidas corporais tomadas, doadoras pertenciam a seis diferentes rebanhos no Brasil. Uma herdabilidade moderada (0,20 a 0,38) foi observada para as características de IVEP e tipo (0,22 a 0,40). Uma alta correlação genética (0,83 a 0,99) foi observada entre as características de IVEP, e correlações baixas a altas (-0,07 a 0,90) foram demostradas para as características de tipo. Correlações fenotípicas baixas (0,01 a 0,13) entre IVEP e tipo foram identificadas, em contraste a correlações genéticas moderadas entre IVEP e tipo, especialmente para largura do ílio (0,29 a 0,42), área de garupa (0,31 a 0,38) e altura de garupa (0,23 a 0,33). Portanto, nosso estudo indica a possibilidade de afetar indiretamente a seleção para IVEP ao selecionar características de tipo.

Palavras-chave: Reprodução. Gado Gir. Gado Holandês. Genética.

SUMMARY

1. GENERAL INTRODUCTION

A century ago, an unfavorable genetic correlation between reproduction traits and milk yield led to the deterioration of reproductive performance, since selection in dairy cattle was focused on milk production (BRITO et al., 2021; PRYCE et al., 2004; ROXSTRÖM et al., 2001). Currently, the focus of dairy cattle selection has shifted from increasing milk production exclusively to a balanced selection, focusing on longevity, health, and fertility (MIGLIOR et al., 2017). In addition to the widely applied reproductive technique of artificial insemination (AI), biotechniques such as *in vitro* embryo production (IVEP) have provided the opportunity to select high-merit cattle in a short interval of time (FERRÉ et al., 2020). However, a large variability is still observed in the IVEP results, affected by factors such as antral follicle population (ZANGIROLAMO et al., 2018), donors' age (BARUSELLI et al., 2022), and breed (BATISTA et al., 2014; GIMENES et al., 2015).

Brazil is the second largest country in embryo production in the world (VIANA, 2022), and the *Bos indicus* donors have a great importance on these numbers (SILVA et al., 2018; VIANA; FIGUEIREDO; SIQUEIRA, 2017). Gyr cattle, the most important *Bos indicus* dairy breed in Brazil, is resistant to heat and parasites and has been widely used to produce Gyr and Crossbred Holstein x Gyr embryos (MADALENA; PEIXOTO; GIBSON, 2012). The IVEP market is constantly growing in Brazil and the world (FERNANDES, 2022), thus studies of traits that can improve the efficiency of the IVEP technique are of high importance to increase the procedure efficiency. Additionally, the traditional reproductive rates usually used to estimate fertility, for example: calving to first service, days open to conception, calving interval, etc., are associated to low heritability (0.02 to 0.10; (BERRY et al., 2016; BERRY; WALL; PRYCE, 2014), and reliability of the breeding values (FLEMING et al., 2019). The inclusion of genomic data such as SNP markers and genome sequence has provided additional opportunities to improve reproductive breeding values reliability (FELTES et al., 2022; ORTEGA et al., 2016; VANRADEN et al., 2017). However, to increase genetic progress on IVEP and fertility it is necessary to improve the quality of the phenotypes and the number of records available, as well as a better record of the herd reproductive management (FLEMING et al., 2019; VIZONÁ et al., 2020).

Phenotypes that better express reproductive physiology with minimal influence on management decisions have the potential to complement the current phenotypes used to determine fertility in dairy cattle. Studies have demonstrated phenotypes such as Anti-Mullerian hormone (AMH), antral follicle count (AFC), and anogenital distance (AGD) with heritability values of 0.46 (GOBIKRUSHANTH et al., 2019), 0.31 (WALSH et al., 2014) and 0.37 (GOBIKRUSHANTH et al., 2019), respectively, associated to IVEP and fertility. Furthermore, the body condition score (BCS), of heritability ranging from 0.14 to 0.33 (LOKER et al., 2011), has been used as an indicator of a cow's energy balance, health, and fertility (ROCHE et al., 2009). Finally, type traits, as the rump measures, easy to obtain and of heritability ranging from 0.20 to 0.41 (EAGLEN et al., 2013), were associated with calving ease (CUE; MONARDES; HAYES, 1990) and more currently with puberty in heifers (SCHMIDT, 2021).

The AMH is a glycoprotein produced by the granulosa cells of preantral and small antral follicles, with production starting on the activation of the primordial follicles (LA MARCA; VOLPE, 2006; MCGEE; HSUEH, 2000; MOSSA et al., 2017). A high correlation (0.89) between AMH, AFC, and the total number of morphological health oocytes and follicles exists (IRELAND et al., 2011, 2008), indicating that AMH and AFC are good predictors of the ovarian reserve in cattle (IRELAND et al., 2008). Additionally, AMH measured at different days of the cycle and different cycles is highly correlated (r = 0.97), suggesting that a single blood sample would be a good predictor of the real AMH concentration (IRELAND et al., 2011). In the literature, AMH has been associated with superovulatory, IVEP and reproductive results. AMH was positively associated with *in vivo* embryo production followed of a superovulation protocol, a greater number of larger follicles, corpus luteum, and produced embryos were obtained at higher AMH concentrations cows (MONNIAUX et al., 2010; SOUZA et al., 2015). Likewise, the number of recovered and cultured oocytes and the number of *in vitro* produced embryos were superior in *Bos taurus* and *Bos indicus* donors with superior concentration of AMH (GUERREIRO et al., 2014). Nevertheless, the relationship between AMH and fertility is still variable, AMH was only associated to pregnancy/AI on estrus detection but not on ovulation synchronized cows (RIBEIRO et al., 2014). AMH was not associated to pregnancy/AI or pregnancy loss on dairy cows (GOBIKRUSHANTH et al., 2018), and a quadratic association between AMH concentration, fertility and longevity was suggested for dairy cows and nulliparous heifers, as cows and heifers with moderate-high AMH concentration showed greater reproductive performance and stayed longer in the herd, respectively (AKBARINEJAD et al., 2020; JIMENEZ-KRASSEL et al., 2015).

The AFC refers to the total number of antral follicles in the ovaries. The follicles of 3 mm or greater on both ovaries are counted by ultrasonography to determine the AFC (ALWARD; COCKRUM; EALY, 2023; BURNS et al., 2005). Although variations on AFC based on day of the estrus cycle (IRELAND et al., 2008), age (BURNS et al., 2005) and body condition exists (DE MORAES et al., 2019), AFC is highly repeatable within the same animal (IRELAND et al., 2007, 2011). AFC is a phenotypic marker positively associated with ovarian function (IRELAND et al., 2009; JIMENEZ-KRASSEL et al., 2009), number of transferable embryos, and the *in vitro* embryo production (CUSHMAN et al., 2009). Greater AFC at weaning was highly (r = 0.90) correlated with greater AFC in older ages (SILVA-SANTOS et al., 2014), and with the number of recovered oocytes, viable oocytes, and produced *in vivo* and *in vitro* embryos (CENTER et al., 2018; SANTOS et al., 2016; SILVA-SANTOS et al., 2014). Nonetheless, as the results observed for AMH the association between AFC and fertility is still not well established. Studies indicated a positive association between AFC and reproductive outcomes in dairy cows (MARTINEZ et al., 2016; MOSSA et al., 2012), whereas heifers with ≥25 follicles with ≥3 mm of diameter presented a reduced survival rate in the herd and reduced fertility compared to heifers with ≤15 follicles (Jimenez-Krassel et al., 2017). In the same way, pregnancy rate of Nellore cows submitted to timed insemination was influenced by AFC, as cows in the low AFC category presented greater pregnancy rate than intermediate or high AFC groups (DE MORAES et al., 2019).

The AGD is defined as the distance from the center of the anus to the base of the clitoris (GOBIKRUSHANTH et al., 2017). AGD variation is related to the fetus's exposure to androgens during the reproductive programming window in prenatal life (DEAN et al., 2012), influencing the genital tubercule migration (BOWMAN et al., 2003). The excessive exposure of female fetuses to androgens leads to underdevelopment of the reproductive system, which results in long AGD and low fertility rates in rodents and humans.(MENDIOLA et al., 2012; WU et al., 2017; ZEHR; GANS; MCCLINTOCK, 2001). AGD in cattle was identified as highly variable, normally distributed, influenced by postnatal factors, such as age and height (GOBIKRUSHANTH et al., 2017), and inversely related to fertility in dairy Holstein cows (CARRELLI et al., 2022; GOBIKRUSHANTH et al., 2017) and nulliparous heifers (CARRELLI et al., 2021). Cows with short AGD at first and second lactation had greater probability to became pregnant at first insemination compared to long AGD cows. In addition, nulliparous heifers with short AGD have a reduced age at first pregnancy, a greater pregnancy rate at first insemination and a lower number of inseminations per conception compared to long AGD heifers. AGD was also associated with a greater number of follicles in response to FSH treatment, fertilized ova, and viable embryos in Holstein cattle (RAJESH et al., 2023), and *in vitro* embryo production in Gyr cattle (MACHADO et al., 2023). Likewise, short AGD cows showed earlier commence and greater duration of estrous, larger preovulatory follicle, reduced concentration of progesterone at estrous, and greater ovulation rates (MADUREIRA et al., 2022). Nonetheless, AGD measured more than once in the same animal indicated a high to moderate genetic correlation between the AGD and the fertility traits, while a low phenotypic correlation was observed between the same features (STEPHEN et al., 2023). On top of that, the absence of an association between AGD and fertility was observed for Holstein heifers (BECI et al., 2023), suggesting that more studies must explore the association between these variables in animals of the same age and throughout the productive life of cattle.

The BCS, an indirect measure of the subcutaneous fat, is an indicator of the animal energy status (LEÓ et al., 2004; ROCHE et al., 2009). The effect of BCS on reproduction has been demonstrated by the reduced quality of embryos produced by superovulated heifers with a BCS of 3.5, in a 1 to 5 scale (KADOKAWA et al., 2008). In addition, cows with low BCS and high variation of the BCS have reduced pregnancy per artificial insemination (CARVALHO et al., 2014; PINEDO et al., 2022a) and increased chances of losing pregnancy (PINEDO et al., 2022b; STEVENSON; ATANASOV, 2022). Lastly, type traits such as the rump measures are historically associated with calving ease (CUE; MONARDES; HAYES, 1990), and unfavorable genetic correlated to fertility (HAILE-MARIAM; BOWMAN; GODDARD, 2004; WALL et al., 2005). More recent results of estimated breeding values indicated a negative correlation between IVEP results and rump width (-0.11) and angle (-0.29, VIZONÁ et al., 2020), while a low genetic correlation (0.08) between anogenital distance and viable oocytes (MACHADO et al., 2023), and a low phenotypic correlation (0.07) between vulva width and antral follicle count (MACULAN et al., 2018; MESQUITA et al., 2016) was suggested in dairy Gyr donors and Tabapuã cows, respectively. Similarly, vulva width and antral follicle count were also phenotypically associated with oocyte viability and in vitro embryo production efficiency in *Bos indicus* and *Bos taurus* breeds (DE VASCONCELOS et al., 2020), as a positive phenotypic correlation between body length (0.09) and thoracic depth (0.10) has been observed with AFC (MACULAN et al., 2018).

Therefore, this thesis was prepared in three chapters, where the objectives and hypothesis were to evaluate in Chapter 1 the association of Anti-Mullerian hormone and anogenital distance with in vitro embryo production in *Bos indicus* Gyr cattle, our biological hypothesis was that AGD is phenotypically and genetically associated with AMH and IVEP outcomes. Specifically, that the number of recovered oocytes, in vitro produced embryos, and AMH concentration would increase as the AGD decreases. Chapter 2 the objectives were to evaluate the association of body condition score, Anti-Mullerian hormone, antral follicle count, and anogenital distance with fertility in nulliparous Holstein heifers, we tested the hypothesis that BCS, weight, AMH, AFC, and AGD are associated with fertility outcomes in nulliparous Holstein heifers. In Chapter 3 our objectives were to assess the genetic and phenotypic parameters of oocytes and embryo production and their association with linear type traits on dairy Gyr cattle, our hypothesis was based on the possibility of a positive genetic and phenotypic correlation between type traits and IVEP.

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2. CHAPTER 1

This paper has been accepted for publication in the Journal of Dairy Science.

Phenotypic and genetic relationships among anogenital distance, Anti-Müllerian hormone, and *in vitro* embryo production in Gyr dairy cattle

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Running head: Relationship between AGD and IVP

Interpretative Summary: Anogenital distance has been shown to be a proxy inversely related to the fertility of artificially inseminated Holstein dairy cattle. Herein, we aimed to evaluate the phenotypic and genetic associations among anogenital distance, Anti-Müllerian hormone concentration, number of recovered oocytes, and *in vitro* embryo production in *Bos indicus* Gyr oocyte donors. A weak inverse phenotypic association was observed between anogenital distance and the total number of produced embryos. However, a weak positive genetic correlation was observed between these two parameters. Thus, phenotypically, the relationship was opposite to the genetic results. Further research is warranted.

ABSTRACT

Anti-Müllerian hormone **(AMH)** concentration and number of recovered oocytes **(ROOC)** are phenotypic parameters associated with *in vitro* embryo production. More recently, anogenital distance (**AGD**) has been proposed as a proxy for fertility in dairy cattle that is easy to collect at a low cost. The aim of the present study was to characterize the AGD and its phenotypic and genetic associations with AMH and *in vitro* embryo production in *Bos indicus* Gyr dairy cattle. Hypothesis was that the number of ROOC, *in vitro* produced embryos, and AMH concentration would increase as the AGD decreases. From July to December 2021, a single morphometrical measurement of AGD was collected in 552 donors from six herds in Brazil. A subset of donors had AMH assayed on the same day. Only OPU events that occurred up to 12 months preceding, and 7 months succeeding the AGD measurement were used to assess the association between AGD, AMH, and *in vitro* embryo production. Thus, 472 donors (1,551 ovum pick-up events, and 140 donors with AMH) were considered in the analysis. A raw average was calculated for each individual donor's ROOC, viable oocytes, total produced embryos, viability rate, and embryo rate (defined as total produced embryos/viable oocytes). Comparisons were conducted within age categories: 3 to <6 years or 6 to <10 years. Phenotypic associations were performed in SAS software. Genetic correlations were estimated using BLUPF90 family programs. The AGD (128.7 mm ± 14; Mean ± SD) had a normal distribution and was highly variable (83 to 172 mm) among the Gyr population. Our experimental hypothesis was partially supported by a phenotypic association of a greater number of total produced embryos ($R^2 = 0.023$; $P = 0.03$) as AGD decreased. Our results failed to support an increase in AMH concentration along with a decrease in AGD. In addition, positive and low genetic correlations were observed between AGD and viable oocytes ($r = 0.08$), and embryo rate ($r = 0.20$). Greater ($P < 0.001$) number of viable oocytes and embryos were observed in donors in the High compared to Intermediate and Low recovered oocytes categories within both ages categories. The age interval of 3 to < 6 years showed a greater number of recovered and viable oocytes for the High AMH compared to the Low category, but no differences were observed among the AGD categories. In summary, for the Gyr breed, AGD was phenotypically inversely associated with a quantity-related parameter, such as the total number of produced embryos. In contrast, AGD showed a low genetic correlation with qualitative-related outcomes such as viable oocytes and embryo rate. Further studies should be performed to validate these retrospective analyses and to better understand the association between anogenital distance and *in vitro* embryo production.

Keywords: Ruminant, *Bos indicus*, reproduction, ovum pick-up

INTRODUCTION

Reproductive biotechniques have played a key role in increasing reproductive efficiency, productivity, and genetic merit of cattle (Pellegrino et al., 2016; Ferré et al., 2020). *In vitro* embryo production **(IVEP)** has provided the opportunity to increase genetic values at shorter intervals from both dam and sire lines (Wiggans et al., 2017). One of the challenges to increasing the use of IVEP is the large embryo production variability among donors. For example, IVEP can be affected by factors such as antral follicle count **(AFC)** (Zangirolamo et al., 2018), age of the donor (Baruselli et al., 2022), nutrition (Armstrong et al., 2001; Sales et al., 2015), synchronization protocol (Elliff et al., 2019; Garcia et al., 2020), and breed (Batista et al., 2014; Gimenes et al., 2014). Thus, the identification of low-cost, easily measurable, and highly repeatable traits that could be used to indirectly improve donor selection for IVEP is a feasible practice and thus warrant further investigation.

Circulating concentrations of Anti-Müllerian hormone **(AMH)** (Rico et al., 2012; Vernunft et al., 2015) and AFC (Pontes et al., 2011; Gimenes et al., 2015; Santos et al., 2016) have been identified as biomarkers for reproductive efficiency, including IVEP. Both parameters are positively associated with ovarian reserves and reproductive success in humans and cattle (Ireland et al., 2011; Sermondade et al., 2019). For example, AMH in cattle has been shown to be positively associated with the number of recovered and cultured oocytes and the number of embryos produced but not with the efficiency of oocytes to fertilize and become embryos (Guerreiro et al., 2014). Moreover, greater AFC at weaning is highly associated with a greater AFC at older ages (Silva-Santos et al., 2014; Morotti et al., 2017) and with the number of recovered oocytes **(ROOC)**, viable oocytes, and embryos produced later in life (Silva-Santos et al., 2014). Additionally, AMH and AFC are highly repeatable within the same animal (Ireland et al., 2007, 2008). These findings make AMH and AFC reliable proxies to determine the number of viable oocytes and embryos produced per ovum pick-up **(OPU)** in cattle. Nonetheless, assessment of AMH and AFC requires collecting blood samples and conducting an assay or an ultrasound exam, respectively, which could potentially make it challenging to be obtained on commercial operations and in less developed countries.

Anogenital distance **(AGD)**, defined as the distance from the center of the anus to the base of the clitoris (Gobikrushanth et al., 2017), has been recently proposed as a proxy for fertility that is easily collected at a low cost and inversely associated with reproductive efficiency in dairy cattle (Gobikrushanth et al., 2017; Carrelli et al., 2021; Grala et al., 2021). For example, shorter AGD was associated with greater pregnancy per insemination at first service in nulliparous heifers (Carrelli et al., 2021) and lactating Holstein cows (Gobikrushanth et al., 2017; Carrelli et al., 2022). In a seasonal system, short AGD primiparous cows were more likely to re-calve within the first 6 weeks postpartum during the second lactation when compared to long AGD animals (Grala et al., 2021). Moreover, AGD has been identified as a highly variable, normally distributed trait (Gobikrushanth et al., 2017, 2018), unlikely to cause a significant decline in milk production when selecting for short AGD (Carrelli et al., 2022), and inversely associated with estrous activity, ovulation rate, and progesterone concentrations in dairy cattle (Madureira et al., 2022). More recently, AGD has been associated with a greater number of follicles in response to FSH treatment, fertilized ova, and viable embryos in superovulated Holstein cattle (Rajesh et al., 2023).

The initial studies using AGD were conducted in mice (Vom Saal and Bronson, 1978; Zehr et al., 2001) and humans (Callegari et al., 1987) in which excessive exposure of female fetuses to androgens during the reproductive programming window was associated with underdevelopment of the female reproductive tract, longer AGD, and lower fertility. Thus, AGD length is determined during prenatal life by the migration of the genital tubercle, which is androgen-dependent (Bowman et al., 2003; Dean et al., 2012). Similarly, sheep fetuses exposed to androgens have shown impaired AMH concentration in their antral follicles (Veiga-Lopez et al., 2012), reduced number of primordial and total follicles, and an increased percentage of primary, preantral, and antral follicles (Steckler et al., 2005; Smith et al., 2009). Likewise, sheep fetuses exposed to testosterone during fetal life showed absent or longer estrous cycles, follicular persistence, and luteal defects that impaired fertility (Steckler et al., 2007). However, the association between AGD, AFC, and AMH remains to be fully elucidated, especially at the genetic level.

The objective of this study was to characterize AGD as a proxy for AMH, and IVEP in *Bos indicus* Gyr cattle. Our biological hypothesis was that AGD is phenotypically and genetically associated with AMH and IVEP outcomes. Specifically, the experimental hypothesis was that the number of ROOC, *in vitro* produced embryos, and AMH concentration would increase as the AGD decreases. The current study was conducted using *Bos indicus* Gyr animals which are one of the predominant dairy breeds in Brazil, a country that has been key in developing and conducting IVEP in livestock (Viana et al., 2017, 2018). Moreover, the Gyr breed is an important dam line, extensively used in IVEP programs to produce replacement Gyr and crossbred Holstein x Gyr heifers for the tropical dairy systems (Rocha et al., 2022).

MATERIALS AND METHODS

All animal handling was performed according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research and approved by the Animal Care and Use Committee of the Universidade Federal de Viçosa (Viçosa, MG, Brazil) under protocol # 010/2022.

Donors, Anogenital Distance, Location, and Records of In Vitro Embryo Production

From July to December 2021, a single morphometrical measurement of AGD in millimeters (mm) and height at the hip, measured in centimeters (cm), were collected from 552 *Bos indicus* Gyr dairy cattle that were oocyte donors for commercial *in vitro* embryo production. Briefly, each donor was restrained in a chute, allowing the technicians to conduct the measurements. First, AGD was measured from the center of the anus to the base of the clitoris **(Figure 1)** by a single experienced technician using a 200 mm stainless steel caliper as described by Gobikrushanth et al. (2017). Donors with apparent vulvar swelling or laceration and cows 30 days prior to or after calving were not enrolled in the study (Rajesh et al., 2022). Subsequently, a livestock measuring stick was used to measure the height at the highest point of the hip (usually the sacrum bone) (Gobikrushanth et al., 2019).

Donors were raised and maintained in the states of Minas Gerais (five herds) and Rio de Janeiro (one herd), Brazil. The raising and management strategies varied among herds, but in general, donors were housed in grazing systems with *ad libitum* access to water and mineral salt and were supplemented with corn silage and concentrate.

Records of the commercial IVEP of each donor from May 2010 until February 2022 were retrieved from the dairy management software used in each herd. The records included the OPU date, ROOC, viable oocytes, and embryos produced per donor on each transvaginal OPU session. Produced embryos denote the total number of recovered embryos, regardless of quality or stage of development. In addition, the viability rate (number of viable oocytes/recovered oocytes) and embryo rate (defined as the number of produced embryos/viable oocytes) were calculated.

Considering the 12 years interval of OPU records in the initial dataset and the association of both IVEP outcomes and AGD with age (Gobikrushanth et al., 2017, 2018), Only OPU events that occurred up to 12 months preceding, and 7 months succeeding the AGD measurement were used to assess the association between AGD, AMH, and IVEP. The final dataset consisted of 472 donors and a total of 1,551 OPU events.

Due to the association of AGD with age and the large range of age in AGD collection observed in our study, the dataset was segregated into age categories, and analyses were performed within categories. The age categories were identified based on **Figure 2,** which shows the AGD, oocyte production, and AMH by donor's age in years. Based on these results, the dataset was divided into four categories: <3 years, 3 to <6 years, 6 to <10 years, and ≥10 years. Only results for categories 3 to <6 years and 6 to <10 years are being reported due to the low sample size in the other two categories.

Anti-Müllerian Hormone Assay

A subset of donors had blood samples collected from the coccygeal blood vessels using EDTA evacuated tubes on the same day of the AGD measurement. Only OPU events that occurred up to 12 mo preceding, and 7 mo succeeding the AGD measurement were used to assay AMH concentrations. Thus, 140 donors randomly selected from each farm were used to assay AMH concentrations **(Supplementary Table 1)**. Samples were placed on ice upon collection and centrifuged at 3,000 g for 15 min. Harvested plasma was placed into 1.5 mL Eppendorf® and frozen at – 20°C until assayed. Plasma concentrations of AMH (ng/mL) were determined using a bovine

AMH Elisa Kit (Ansh Labs, TX, USA), following instructions from the manufacturer. The intra- and inter-assay coefficients of variation were 2.37% and 2.47%, respectively.

Statistical Analysis for Phenotypic Associations

The information retrieved from the herds contained donor ID, herd, age at AGD measurement in years, AGD in mm, number of OPU recorded, age at OPU in years, ROOC, number of viable oocytes, total produced embryos, viability rate, embryo rate, AMH concentration, and hip height. The age of the donor in years at the time of AGD measurement was calculated by the difference between the date of birth and the date of measurements, and the age at OPU was calculated by the difference between the date of birth and the date of the OPU. Donor was considered as the experimental unit, and thus, a raw average was calculated for each individual donor's age and number of (1) recovered oocytes, (2) viable oocytes, (3) total produced embryos, (4) viability rate, and (5) embryo rate to account for donors with differences in the number of OPU sessions and OPU intervals. Viable oocytes were defined according to Leibfried and First (1979).

Phenotypic characterization and associations were performed using SAS version 9.4 (SAS Institute Inc.). The descriptive statistics, such as mean and standard deviation, were determined using the Means procedure. The associations of cow age and height with AGD were assessed by Mixed procedure, whereas their coefficients of determination (R^2) were assessed using the Reg procedure.

To assess the associations of AGD, and AMH with recovered oocytes, viable oocytes, total produced embryos, viability rate, and embryo rate, a mixed linear model was performed within each age category (3 to < 6 years and 6 to < 10 years). Herd and OPU age were included in the model as a random effect and fixed covariate, respectively. The coefficient of determination (R^2) of each model was assessed using the Reg procedure.

The associations among the variables were also evaluated based on tertiles within age groups of 3 to < 6 years and 6 to < 10 years using the Glimmix procedure. For that, donors were classified into three groups (i.e., 33% bottom, 33% intermediate, and 33% top values) of AGD, AMH, and ROOC: Short, Intermediate, and Long AGD, Low, Intermediate and High AMH concentration, and Low, Intermediate and High ROOC. The response variables evaluated were AGD, recovered oocytes, viable oocytes, total produced embryos, embryo rate, viability rate, and AMH. Herd and age at OPU were included in the model as a random effect and fixed covariate, respectively. Statistical differences were considered at $P < 0.05$, and a tendency to significance at $P > 0.05$ and $P \leq 0.1$.

Pedigree Data and Genetic Correlations

A pedigree file was retrieved from the herd management software of each herd and from the Brazilian Association of Zebu Breeders (i.e., Associação Brasileira dos Criadores de Zebu; ABCZ) containing records of 1,151 animals and up to five generations of each donor. The pedigree file was used to create the relationship matrix for the estimation of heritabilities and genetic correlations.

Uni and bivariate animal mixed models were performed for AGD, recovered oocytes, viable oocytes, total produced embryos, viability rate and embryo rate. Genetic analyses were not performed for AMH due to the low sample size. Heritabilities and genetic correlations were calculated according to Falconer and Mackay (1996). Variance components were obtained by the restricted maximum likelihood (REML) method using BLUPF90 family programs (Misztal et al. 2002). The model used was:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Za} + \boldsymbol{e}$$

where $\boldsymbol{y}$, $\boldsymbol{\beta}$, $\boldsymbol{a}$, and $\boldsymbol{e}$ are the vectors of observations, fixed effects, additive genetic random effects, and residual effects, respectively; $\boldsymbol{X}$ and $\boldsymbol{Z}$ are the incidence matrices of fixed and random effects, respectively. The model included effects of herd, age at AGD measurement (only for AGD), and age at OPU (only for IVEP traits). In the single-trait model, it was assumed that $\boldsymbol{a} \sim N(0, \boldsymbol{A}\sigma_a^2)$ and $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$, where σ_a^2 and σ_e^2 are the additive genetic, and residual variances, respectively; **A** is the relationship matrix, and **I** is the identity matrix. In the multi-trait model, it was assumed that $\boldsymbol{a} \sim N(0, G_0 \otimes \boldsymbol{A})$ and $\boldsymbol{e} \sim N(0, R_0 \otimes \boldsymbol{I})$, in which G_0 and R_0 are the additive genetic and residual (co)variance matrices, respectively.

RESULTS AND DISCUSSION

The current study assessed the associations of IVEP outcomes with AGD, recovered oocytes, and AMH. The rationale for this objective was based on observations that AFC, AMH, and AGD can be affected during prenatal life, influenced by androgens (Wolf et al., 2002; Steckler et al., 2005; Veiga-Lopez et al., 2012), and associated with fertility (Guerreiro et al., 2014; Monteiro et al., 2017; Carrelli et al., 2022, Springman et al., 2022). Nevertheless, other factors such as lactation status, environmental temperature (Akbarinejad et al., 2018; Succu et al., 2020), maternal nutrition (Sullivan et al., 2009; Mossa et al., 2013; Weller et al., 2016), and heritability (Walsh et al., 2014; Nawaz et al., 2018) are known for affecting the offspring AFC and AMH and should also be considered as potential parameters influencing fertility.

Indirect traits that are easily measured, highly repeatable, and collected at a low cost will help to enhance dairy cattle fertility, especially in breeds that have not been submitted to selection for fertility, such as the *Bos indicus* Gyr cattle. In that sense, AFC (Silva-Santos et al., 2014; Santos et al., 2016; Monteiro et al., 2017) and AMH (Guerreiro et al., 2014; Vernunft et al., 2015) have been associated with the recovered oocytes and IVEP outcomes in dairy cattle. However, the association between AGD, recovered oocytes, and IVEP outcomes remains to be elucidated in the literature.

Phenotypic Characterization of AGD in Gyr Cattle

One of the objectives of our study was to characterize the AGD distribution in a dairy Gyr cattle population (**Figure 3**). The AGD in the Gyr cattle population of the current study was normally distributed and highly variable (128.7 ± 14 mm; Mean ± SD). The bottom quartile ranged from < 101 to 110 mm, the two middle quartiles from 111 to 150 mm, and the top quartile from 151 to > 160 mm. The AGD distribution and variability identified in the current study are similar to those identified in previous studies using Holstein heifers (Carrelli et al., 2021) and lactating cows (Gobikrushanth et al., 2017, 2019; Carrelli et al., 2022). Nonetheless, the mean Gyr cattle AGD in the current study was slightly shorter than those observed in North American and Canadian Holstein cows (Carrelli et al., 2022; Gobikrushanth et al., 2017).

The descriptive statistics for the 472 donors and the 1,551 OPU events used for the statistical analysis are presented in **Table 1**. The table includes the number of donors, AGD, age at AGD measurement, average number of OPU sessions per donor, as well as the recovered oocytes, viable oocytes, embryos, viability, and embryo rate for each of the six herds participating on the current study. The number of recovered oocytes was similar among the farms participating in the study, while the total number of produced embryos presented more variation. The minimum and maximum ranges for each of the parameters were 83 to 172 mm for AGD, 1.5 to 15 years for the age at AGD, 1 to 14 OPU sessions per donor, 0 to 78 recovered oocytes per OPU session, 0 to

63 viable oocytes for OPU session, 0 to 25 embryos per OPU session, 0 to 100% viability rate and 0 to 100% embryo rate. Overall, the number of viable oocytes was 73% of the recovered oocytes, the number of embryos was 25.6% of the viable oocytes, and the number of embryos was 18.7% of the total recovered oocytes. These results are below the average (32%) of the embryos/recovered oocyte rates reported from IVEP for *Bos indicus* in Brazil (Viana et al., 2012). In agreement with previous studies (Vizoná et al., 2020; Feltes et al., 2022), IVEP outcomes can be highly variable, as evidenced in the current results. The IVEP outcomes can be affected by several factors such as nutrition, oocyte quality (Lonergan and Fair, 2016), day of the estrous cycle at OPU (de Wit et al., 2000), technician experience, medium used in the cultures, etc.

Association Between AGD and IVEP Outcomes

A positive association ($R^2 = 0.10$, $P < 0.01$) between age and AGD in the Gyr oocyte donors was identified **(data not shown)**. A similar association ($R^2 = 0.09$) has been reported for Canadian Holstein cows (Gobikrushanth et al., 2017) and Irish Holstein Friesians cows (Gobikrushanth et al., 2019). The height at the hip in the Gyr cattle of the current study also had a positive association ($R^2 = 0.10$, $P < 0.01$) with AGD, which agrees with previous studies in Canadian Holstein cows (Gobikrushanth et al., 2017) and Irish Holstein Friesians cows (Gobikrushanth et al., 2019), indicating an increase on AGD as the animal's height increase. In general, the phenotypic variation in AGD explained by age and height in Gyr donors was small.

Our experimental hypothesis was partially supported, that the number of recovered oocytes, *in vitro* produced embryos, and AMH concentration would increase as AGD decreases. Support for the hypothesis can be evidenced by a negative phenotypic association of the total number of produced embryos, although of small magnitude (**Table 2**; $R^2 = 0.023$, $P = 0.03$) with

AGD in the Gyr oocyte donors between 3 to < 6 years old. Our results are in agreement with Rajesh et al. (2023), who superstimulated lactating cows and heifers to compare the superovulation response, fertilization rate, embryo production, and embryo quality between short vs long AGD cows or heifers. The authors were able to demonstrate a tendency for a greater fertilization rate and a greater proportion of viable embryos when comparing short vs long AGD cows but not in heifers. Moreover, the superovulated lactating cow results are in agreement with the fertility results on dairy heifers (Carrelli et al., 2021) and cows (Gobikrushanth et al., 2017; Carrelli et al., 2022), and agree with the phenotypic results of a greater number of IVP embryos on short AGD Gyr donors in this study. However, our results failed to support a similar association of AGD and IVEP outcomes in donors in the 6 to < 10 years age category **(Table 2)**. The associations between AGD, AMH, and recovered oocytes with IVEP were also assessed by classifying donors into Long, Intermediate, and Short AGD categories in the 3 to < 6 years **(Table 3A)** and 6 to < 10 years categories **(Table 4A)**. In agreement with recent studies on superovulated Holstein cattle (Rajesh et al., 2023), no differences in IVEP were observed when donors in the Long AGD category were compared to donors in the Intermediate and Short AGD category. Moreover, no associations ($P > 0.05$) were identified between recovered oocytes, viable oocytes, and viability rate **(Table 2)** with AGD, while a tendency of association of small magnitude was observed between embryo rate (**Table 2**; $R^2 = 0.012$, $P = 0.06$) and AGD. The results of the current study also failed to support an association between AMH concentration and AGD **(Table 2)**, whereas previous studies identified a greater AMH concentration in Long AGD cows compared to Short AGD cows (Akbarinejad et al., 2020; Rajesh et al., 2023). A limitation of our study was the limited sample size for AMH concentration. Future prospective studies should be conducted to associate AGD with future IVEP performance.

Genetic Correlation among AGD and IVEP Outcomes

The genetic correlations of AGD with recovered oocytes, viable oocytes, total produced embryos, and embryo rate were low **(Table 5)**. The heritability estimated for AGD in the current study using Gyr donors (0.510 ± 0.13) was slightly greater than the one previously described of 0.37 ± 0.08 for Irish Holstein-Friesian cows (Gobikrushanth et al., 2019). The heritability estimates for IVEP outcomes ranged from 0.06 ± 0.09 to 0.48 ± 0.15 and is in the upper limit of estimates reported in the literature (Cornelissen et al., 2017).

To the best of our knowledge, this is the first report of the genetic correlation of AGD with IVEP outcomes. In general, the correlations among IVEP outcomes were high, as expected, given the derivation of some traits from others. The genetic correlations of AGD with IVEP outcomes were low and associated with a large standard error, most likely due to the limited sample size. A low to moderate positive genetic correlation was observed between AGD and embryo rate. Although this result could give us an idea of the direction of the genetic relationship between AGD and IVEP, the interpretation of this result should be considered cautiously, given the high standard deviation associated with the estimate. Grala et al. (2021) have reported a negative correlation of phenotypic AGD values with estimated breeding values for fertility traits (based on parent averages). In the current study, the correlations of phenotypic values of AGD with estimated breeding values for IVEP outcomes were slightly negative and very low (results not shown). Even though the genetic correlation was not as high as desirable for indicator traits, given the higher heritability and the possibility of obtaining earlier in life, AGD could still be beneficial to use to indirectly improve the success of genetic improvement in fertility. Thus, future work is needed to validate these results and evaluate the benefit of using AGD as a proxy for fertility.

Phenotypic Association among IVEP Outcomes

The associations among IVEP outcomes were assessed by regression (**Table 2**) and by classifying donors into Low, Intermediate, and High AMH categories **(Table 3B; Table 4B)** and Low, Intermediate, and High recovered oocytes **(Table 3C; Table 4C)** at the ages of 3 to < 6 and 6 to < 10 years old. Donors classified in the High AMH in the 3 to < 6 years age category had greater ($P \leq 0.01$) number of recovered and viable oocytes when compared to donors in the Low AMH category, no other differences were identified for AMH in both age ranges. High recovered oocytes category donors in both age ranges had a greater ($P \leq 0.01$) number of viable oocytes and total produced embryos compared to the Intermediate and Low categories, while the Intermediate category showed greater ($P \leq 0.01$) values for the same parameters compared to the Low recovered oocytes category. At the age of 3 to < 6 years AMH concentration and viability rate were greater ($P \leq 0.01$) for the High and Intermediate oocyte categories compared to the Low category. Moreover, in the category 6 to < 10 years, viability rate was greater for the High compared to the Low recovered oocytes category. Our results agree with previous reports in which donors with high AMH produce more recovered and viable oocytes (Guerreiro et al., 2014), however we failed to show associations in the age category of 6 to < 10 years, probably due to the small number of donors in each category (Low, Intermediate and High). Similarly, donors producing more recovered oocytes (Monteiro et al., 2017; Watanabe et al., 2017) produced a greater number of viable oocytes and embryos. However, controversial results have been shown about the relationship between recovered oocytes and oocyte viability rate. Some studies (Silva-Santos et al. 2014) have not found differences in oocyte viability on animals with high vs low recovered oocytes, while others (Santos et al. 2016) have been able to detect differences among donors with high, intermediate, and low oocyte production. Finally, no statistical difference was observed for the embryo rate between AMH or oocyte categories in the current study. Similarly, previous studies (Silva-Santos et al., 2014; Monteiro et al., 2017) also failed to demonstrate differences in the embryo rate associated with oocyte production and AMH (Guerreiro et al., 2014).

CONCLUSION

Anogenital distance was normally distributed and highly variable in the *Bos indicus* Gyr dairy cattle population used in this study. Oocyte donors with a greater ROOC per OPU presented, as expected, a greater number of viable oocytes and total number of produced embryos compared to donors with lower ROOC, while the same results were not confirmed for AMH. An inverse phenotypic association between anogenital distance and *in vitro* embryo production was evidenced in the total number of produced embryos. Genetically, the correlation of anogenital distance with *in vitro* embryo production was low and positive. Although the heritability of anogenital distance is high, the low phenotypic association and genetic correlations of anogenital distance with *in vitro* embryo production indicate that the possibility of using AGD as proxy for fertility in Gyr dairy cattle should be considered cautiously. Thus, future work is needed to validate these results, better understand the association between anogenital distance and *in vitro* embryo production, and evaluate the benefits of using AGD as a potential proxy for fertility.

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Table 1. Descriptive statistics (Mean ± SD) for anogenital distance (AGD) and *in vitro* embryo production parameters in 472 Gyr oocyte donor from six herds located in Brazil.

Herd[1]	n[2]	AGD[3]	Age at AGD[4]	OPU[5] Sessions/donor	ROOC[6]	Viable oocytes[7]	Embryo[8]	Viability Rate[9]	Embryo Rate[10]
MG1	124	131.9 ± 13.1	5.9 ± 2.4	3.0 ± 2.2	19.2 ± 11.4	14.7 ± 9.2	3.8 ± 2.9	76.6 ± 15.9	25.8 ± 17.2
MG2	160	124.3 ± 15.5	5.0 ± 2.4	4.2 ± 2.4	23.5 ± 12.3	18.2 ± 10.4	4.1 ± 3.5	77.4 ± 9.0	22.4 ± 16.0
MG3	79	128.9 ± 12.1	6.6 ± 2.2	2.3 ± 1.1	24.9 ± 13.0	19.4 ± 11.3	5.0 ± 4.0	77.9 ± 8.7	25.8 ± 17.7
MG4	37	128.4 ± 16.2	7.1 ± 3.6	2.5 ± 1.2	27.2 ± 16.5	17.4 ± 11.4	6.4 ± 5.3	64.0 ± 9.5	36.8 ± 17.4
MG5	46	132.2 ± 13.8	7.6 ± 2.6	3.4 ± 2.2	*	10.8 ± 4.6	3.6 ± 2.0	*	33.3 ± 18.4
RJ1	26	127.6 ± 12.5	6.3 ± 3.6	3.0 ± 2.0	17.1 ± 7.2	13.2 ± 7.0	2.3 ± 1.6	77.2 ± 10.9	17.4 ± 12.7
Overall	472	128.4 ± 14.4	6.0 ± 2.7	3.3 ± 2.2	22.4 ± 12.6	16.4 ± 10.1	4.2 ± 3.5	74.5 ±12.3	27.0 ± 17.4
Range[11]	472	83 - 172	1.5 - 15.7	1 - 14	0 - 78	0 - 63	0 - 25	0 - 100	0 -100

[1] Herds located in the states of Minas Gerais (MG), and Rio de Janeiro (RJ), Brazil;
[2] n = number of evaluated donors;
[3] AGD = Anogenital distance measured in mm;
[4] Age at AGD in years;
[5] OPU = Ovum pick-up;
[6] ROOC = Number of recovered oocytes per OPU session;
[7] Viable oocytes include the total number of oocytes classified as I, II, and III degrees (Leibfried and First, 1979);
[8] Total number of *in vitro* produced embryos per OPU session, regardless of quality or stage of development;
[9] Calculated as the division of viable oocytes (7)/recovered oocytes (6);
[10] Calculated as the division of produced embryo (8)/viable oocytes (7);
[11] Minimum – maximum.

Table 2. Regression coefficients and SEM of *in vitro* embryo production parameters on Anogenital distance (AGD) in Gyr oocyte donors classified by age

Endpoint	n[1]	Age category	AGD coefficient	SEM	*P*-value	R^2
Recovered oocytes	215	3 to < 6	-0.003	0.062	0.962	0.001
	118	6 to < 10	0.026	0.067	0.696	< 0.001
Viable oocytes	215	3 to < 6	0.001	0.05	0.989	< 0.001
	151	6 to < 10	0.047	0.047	0.315	0.002
Embryo	215	3 to < 6	-0.036	0.016	0.03	0.023
	151	6 to < 10	0.025	0.02	0.213	0.003
Viability rate	215	3 to < 6	< 0.001	0.001	0.853	< 0.001
	118	6 to < 10	< 0.001	0.001	0.82	0.002
Embryo rate	215	3 to < 6	0.001	0.001	0.068	0.012
	151	6 to < 10	< 0.001	0.001	0.927	< 0.001
AMH (ng/mL)	69	3 to < 6	0.001	0.003	0.718	0.002
	43	6 to < 10	< 0.001	0.002	0.879	< 0.001

[1] A total of 225 donors in the 3 to < 6 years old category had AGD measured. A subset of 69 donors had blood samples, and AMH assayed. Moreover, only 215 out of the 225 donors had ROOC information.

A total of 151 donors in the 6 to < 10 years old category had AGD measured. A subset of 43 donors had blood samples, and AMH assayed. Moreover, only 118 out of the 151 donors had ROOC information.

Table 3. Mean ± SEM of anogenital distance **(AGD)**, plasma AMH concentration, number of recovered oocytes **(ROOC)**, and in vitro embryo production in Gyr oocyte donors with 3 to < 6 years old classified into **(A)** Short (≤ 120 mm), Intermediate (121 - 132 mm), and Long AGD (≥ 133 mm), **(B)** Low (≤ 0.32 ng/mL), Intermediate (0.33 - 0.59 ng/mL), and High AMH (≥ 0.60 ng/mL), and **(C)** Low (≤ 17), Intermediate (18 - 26), and High (≥ 27) ROOC.

Parameter	**A) AGD**			**B) AMH**			**C) ROOC**		
	Short	Intermediate	Long	Low	Intermediate	High	Low	Intermediate	High
n[1]	73	76	76	24	23	22	72	71	72
AGD	112.7 ±0.7^{C}	126.2 ±0.7^{B}	142.3 ±0.7^{A}	126.9 ±2.8	128.4 ±2.9	130.0 ±2.9	128.6 ±1.9	125.0 ±1.8	128.6 ±1.8
Plasma AMH ng/mL	0.4 ±0.1	0.6 ±0.1	0.6 ±0.1	0.2 ±0.0^{C}	0.5 ±0.0^{B}	0.9 ±0.0^{A}	0.3 ±0.1^{B}	0.6 ±0.1^{A}	0.6 ±0.1^{A}
Recovered oocytes	25.5 ±2.9	24.8 ±2.9	25.2 ±2.9	18.8 ±2.1^{B}	23.0 ±2.0^{AB}	29.2 ±2.1^{A}	12.5 ±1.4^{C}	22.3 ±1.4^{B}	36.0 ±1.3^{A}
Viable oocytes	18.1 ±1.8	16.9 ±1.8	18.3 ±1.8	14.1 ±1.7^{B}	18.1 ±1.8^{AB}	22.9 ±1.8^{A}	8.5 ±0.8^{C}	16.5 ±0.8^{B}	29.1 ±0.1^{A}
Produced embryos	4.9 ±0.6	4.4 ±0.6	4.0 ±0.6	3.5 ±0.5	4.4 ±0.6	5.0 ±0.6	2.4 ±0.6^{C}	4.0 ±0.6^{B}	6.5 ±0.6^{A}
Viability rate (%)	73.2 ±3.1	70.9 ±3.1	74.5 ±3.1	72.3 ±2.8	79.3 ±2.8	79.2 ±2.8	67.7 ±3.2^{B}	73.5 ±3.2^{A}	76.2 ±3.2^{A}
Embryo rate (%)	28.0 ±3.1	26.5 ±3.1	24.5 ±3.2	26.3 ±3.1	25.8 ±3.2	23.2 ±3.2	26.0 ±3.1	25.2 ±3.0	22.3 ±3.0

Different letters within a row indicate a significant difference (P<0.001) within AGD, AMH, or recovered oocytes category.

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[1] A total of 225 donors in the 3 to < 6 years old category had AGD measured. A subset of 69 donors had blood samples, and AMH assayed. Moreover, only 215 out of the 225 donors had ROOC information.

Table 4. Mean ± SEM of anogenital distance **(AGD)**, plasma AMH concentration, number of recovered oocytes **(ROOC)**, and in vitro embryo production in Gyr oocyte donors with 6 to < 10 years old classified into **(A)** Short (≤ 125 mm), Intermediate (126- 136 mm), and Long AGD (≥ 137 mm), **(B)** Low (≤ 28 ng/mL), Intermediate (0.29 - 0.43 ng/mL), and High AMH (≥ 0.44 ng/mL), and **(C)** Low (≥15), Intermediate (16 - 22), and High (≥ 23) ROOC.

Parameter	**A) AGD**			**B) AMH**			**C) Recovered oocytes**		
	Short	Intermediate	Long	Low	Intermediate	High	Low	Intermediate	High
n[1]	52	49	50	14	14	15	39	39	40
AGD	115.9 ±1.0^{C}	130.7 ±1.0^{B}	145.8 ±1.0^{A}	135.6 ±4.9	129.0 ±4.9	133.5 ±4.8	127.1 ±3.0	132.2 ±3.0	130.1 ±2.9
Plasma AMH ng/mL	0.3 ±0.1	0.4 ±0.0	0.4 ±0.1	0.2 ±0.0^{C}	0.4 ±0.0^{B}	0.6 ±0.0^{A}	0.3 ±0.1	0.4 ±0.0	0.4 ±0.0
ROOC	21.6 ±1.9	19.3 ±2.0	22.4 ±2.0	19.2 ±2.5	23.1 ±2.6	22.6 ±2.6	10.8 ±0.7^{C}	19.7 ±0.7^{B}	32.1 ±0.7^{A}
Viable oocytes	15.2 ±1.4	14.1 ±1.5	16.0 ±1.5	13.4 ±2.1	16.2 ±2.1	16.4 ±2.1	7.8 ±0.9^{C}	14.1 ±0.9^{B}	25.2 ±0.9^{A}
Total embryos	4.4 ±0.7	4.3 ±0.7	5.0 ±0.7	4.9 ±1.1	4.8 ±1.1	5.6 ±1.1	2.1 ±0.7^{C}	4.7 ±0.7^{B}	7.2 ±0.6^{A}
Viability rate (%)	74.9 ±2.9	75.3 ±3.0	74.9 ±3.0	74.5 ±4.5	77.0 ±4.7	75.1 ±4.7	73.7 ±3.3^{B}	71.9 ±3.3^{AB}	78.8 ±3.3^{A}
Embryo rate (%)	32.5 ±3.1	30.0 ±3.1	33.6 ±3.2	36.0 ±5.0	36.2 ±5.0	35.7 ±4.9	28.4 ±3.9	34.4 ±3.9	28.2 ±3.8

Different letters within a row indicate a significant difference ($P<0.001$) within AGD, AMH, or recovered oocytes category

[1] A total of 151 donors in the 6 to < 10 years old category had AGD measured. A subset of 43 donors had blood samples, and AMH assayed. Moreover, only 118 out of the 151 donors had ROOC information.

Table 5. Genetic correlation[1] (above diagonal), phenotypic correlation[2] (below-diagonal), and heritability[3] (diagonal) of anogenital distance **(AGD)**, number of recovered oocytes **(ROOC)**, and *in vitro* embryo production in Gyr dairy cattle. Values within parenthesis depict SD.

Parameter	AGD	Recovered oocytes	Viable oocytes	Produced embryo	Viability rate	Embryo rate
AGD	**0.51 (0.13)**	–0.01 (0.33)	0.08 (0.29)	0.02 (0.27)	0.02 (0.89)	0.20 (0.79)
ROOC	0.01 (0.05)	**0.46 (0.15)**	0.99 (0.09)	0.84 (0.22)	0.99 (0.01)	NC
Viable oocytes	0.03 (0.06)	0.97 (0.003)	**0.48 (0.15)**	0.81 (0.15)	0.99 (0.09)	0.10 (0.84)
Total embryos	–0.06 (0.06)	0.62 (0.04)	0.61 (0.04)	**0.56 (0.16)**	0.40 (0.52)	NC
Viability rate	0.02 (0.05)	0.30 (0.04)	0.43 (0.04)	0.20 (0.06)	**0.31 (0.16)**	NC
Embryo rate	–0.08 (0.05)	–0.04 (0.05)	–0.10 (0.05)	0.57 (0.03)	–0.10 (0.05)	**0.06 (0.09)**

NC = not converged;

[1] Genetic correlations indicate the overall genetic similarity between two parameters;

[2] Phenotypic correlations indicate the degree to which two parameters co-vary among individuals in a population (i.e., they describe if animals with high values for one parameter also have high (or low) values for another parameter);

[3] Heritabilities indicate how much of the phenotypic variation of a given parameter is explained by the individual’s genetics.

Supplementary Table 1. Descriptive statistics (Mean ± SD) for anogenital distance (AGD) and in vitro embryo production parameters of the Gyr oocyte donor randomly selected for blood collection and AMH concentration.

Herds[1]	n[2]	AGD[3]	Age at AGD[4]	AMH[5]	OPU[6]	ROOC[7]	Viable oocytes[8]	Embryo [9]	Viability Rate[10]	Embryo Rate[11]
MG1	33	132.6 ± 14.1	5.4 ± 1.8	0.50 ± 0.36	3.1 ± 2.5	20.8 ± 10.3	16.5 ± 9.2	3.9 ± 2.6	79.3 ± 15.6	23.6 ± 15.6
MG2	52	124.4 ± 14.1	4.8 ± 2.2	0.47 ± 0.22	4.2 ± 2.4	26.1 ± 11.1	20.3 ± 9.5	4.8 ± 3.3	77.8 ± 9.7	23.6 ± 16.3
MG3	27	129.1 ± 11.7	6.2 ± 2.2	0.43 ± 0.26	5.6 ± 2.2	23.5 ± 10.6	17.9 ± 9.1	4.6 ± 3.6	76.2 ± 7.7	25.7 ± 19.8
MG4	7	142.4 ± 18.4	8.5 ± 3.5	0.22 ± 0.10	2.7 ± 1.2	19.9 ± 10.7	12.5 ± 8.5	5.1 ± 3.5	62.8 ± 12.1	40.8 ± 6.9
MG5	13	133.7 ± 15.8	8.1 ± 1.9	0.46 ± 0.20	3.1 ± 1.9	*	11.1 ± 6.2	3.4 ± 1.4	*	30.6 ± 20.8
RJ1	8	123.0 ± 11.3	5.2 ± 3.1	0.51 ± 0.35	2.9 ± 1.4	18.4 ± 7.8	12.7 ± 5.9	2.9 ± 1.6	69.0 ± 6.2	22.8 ± 9.6
Overall	140	130.9 ± 14.6	6.4 ± 2.5	0.43 ± 0.28	3.6 ± 2.2	21.7 ± 10.8	15.2 ± 9.3	4.3 ± 3.0	73.0 ± 12.5	27.8 ± 17.0
Range[12]	140	100 - 172	1.5 - 13	0.10 – 1.62	1 - 13	4 - 52	1 - 40	0 -17	25 -100	0 -100

[1] Herds located in the states of Minas Gerais (MG), and Rio de Janeiro (RJ), Brazil;

[2] n = number of evaluated donors;

[3] AGD = Anogenital distance measured in mm;

[4] Age at AGD = age at AGD measurement in years;

[5] Plasma Anti-Müllerian hormone in ng/mL;

[6] Number of Ovum pick-up sessions per donor;

[7] Number of recovered oocytes per OPU session;

[8] Number of viable oocytes include oocytes classified as I, II, and III degrees (Leibfried and First, 1979);

[9] Total number of in vitro produced embryos per OPU session, regardless of quality or stage of development;

[10] Calculated as the division of viable oocytes (8)/recovered oocytes (7);

[11] Calculated as the division of produced embryos (9)/viable oocytes (8);

[12] Minimum – maximum.

* Data not provided by the farm or not able to be calculated.

Figure 1. Positioning the digital caliper to measure anogenital distance: the distance from the center of the anus to the base of the clitoris.

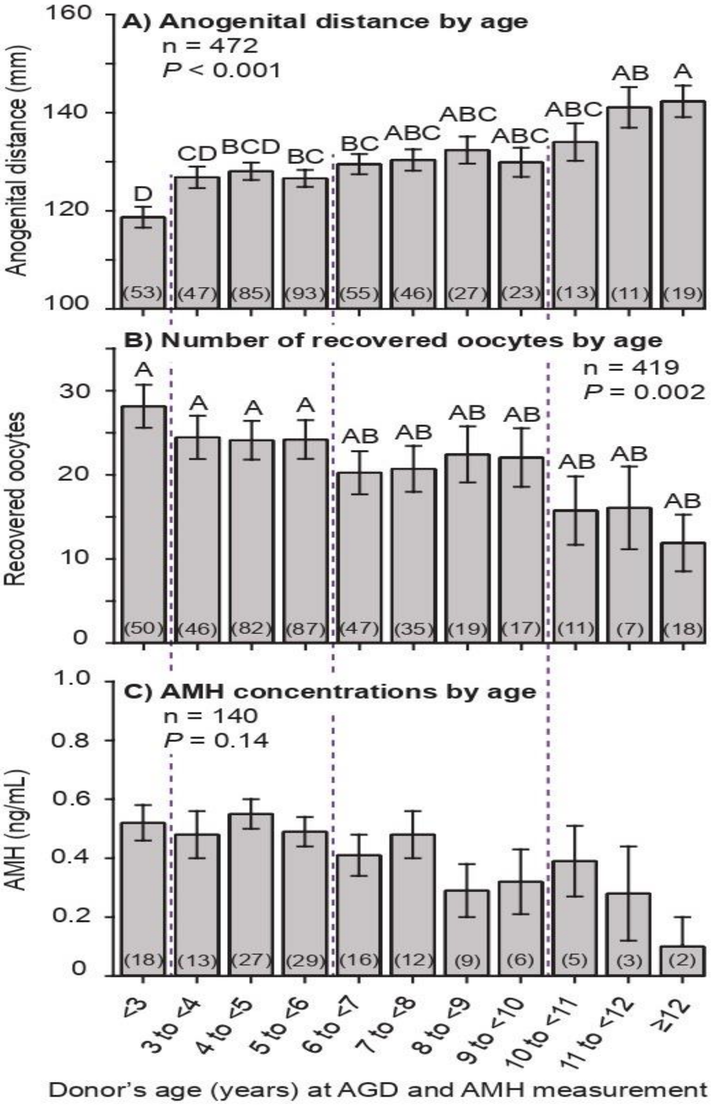

Figure 2. Mean ± SEM of anogenital distance **(AGD)**, plasma AMH concentration, and number of recovered oocytes **(ROOC)** depicted by donor's age. Different capital letters indicate significant differences ($P < 0.05$) among different ages. Numbers within the bars indicate the number of donors in each age classification. Purple dashed lines indicate the age categories that were selected for subsequent analysis: <3 years, 3 to <6 years, 6 to <10 years, and ≥10 years.

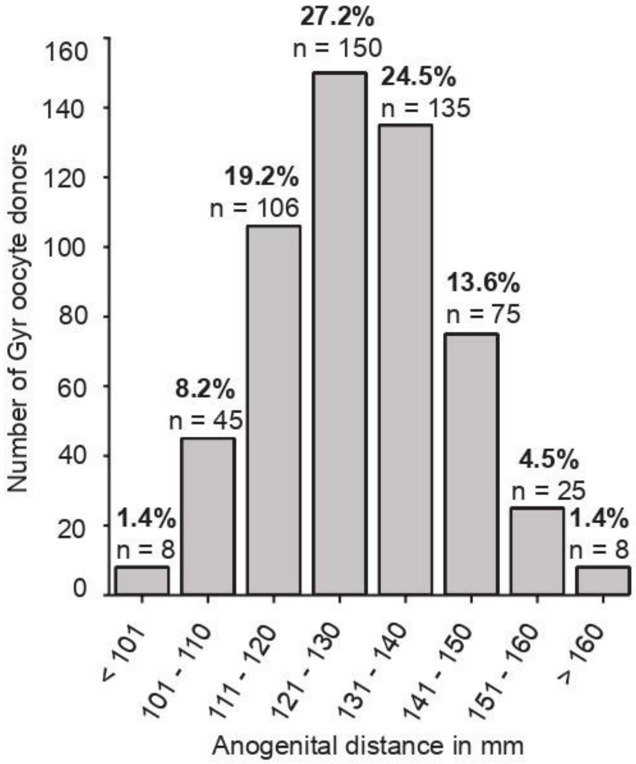

Figure 3. Distribution of anogenital distance **(AGD)** in mm in Gyr oocyte donors (n = 552) used for commercial *in vitro* embryo production in six herds located in Brazil.

3. CHAPTER 2

This manuscript was written to be submitted to the Journal of Dairy Science.

Association of body condition score, anogenital distance, and ovarian reserve-related traits with fertility in nulliparous Holstein heifers

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Running head: BCS, anogenital distance, and ovarian reserve-related traits in Holstein heifers

Interpretative Summary: Body condition score, Anti-Müllerian hormone, antral follicle count, and anogenital distance have been related to fertility in Holstein dairy cattle. Herein, we aimed to evaluate the correlation between these phenotypes and their association with fertility in nulliparous Holstein heifers. A tendency of correlation was observed between AMH, AFC, and AGD. Moreover, heifers with greater body condition score and moderate Anti-Müllerian hormone showed enhanced fertility (pregnancy at first AI and pregnancy of all services). However, no association between antral follicle count and anogenital distance with reproductive outcomes was observed.

ABSTRACT

The aim of our study was to associate the fertility of nulliparous Holstein heifers with (1) phenotypic traits that can be manipulated through nutrition and management, such as Body condition score **(BCS)** and weight; (2) intrinsic ovarian reserved-related traits, such as Anti-Müllerian hormone **(AMH)** concentration, antral follicle count **(AFC)**; and (3) novel indirect traits, such as anogenital distance **(AGD)** and vulva length **(VULVA)**. A second objective was to assess the correlation among those traits. From August 2022 to April 2023, 698 Holstein heifers had BCS, blood samples to measure AMH, an ultrasound evaluation to estimate AFC, and the AGD and vulva length measures taken. Data from weight measure and fertility outcomes were retrieved from the on-farm herd management system weekly backups. All the phenotypic traits were collected around 370 days of age, when the heifers started their breeding program, and were associated with the fertility outcomes until 520 days of age or five breedings. Correlations and comparisons were performed in SAS software. Our experimental hypothesis was partially supported by a tendency of correlation ($r = 0.07$; $P = 0.08$) between AMH and AGD and a correlation ($r = 0.08$; $P = 0.03$) between AFC and AGD. In addition, correlations between BCS and weight ($r = 0.08$; $P = 0.04$), weight and vulva length ($r = 0.15$; $P < 0.001$), and AMH and vulva length ($r = 0.09$; $P = 0.03$) were also identified. Data were ranked into tertiles (Bottom, Middle, Top) of BCS, weight, AMH, AFC, and AGD for further analysis. The BCS Bottom tertile showed lower weight (357.84 ± 0.59) compared to the Middle (359.91 ± 0.51) and Top BCS (360.29 ± 0.64) tertiles, while Bottom (94.86 ± 0.18) and Top BCS (94.62 ± 0.20) tertiles displayed a longer AGD. Similarly, Bottom weight tertile heifers exhibited lower BCS (3.48 ± 0.01) than Middle (3.51 ± 0.01) and Top (3.52 ± 0.01) weight groups. Heifers in the Top AMH tertile showed greater AFC (16.88 ± 0.17) compared to Middle (15.21 ± 0.16) and Bottom (14.93 ± 0.17) AMH tertiles, while heifers in the Top AFC tertile displayed greater AMH concentration (401.47 ± 7.10) than Middle (375.75 ± 6.34) and Bottom (356.51 ± 6.86) groups, and Middle AFC exhibited greater AMH compared to the Bottom AFC tertile. Our results failed to support an association of AGD with fertility outcomes in nulliparous Holstein heifers. In contrast, our hypothesis was supported by an association of AMH and BCS with fertility. That is, heifers in the Top BCS and the Middle AMH tertile have greater P/AI at first service and P/AI on all the services than their counterparts on the Bottom and Top tertile, respectively.

Keywords: Reproduction, ruminant, fertility, Anti-Müllerian hormone, antral follicle count

INTRODUCTION

Advancements in reproductive strategies, nutrition, and general management, along with the use of genetic selection, have led to an increased use of sex-sorted semen. Altogether, these factors have contributed to an oversupply of dairy replacements (Overton and Dhuyvetter, 2020). Despite the improvements in fertility, the life expectancy of dairy cattle currently averages 3 years after the first calving. Health problems, including failure to conceive, are major culling reasons (De Vries, 2020), with reproduction representing about 21% of those (USDA, 2018). Selection indices, such as net merit (**NM$**), aim to breed productive dairy cattle with greater health, fertility, and functional type (VanRaden et al., 2018). Genetic parameters related to fertility as days to first breeding, number of inseminations, nonreturn rate, calving interval although of low heritability and reliability of the estimated breeding values, were added to the NM$ in 2004 (VanRaden et al., 2004, 2017). Therefore, to advance the genetic progress for fertility and to help producers select more profitable replacement heifers in the herd, it is necessary to improve the number and quality of phenotypes associated with reproductive outcomes. Ideally, new fertility traits should be easily collected at a low cost and result in continuous rather than binary data.

Body condition score (**BCS**), which is an indirect measure of the subcutaneous fat associated with the animal's energy status (Leó et al., 2004), and BCS change in lactating dairy cows have been associated to pregnancy per AI (**P/AI**) (Carvalho et al., 2014; Pinedo et al., 2022a), and pregnancy losses (Pinedo et al., 2022b; Stevenson and Atanasov, 2022). For example, cows with low BCS and/or great variation in BCS change have been associated with lower P/AI and increased risk of pregnancy loss. Nevertheless, Holstein heifers submitted to a multiple ovulation protocol with a BCS of 3.5 produced fewer excellent-grade embryos compared to heifers classified with a BCS of ≤ 3 (Kadokawa et al., 2008). The assessment of the association of BCS and BCS change in reproductive management has been better elucidated in dairy cows than in heifers. Thus, assessing heifer BCS and its association with reproductive outcomes could be beneficial for the dairy cattle industry, as BCS is a phenotypic trait that can be manipulated.

Traits that are intrinsic to the animal, such as those related to their ovarian reserve, have also been associated with fertility. Specifically, Anti-Müllerian hormone **(AMH)** concentration and antral follicle count **(AFC)** have been shown to be reliable markers of the size of the ovarian reserve (Ireland et al., 2011; Monniaux et al., 2013), of moderate heritabilities (Nawaz et al., 2018; Walsh et al., 2014), and associated with oocyte and embryo production (Guerreiro et al., 2014; Silva-Santos et al., 2014; Morotti et al., 2017). However, the relationship of AMH and AFC with reproductive performance in dairy cattle is variable and still needs to be fully understood. Ribeiro et al. (2014) indicated a positive linear association of AMH concentration and pregnancy rate at spontaneous estrous detection, while no association of circulating AMH and reproductive variables was observed by Gobikrushanth et al. (2018). Moderate-high AMH cows (Akbarinejad et al., 2020) and dairy heifers (Jimenez-Krassel et al., 2015) had greater reproductive performance and stayed longer in the herd, respectively, compared to the least or greatest AMH herd mates, indicating a quadratic association between AMH and fertility. The inconsistency of results observed for AMH is also observed for AFC. Some studies demonstrated a positive association between AFC and reproductive outcomes in dairy cows (Mossa et al., 2012; Martinez et al., 2016), while heifers classified as ≥ 25 follicles were associated with suboptimal fertility and short herd lifetime (Jimenez-Krassel et al., 2017).

In recent years, anogenital distance (**AGD**, defined as the distance from the center of the anus to the clitoris) has been proposed as a proxy for fertility in dairy cattle. Despite some inconsistency across populations (Gobikrushanth et al., 2019b; Beci et al., 2023) and parity influence (Gobikrushanth et al., 2017a; Carrelli et al., 2022), AGD was inversely associated with reproductive outcomes in Holstein cows (Gobikrushanth et al., 2017a; Grala et al., 2021; Carrelli et al., 2022) and nulliparous heifers (Carrelli et al., 2021; Beci et al., 2023; Stephen et al., 2023). In addition, short AGD cows showed earlier commence and greater duration of estrous, larger preovulatory follicle, reduced concentration of progesterone at estrous, greater ovulation rates (Madureira et al., 2022), and greater percentage of fertilized ova, viable embryos (Rajesh et al., 2023), and *in vitro* embryo production (Machado et al., 2023). Anogenital distance is easy to measure, of moderate heritability (Gobikrushanth et al., 2019b; Stephen et al., 2023; Machado et al., 2023), and not influenced by the stages of the estrous cycle or lactation (Carrelli et al., 2022; Rajesh et al., 2022). Even so, a positive association between AGD and AMH has been described (Akbarinejad et al., 2019; Rajesh et al., 2023), no association between AGD and AFC has been demonstrated (Grala et al., 2021; Rajesh et al., 2023).

The aim of our study was to associate the fertility of nulliparous Holstein heifers with (1) phenotypic traits that can be manipulated through nutrition and management, such as BCS and weight; (2) intrinsic ovarian reserved-related traits, as AMH and AFC; and (3) novel indirect traits, as AGD and vulva length **(VULVA).** Thus, we tested the hypothesis that BCS, weight, AMH, AFC, and AGD are associated with fertility outcomes in nulliparous Holstein heifers. All the phenotypic traits were collected around 370 days of age when Holstein heifers started their breeding program and were associated with the reproductive outcomes until 520 days of age or five breedings.

MATERIALS AND METHODS

All animal handling was performed according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research and approved by the Institutional Animal Care and Use Committee of Kansas State University, under protocol #4784.

Heifers and Reproductive Management

The current study was conducted using 698 Holstein heifers (12.5 ± 0.16 month-old) from a single heifer yard in the State of Kansas (USA). Only heifers with Holstein sires and Holstein maternal grandsires were enrolled in the study. Heifers were house in a dry lot system, with *ad libitum* access to water, and fed a TMR formulated according to NRC (2001) guidelines to meet a gain of 1 kg per day.

Figure 1A displays the reproductive management of the regular heifer yard breeding program. Briefly, breedings started when heifers reached 370 days of age and weighed 300 kg. Heifers that did not reach the weight criteria at 370 days of age, were enrolled on the breeding program one month later. Heifers were moved to the breeding pen three days before they started reproductive management and were subsequently bred for seven days based on alerts from automatized activity monitors (**AAM**, eSense, SCR by Allflex, Netanya, Israel). Heifers that did not show estrus during this period received a prostaglandin F2α shot (**PGF1**) and breedings based on AAM continued for another week. Not-bred heifers received a second prostaglandin F2α shot (**PGF2**) seven days later so that breedings based on AAM could continue for another week. At this time, nonbred heifers were enrolled on a 5-day CIDR-synch timed AI protocol. Heifers had three chances to be bred on the timed AI, and AAM estrus breedings were allowed at any time during the breeding program. All breedings were conducted once a day in the morning, and mattings consisted of three chances to receive Holstein or Jersey sexed semen and two more chances to receive conventional Angus semen. Not pregnant heifers at 520 days of age or those not pregnant after the fifth breeding were flagged as “do not breed” and removed from the herd.

Pregnancy diagnoses were carried out via ultrasonography by the herd veterinarian weekly at 36 and 80 ± 3 days post-AI. Pregnancy was confirmed by the presence of a conceptus with a positive heartbeat. All the information on breeding and pregnancy diagnosis was recovered from weekly backups from the farm DairyComp 305 software (Valley Agricultural Software Inc.).

Experimental design, BCS, Weight, AMH, AFC, AGD, and VULVA

Figure 1B displays the experimental design. Briefly, heifers were enrolled in the study weekly as they reached the age (370 days) and the weight (300 Kg) to start on the breeding program. Collections were conducted every other week. Thus, heifers evaluated at each collection date were 11 and 4 days from the start of their breeding program. The experiment was conducted between August 2022 and April 2023.

On each collection date, heifers were maintained in a headlock system so that a single experienced technician could obtain the BCS, collect a blood sample, and measure the AGD and the vulva length distances. A 1 to 5 BCS scale was used in increments of 0.25 units (Edmonson et al., 1989). Blood samples were collected from the coccygeal blood vessels using EDTA evacuated tubes, and samples were placed on ice upon collection and centrifuged at 1,800 × g (J-6B centrifuge, Beckman Coulter Inc, Brea, CA). Harvested plasma was placed into 1.5 mL tubes and frozen at – 20°C until plasma concentrations of AMH (pg/mL) were determined using a bovine

AMH Elisa Kit (Ansh Labs, TX, USA), following instructions from the manufacturer. Intra- and inter-assay coefficients of variation were 2.85% and 10.04%, respectively.

The distance from the center of the anus to the base of the clitoris was measured using a 150 mm stainless steel digital caliper, as described by Gobikrushanth et al. (2017). Subsequently, the same technician using the same caliper took a measure of the vulva length, the distance from the center of the anus to the end of the ventral commissure of the vulva.

Three experienced technicians recorded ultrasound video of the left and right ovaries of each heifer using an Ibex-Evo II (E.I. Medical Imaging, Loveland, CO, US), equipped with a multilinear-array transducer set up at 7.5 MHz to access AFC. A single technician used recorded videos to count all the follicles ≥ 3 mm and determine the AFC for each heifer.

The weight of each heifer three days before starting the breeding program was collected by the farm crew and retrieved from DairyComp 305 backups (Valley Agricultural Software Inc.).

Data Handling and Statistical Analysis

The collected data and the information retrieved from the farm system were organized in a spreadsheet that contained information about heifer ID, birth date, AI start date, collection date, AI start age, collection age, season, BCS, weigh, AMH concentration, AFC, AGD, VULVA, $PGF_{2\alpha}$ date, number of services, AI date, AI age, days to breed, age at first AI, age at conception, AI bull, AI semen type, AI technician, pregnancy check at 36 days, pregnancy check at 80 days. During the analysis, 57 heifers that were enrolled late (> 380 months old) in the breeding program due to low weight were removed from the data. One heifer died during the experiment and before receiving the first AI, this animal was also completely removed from the dataset. Therefore, we collected 756 heifers, and we removed 1 dead and 57 old heifers from the data. Thus, the final number of heifers used in the analysis was 698.

The service of heifers that were removed from the herd before they had their pregnancy diagnosis were removed from the analysis. The numbers were: five heifers in the second service, eight heifers in the third service, five heifers in the fourth service, and seven heifers in the fifth service. For heifers that were pregnant from one given service, lost the pregnancy until 80 days, and were bred two or more times, the positive pregnancy was counted in the first service, and then, the other services were removed from the dataset.

Associations were performed using SAS version 9.4 (SAS Institute Inc.). The descriptive statistics were determined using the Means procedure. Before starting the analyses, we checked the association of AGD with age at the AGD measurement, season was added to the model as a fixed effect. The associations of body condition score, weight, AMH, AFC, AGD, and vulva length, and their coefficients of Pearson correlation were assessed using the Corr procedure.

The associations among the variables were also evaluated based on tertiles. For that, the Rank procedure was used to classify heifers into three groups (i.e., Bottom: 33% bottom, Middle: 33% intermediate, and Top: 33% top values) of BCS, weight, AMH, AFC, and AGD. Then, the Glimmix procedure was used to evaluate BCS, weight, AMH, AFC, AGD, P/AI at first service, P/AI for all services, and age at conception as response variables. Fixed effects were tertiles of BCS, weight, AMH, AFC, AGD, as well as season (June to September: season 1; October to May: season 2), PGF (yes or no), and service # (only for all P/AI) whereas random effects were AI tech and AI bull.

PGF (*P*-value = 0.41) and season (*P*-value = 0.48) were not significant for the P/AI at first service but were forced to stay in the model. Service # (*P*-value = 0.3), PGF (P-value = 0.24), and season (*P*-value = 0.64) were not significant for the P/AI at all services but were forced to stay in the model. Data is expressed as LS means ± SEM unless specified otherwise. Statistical differences were considered at $P < 0.05$, and a tendency to significance at $P > 0.05$ and $P \leq 0.1$.

RESULTS

The distribution of BCS, AMH, AFC, and AGD in nulliparous Holstein heifers is presented in **Figure 2**. The BCS in the nulliparous heifers was variable (3.50 ± 0.29; Mean ± SD), ranging from 2 to 4.5. The AMH concentration ranged from 13.40 to 1,711.40 pg/mL, with a greater number of heifers with AMH varying from 100 to 303 pg/mL. AFC distribution had a greater concentration of animals between 10 to 18 follicles (16.10 ± 6.41; Mean ± SD), while AGD was highly variable and normally distributed, ranging from 70.40 to 125.00 mm, (94.50 ± 7.84; Mean ± SD). The descriptive data of the 698 Holstein heifers classified according to the week of enrollment is available in **Supplementary Table 1**.

One of the objectives of the study was to assess the correlation coefficient between the phenotypic traits BCS, weight, AMH, AFC, AGD, and VULVA **(Table 1)**. Most of the correlations were of low intensity ($r < 0.10$), except for the correlation between AMH and AFC ($r = 0.53$; $P < 0.001$) and AGD and VULVA ($r = 0.66$; $P < 0.001$). Although of low magnitude, the correlations between BCS and weight ($r = 0.08$; $P = 0.04$), weight and vulva length ($r = 0.15$; $P < 0.001$), AMH and vulva length ($r = 0.09$; $P = 0.03$), and AFC and AGD ($r = 0.08$; $P = 0.03$) were significant. While a tendency was observed for a correlation between AMH and AGD ($r = 0.07$; $P = 0.08$).

The association between the phenotypes was assessed by regression and by classifying heifers into Bottom, Middle, and Top tertiles of BCS, weight, AMH, AFC, and AGD. For the BCS tertiles **(Table 2)**, heifers on Bottom BCS had lower weight (357.84 ± 0.59) and longer VULVA (123.94 ± 0.19) compared to the Middle (359.91 ±0.51; 123.16 ±0.17) and Top (360.29 ± 0.64; 123.67 ± 0.21) BCS groups. In addition, the weight did not differ between the Middle and Top BCS. Heifers in the Bottom (94.86 ± 0.18) and Top (94.62 ± 0.20) BCS categories showed longer AGD than the Middle group (94.0 ± 0.16). Additionally, heifers in the Top BCS category showed greater ($P = 0.02$) P/AI for all services (63.6 ± 3.0) than the Middle (53.4 ± 2.5) and Bottom (52.6 ± 2.9) groups, while a greater P/AI at first service ($P = 0.05$) was observed for the Top BCS (64.0 ± 3.8) compared to the Bottom (51.0 ± 3.8) BCS category. No differences were observed for AMH, AFC, or age at conception among the BCS tertiles.

For the weight tertiles (Bottom weight tertile [331.11 ± 0.57 Kg], Middle [359.11 ± 0.56 Kg], Top [387.82 ± 0.60 Kg]; **Table 3**), heifers in the Top (3.52 ± 0.01) and Middle (3.51 ± 0.01) weight tertiles showed greater BCS compared to the Bottom (3.48 ± 0.01) weight tertile. A tendency of association between AMH, and AFC to weight groups was observed, while no association between weight category and P/AI or age at conception was detected ($P > 0.05$). Results for the AMH concentration tertiles (Bottom [169.60 ± 6.94 pg/mL], Middle [319.70 ± 6.38 pg/mL] and Top [644.44 ± 6.94 pg/mL]) are displayed in **Table 4**. Heifers in the Top AMH category showed greater BCS (3.52 ± 0.01) compared to the Middle and Bottom AMH (3.49 ± 0.01) groups. In addition, heifers classified in the Top AMH category had on average 1.95 and 1.67 more follicles in the ovaries than the Bottom (14.93 ± 0.17) and Middle (15.21 ± 0.16) groups, respectively. Heifers on Middle AMH showed a greater P/AI at first service ($P = 0.04$) and P/AI on all services ($P = 0.02$) compared to the Top AMH heifer's category. No difference between

Bottom (56%) and Top (50%) AMH groups was observed for P/AI at the first service or all services. **Table 5** displays the results for the AFC tertiles. AMH concentration followed a linear effect, as heifers in the Top AFC category had greater AMH concentration (401.47 ± 7.11) compared to the Middle (375.75 ± 6.34) and Bottom (356.51 ± 6.86) categories, while heifers in the Middle category had greater AMH concentration than the Bottom AFC group. Heifers in the Top AFC category also showed longer AGD (P <0.001) compared to the Middle and Bottom AFC groups. No differences were observed for the AFC category and the fertility outcomes (P > 0.05). **Table 6** displays heifers classified as Bottom (86.99 ± 0.20 mm), Middle (94.43 ± 0.18 mm), and Top (102.07 ± 0.20 mm) AGD. In that sense, as AGD increased, vulva length also increased (P < 0.01). Also, a tendency of association was observed between BCS, weight, and AGD but no other differences or associations were observed. Furthermore, although age at conception was not significant at any of the tertiles, it was affected by the season (P = 0.02), and $PGF_{2\alpha}$ (P = 0.04), data not shown.

DISCUSSION

The current study assessed the association of the fertility of nulliparous Holstein heifers with (1) phenotypic traits that can be manipulated through nutrition and management, such as BCS and weight; (2) intrinsic ovarian reserved-related traits, such as AMH and AFC; and (3) novel indirect traits, such as AGD and vulva length. Rationale for this objective was two folds, first to associate measures that can be manipulated or are intrinsic to the heifers, and second, to associate historically related fertility traits such as AMH (Ribeiro et al., 2014; Mossa et al., 2017; Mossa and Ireland, 2019) and AFC (Ireland et al., 2007; Mossa et al., 2012; Alward et al., 2023) to new traits that are easy to measure, collected at a low cost, and highly repeatable as AGD. The justification for collecting vulva length was to explore a measurement associated with AGD that would be easier to measure.

The distribution and variability identified in the current study are similar to those identified in previous studies for BCS in beef heifers (Dickinson et al., 2019), AMH in Holstein dairy cows (Akbarinejad et al., 2019, 2020), AFC in Holstein heifers (Jimenez-Krassel et al., 2017) and AGD in Holstein dairy cows and heifers (Gobikrushanth et al., 2017; Carrelli et al., 2021). The correlation identified between AMH and AFC in this study was lower than demonstrated previously ($R^2 = 0.88$) for synchronized animals (Ireland et al., 2008). Nonetheless, a closer association was detected for AMH and AFC correlation ($R^2 = 29$) when it was measured at a random stage of the follicular wave (Gobikrushanth et al., 2017b). Similar to our results, the correlation ($R^2 = 48$) observed in a previous study between AGD measures (Beci et al., 2023) was likewise moderate as the correlation between AGD and vulva length, demonstrating that measures taken at the same region of the animal body are correlated. Additionally, associations between BCS and weight were observed before on dairy cows (Roche et al., 2007), although the association observed here was of lower intensity. Results of vulva measures associated with the weight of the animal were also displayed before. However, no associations between AMH and vulva length were observed (Maculan et al., 2018). Lastly, a tendency ($P = 0.08$) of correlation between AMH and AGD was described (Akbarinejad et al., 2019) before, while AFC and AGD were investigated, but no association ($r = -0.025$; $P = 0.66$) was observed between them (Grala et al., 2021). The correlations identified among AMH, AFC, and AGD, although low, would help explain the association of AGD with fertility in dairy cows (Gobikrushanth et al., 2017a; Carrelli et al., 2022) and heifers (Carrelli et al., 2021). In that sense, it is worth mentioning that these three phenotypes can be affected during prenatal life through the influence of factors such as androgens (Steckler et al., 2005; Veiga-Lopez et al., 2012).

Although BCS has been widely explored in dairy cows, especially concerning the variation during the dry period and lactation (Pinedo et al., 2022a; b; Stevenson and Atanasov, 2022), the acknowledgment of how BCS influences dairy heifer fertility remains to be explored. Body condition score is a subjective measure of the amount of stored fat on the body, usually used to indicate the animal's energetic status. Changes in the energy balance are ordinarily accompanied by body fat mobilization and changes in body weight (van Straten et al., 2009). Those changes make reasonable heifers with greater BCS display a higher weight, as high-weight heifers show greater BCS. However, one has to take into consideration that the animal's frame size, gestation, and breed can influence the measures of BCS and weight (Roche et al., 2009). In a previous study, BCS was negatively associated with AGD (Gobikrushanth et al., 2019b), the Middle BCS category showed a shorter AGD than the Bottom and Top groups, although the correlation between BCS and AGD was not significant and also negative in our data **(Table 2)**. A similar association between the BCS category and vulva length was also observed, considering the association between vulva length and AGD **(Table 2)**.

The association between body energy status and fertility has been reported in the literature (Roche et al., 2007; Carvalho et al., 2014; Pinedo et al., 2022a) and was also demonstrated in here. Top BCS heifers showed greater overall P/AI and a greater P/AI at first service than Bottle BCS groups, respectively. The body's energetic reserve has a direct effect on the hypothalamic-reproductive axis and uterine function (Beam and Butler, 1999; Wathes et al., 2007), cows increase pregnancy in the first AI linearly as BCS increases until 4.25 (scale 1 to 5, Stevenson and Atanasov, 2022). However, we must consider that most studies exploring the effects of BCS on fertility were performed in dairy cows during the post-partum period and negative energy balance. A study evaluating multiparous, non-lactating, and non-pregnant beef cattle showed a reduced ovulation rate and embryo recovery rate in High BCS compared to Moderate BCS cows (Bastos et al., 2023). Those results agree with a reduced quality of embryos produced by 3.5 BCS Holstein heifers compared to heifers with BCS around 3.0 (Kadokawa et al., 2008), that contrast with our current results.

Anti-Müllerian hormone is a marker of the ovarian reserve, involved in avoiding the premature exhaustion of the ovarian follicles, and a modulator of follicular development (Dewailly et al., 2014). Those facts help to explain why the Top AMH category displayed more AFC than the Middle and Bottom categories. As the Top AFC category presented a greater AMH concentration than the Middle and Bottom groups, and the Middle category had a greater AMH concentration compared to the Bottom AFC category. Moreover, heifers in the Top AMH group showed greater BCS than heifers in the Middle and Bottom categories. Additionally, a greater AMH concentration was associated with greater odds of becoming pregnant within 84 days at a seasonal herd (Gobikrushanth et al., 2019a), and a higher pregnancy rate at detected estrus (Ribeiro et al., 2014). Meanwhile a longer productive herd life, increased survival rate after the first calf and a greater percentage of pregnant cows were observed on Moderate-High AMH heifers (Jimenez-Krassel et al., 2015), similar to the greater pregnancy at first service and overall pregnancy rate observed for Middle AMH heifers in the current study.

Lastly, an association of AFC with AGD was tested before (Grala et al., 2021), but no effect was found to help explain the Top AFC category displaying heifers with longer AGD. We failed in our data to support our hypothesis of greater fertility on heifers with moderate to high AFC and short AGD. In the literature, controversial results show an association of greater AFC with fertility in dairy cows. Meantime, another study demonstrates a longer herd life for heifers displaying < 15 follicles of ≥ 3 mm compared to ≥ 25 follicle heifers (Jimenez-Krassel et al., 2017). In recent years, the inverse association of AGD with fertility has been explored (Gobikrushanth et al., 2017a; Carrelli et al., 2021, 2022). However, a more recently published study, also in heifers, reported fertility differences for AGD vulva and AGD rate variations of the AGD measurement but not in different AGD groups (Beci et al., 2023). Similarly, an experiment measuring AGD in the same animals more than once observed a low phenotypic association between AGD and fertility measures, while moderate to high genetic correlations were exhibited for the same traits (Stephen et al., 2023). Therefore, we must consider that changes during the cow's life, especially involving steroidal hormones, as during the estrous cycle, would be involved with changes in the AGD measurements, contributing to differences in fertility as the animal grow older. In that sense, more studies taking AGD measures throughout the animal's life would help to understand those associations.

CONCLUSION

Our results failed to support the hypothesis of an association between the anogenital distance with fertility outcomes in nulliparous Holstein heifers. Similarly, our results partially supported the hypothesis that intrinsic ovarian reserve-related traits would be associated with fertility. That is, although Anti-Müllerian hormone and antral follicle count were correlated, heifers with moderate Anti-Müllerian hormone but not antral follicle count had greater P/AI at first service and all services. In contrast, the results herein demonstrated a strong association between body condition score and P/AI in all services and at first AI: BCS ≥ 3.75 compared to BCS ≤ 3.25. These result did, however, not impact the age at conception.

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Table 1. Correlation coefficients of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), and vulva length are displayed on the upper diagonal. Probabilities (P-values) are displayed in parenthesis below each coefficient. The number of nulliparous heifers used on each correlation is displayed on the lower diagonal.

	BCS	Weight	AMH	AFC	AGD	VULVA
BCS	n = 673	0.08 (*P* = 0.04)	0.04 (*P* = 0.31)	0.03 (*P* = 0.39)	-0.03 (*P* = 0.47)	-0.03 (*P* = 0.46)
Weight	n = 671	n = 696	-0.03 (*P* = 0.48)	0.00 (*P* = 0.96)	0.06 (*P* = 0.12)	0.15 (*P* < 0.001)
AMH	n = 661	n = 684	n = 686	0.53 (*P* < 0.001)	0.07 (*P* = 0.08)	0.09 (*P* = 0.03)
AFC	n = 650	n = 674	n = 663	n = 675	0.08 (*P* = 0.03)	0.04 (*P* = 0.34)
AGD	n = 673	n = 696	n = 686	n = 675	n = 698	0.66 (*P* < 0.001)
VULVA	n = 635	n = 633	n = 625	n = 612	n = 635	n = 635

BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

Table 2. LS Means ± SEM of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), vulva length, pregnancy per AI at first service, pregnancy per AI for all services, and age at conception classified into Low, Moderate and High BCS

	Bottom	Middle	Top	P-Value
n	387	506	311	-
BCS range	≤ 3.25	3.50	≥ 3.75	-
BCS	3.16 ± 0.01^{C}	3.50 ± 0.01^{B}	3.85 ± 0.01^{A}	<0.001
Weight	357.84 ± 0.59^{B}	359.91 ± 0.51^{A}	360.29 ± 0.64^{A}	0.008
AMH	380.40 ± 6.73	377.44 ± 5.79	375.90 ± 7.28	0.90
AFC	15.58 ± 0.17	15.91 ± 0.14	15.53 ± 0.18	0.18
AGD	94.86 ± 0.18^{A}	94.00 ± 0.16^{B}	94.62 ± 0.20^{A}	0.001
VULVA	123.94 ± 0.19^{A}	123.16 ± 0.17^{B}	123.67 ± 0.21^{B}	0.008
P/AI at 1st service (%)	51.0 ± 3.8^{B}	54.7 ± 3.3^{AB}	64.0 ± 3.8^{A}	0.05
P/AI all services (%)	52.6 ± 2.9^{B}	53.4 ± 2.5^{B}	63.6 ± 3.0^{A}	0.02
Age at conception (d)	406.39 ± 6.03	404.71 ± 5.97	406.48 ± 6.07	0.51

[1] Values within a row with different uppercase superscript letters differ at $P < 0.05$; n: number of services; BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

Table 3. LS Means ± SEM of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), vulva length, pregnancy per AI at first service, pregnancy per AI for all services, and age at conception classified into Low, Moderate and High Weight

	Bottom	Middle	Top	P-Value
n	418	434	405	-
BCS	3.48 ± 0.01^{B}	3.51 ± 0.01^{A}	3.52 ± 0.01^{A}	<0.001
Weight	331.11 ± 0.57^{C}	359.11 ± 0.56^{B}	387.82 ± 0.60^{A}	<0.001
AMH	381.65 ± 6.43^{ab}	386.09 ± 6.37^{a}	366.00 ± 6.74^{b}	0.08
AFC	15.41 ± 0.16^{b}	15.70 ± 0.16^{ab}	15.91 ± 0.17^{a}	0.09
AGD	94.47 ± 0.17	94.69 ± 0.17	94.32 ± 0.18	0.33
VULVA	123.35 ± 0.18^{b}	123.51 ± 0.18^{ab}	123.91 ± 0.19^{a}	0.10
P/AI at 1st service (%)	58.8 ± 3.5	54.7 ± 3.6	56.5 ± 3.7	0.69
P/AI all services (%)	59.2 ± 2.7	52.4 ± 2.7	58.1 ± 2.8	0.17
Age at conception (d)	405.93 ± 5.98	406.15 ± 6.02	405.50 ± 6.03	0.93

[1] Values within a row with different uppercase superscript letters differ at $P < 0.05$;

[2] Values within a row with different lowercase superscript letters tended to differ with $0.05 < P < 0.10$.

n: number of services; BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

Table 4. LS Means ± SEM of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), vulva length, pregnancy per AI at first service, pregnancy per AI for all services, and age at conception classified into Low, Moderate and High AMH

	Bottom	Middle	Top	P-Value
n	412	411	412	-
BCS	3.49 ± 0.01^{B}	3.49 ± 0.01^{B}	3.52 ± 0.01^{A}	0.001
Weight	359.21 ± 0.61	360.06 ± 0.56	358.76 ± 0.61	0.28
AMH	169.60 ± 6.94^{C}	319.70 ± 6.38^{B}	644.44 ± 6.94^{A}	<0.001
AFC	14.93 ± 0.17^{B}	15.21 ± 0.16^{B}	16.88 ± 0.17^{A}	<0.001
AGD	94.57 ± 0.19	94.44 ± 0.17	94.48 ± 0.19	0.88
VULVA	123.54 ± 0.20	123.73 ± 0.18	123.50 ± 0.20	0.65
P/AI at 1st service (%)	56.0 ± 3.9^{AB}	63.2 ± 3.3^{A}	50.6 ± 4.1^{B}	0.04
P/AI all services (%)	56.6 ± 2.9^{AB}	62.2 ± 2.6^{A}	50.7 ± 3.0^{B}	0.02
Age at conception (d)	407.27 ± 6.06	403.92 ± 6.00	406.39 ± 6.01	0.13

[1] Values within a row with different uppercase superscript letters differ at $P < 0.05$;
n: number of services; BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

Table 5. LS Means ± SEM of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), vulva length, pregnancy per AI at first service, pregnancy per AI for all services, and age at conception classified into Low, Moderate and High AFC

	Bottom	Middle	Top	P-Value
n	409	397	415	
BCS	3.50 ± 0.01	3.51 ± 0.01	3.49 ± 0.01	0.22
Weight	358.68 ± 0.61	360.25 ± 0.56	359.11 ± 0.63	0.13
AMH	356.51 ±6.86^{C}	375.75 ±6.34^{B}	401.47 ± 7.11^{A}	<0.001
AFC	10.02 ± 0.17^{C}	14.94 ± 0.16^{B}	22.06 ± 0.18^{A}	<0.001
AGD	94.32 ± 0.19^{B}	94.07 ± 0.17^{B}	95.10 ± 0.19^{A}	<0.001
VULVA	123.75 ± 0.20	123.25 ± 0.18	123.77 ± 0.20	0.08
P/AI at 1st service (%)	54.3 ± 3.9	53.8 ± 3.6	61.8 ± 3.7	0.25
P/AI all services (%)	52.3 ± 2.9	56.8 ± 2.7	60.6 ± 2.9	0.17
Age at conception (d)	405.00 ± 6.02	407.82 ± 5.99	404.76 ± 6.05	0.14

[1] Values within a row with different uppercase superscript letters differ at $P < 0.05$;
n: number of services; BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

Table 6. LS Means ± SEM of body condition score (BCS), weight, Anti-Müllerian hormone (AMH), antral follicle count (AFC), anogenital distance (AGD), vulva length, pregnancy per AI at first service, pregnancy per AI for all services, and age at conception classified into Low, Moderate and High AGD

	Bottom	Middle	Top	P-Value
n	420	416	424	-
BCS	3.51 ± 0.01^{a}	3.51 ± 0.01^{a}	3.49 ± 0.01^{b}	0.08
Weight	358.20 ± 0.64^{b}	359.38 ± 0.58^{ab}	360.45 ± 0.64^{a}	0.08
AMH	370.09 ± 7.27	379.02 ± 6.55	384.63 ± 7.29	0.43
AFC	15.53 ± 0.18	15.64 ± 0.16	15.86 ± 0.18	0.49
AGD	86.99 ± 0.20^{C}	94.43 ± 0.18^{B}	102.07 ± 0.20^{A}	<0.001
VULVA	122.66 ± 0.21^{C}	123.32 ± 0.19^{B}	124.80 ± 0.21^{A}	<0.001
P/AI at 1st service (%)	55.8 ± 3.6	57.4 ± 3.6	56.8 ± 3.6	0.94
P/AI all services (%)	55.7 ± 2.7	57.2 ± 2.8	56.9 ± 2.8	0.92
Age at conception (d)	406.71 ± 6.04	404.61 ± 6.01	406.26 ± 6.03	0.45

[1] Values within a row with different uppercase superscript letters differ at $P < 0.05$;

[2] Values within a row with different lowercase superscript letters tended to differ with $0.05 < P < 0.10$.

n: number of services; BCS: Scale 1 to 5 (0.25 increments); Weight in Kg; AMH in pg/mL; AGD and VULVA (vulva length) in mm.

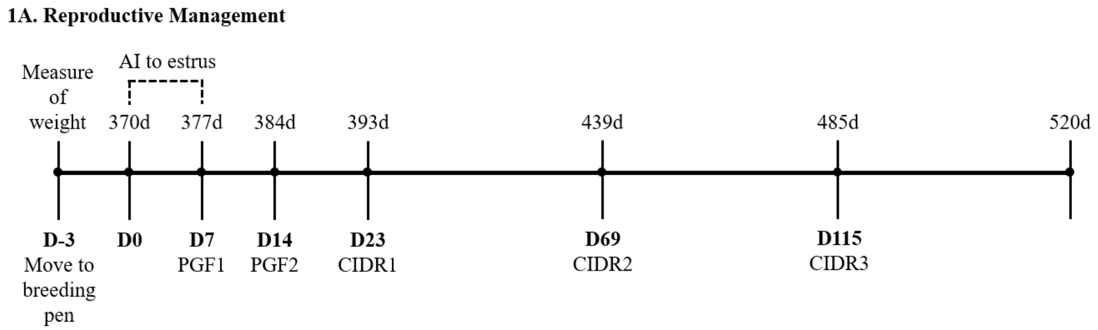
Supplementary Table 1. Descriptive data of nulliparous Holstein heifers by week of enrollment in the study.

Study Week	n (%)	AI start	Age at AI start	Collection date	Age at collection date	Average BCS[1] (min – max)	Average weight (min – max)	Average AMH[2] (min – max)	Average AFC[3] (min – max)	Average AGD[4] (min – max)	Number of pregnant heifers at 520 days (%)	Average age at conception
1	44 (6.3%)	8/22/22	12.3 (12.2-12.4)	8/26/22	12.4 (12.3-12.5)	3.5 (3.0-3.75)	392.1 (333.3-488.4)	343.1 (17.7-693.8)	22.5 (10-38)	96.9 (84.2-125.0)	42.0 (95.5%)	401.9 (370.0-514.0)
2	36 (5.1%)	8/29/22	12.3 (12.0-12.5)	9/2/22	12.4 (12.1-12.6)	3.3 (2.75-3.75)	393.3 (329.7-470.7)	362.8 (71.1-1254.0)	15.9 (5-32)	92.4 (70.4-112.9)	32 (88.9%)	404.3 (372.0-384.0)
3	32 (4.6%)	9/5/22	12.3 (12.2-12.4)	9/9/22	12.4 (12.3-12.5)	3.2 (2.0-3.75)	379.1 (324.7-437.2)	359.0 (65.1-1711.4)	13.8 (4-36)	96.8 (82.5-110.8)	32 (100.0%)	395.3 (370.0-463.0)
4	33 (4.7%)	9/12/22	12.3 (12.2-12.4)	9/16/22	12.4 (12.3-12.5)	3.3 (2.75-3.75)	378.9 (330.2-431.7)	314.3 (49.8-689.8)	13.6 (4-28)	90.4 (81.2-104.4)	33 (100.0%)	395.4 (368.0-468.0)
5	31 (4.4%)	9/19/22	12.3 (12.2-12.4)	9/23/22	12.4 (12.3-12.5)	3.4 (3.0-3.75)	387.7 (321.5-427.2)	458.3 (101.8-838.4)	16.2 (6-29)	93.6 (80-110.7)	31 (100%)	400.2 (367.447.0)
6	9 (1.3%)	9/26/22	12.3 (12.3-12.4)		12.9 (12.9-13.0)	3.5 (3.25-3.75)	362.9 (330.2-401.8)	394.5 (73.3-686.4)	13.1 (5-21)	89.0 (74.7-101.4)	9 (100%)	400.1 (372.0-452.0)
7	1 (0.1%)	10/3/22	12.2 (12.2-12.2)	10/14/22	12.5 (12.5-12.5)	3.2 (3.25-3.25)	403.2 (403.2-403.2)	339.2 (339.2-339.2)	14.0 (14-14)	72.9 (72.9-72.9)	1 (100%)	401 (401.0-401.0)
8	17 (2.4%)	10/10/22	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.5 (3.25-4.0)	375.4 (330.6-415.9	377.7 (90.5-1171.8)	12.6 (5-23)	92.6 (77.9-103.8)	16 (94.1%)	411.8 (376.0-500.0)
9	20 (2.9%)	10/17/22	12.2 (12.2-12.4)	10/28/22	12.6 (12.5-12.7)	3.4 (3.0-4.0)	353.2 (322.9-389.6)	495.9 (24.1-1481.9)	16.4 (6-26)	91.3 (77.1-110.4)	19 (95.0%)	388.3 (367.0-410.0)
10	20 (2.9%)	10/24/22	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.3 (3.00-3.75)	382.0 (317.0-435.4)	406.4 (109.8-1004.3)	15.8 (7-32)	93.5 (81-109.4)	20 (100%)	396.5 (374.0-481.0)
11	16 (2.3%)	10/31/22	12.3 (12.2-12.4)	11/11/22	12.6 (12.5-12.7)	3.7 (3.5-4.0)	372.6 (329.3-421.8)	534.4 (105.0-1544.6)	15.6 (4-35)	94.3 (84.4-103.7)	15 (93.8%)	397.7 (369.0-436.0)
12	22 (3.1%)	11/7/22	12.3 (12.2-12.4)		12.4 (12.3-12.5)	4.0 (3.5-4.5)	367.6 (331.1-405.4)	397 (46.7-1217.4)	14.4 (7-24)	89.7 (71.8-108.0)	21 (95.5%)	388.1 (371.0-449.0)
13	29 (4.1%)	11/14/22	12.3 (12.2-12.4)	12/2/22	12.9 (12.8-13.0)	3.7 (3.25-4.25)	372.9 (327.4-431.3)	383.0 (105.6-884.6)	13.6 (6-25)	96.0 (84.3-108.0)	28 (96.6%)	399.8 (372.0-475.0)

14	24 (3.4%)	11/21/22	12.3 (12.2-12.4)		12.6 (12.5-12.7)	4.0 (3.5-4.25)	392.8 (335.1-440.4)	401.2 (121.1-1076.9)	15.7 (8-40)	97.5 (83.5-107.5)	24 (100%)	397.0 (371.0-472.0)
15	14 (2.0%)	11/28/22	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.6 (3.25-4.25)	373.7 (340.6-407.3)	418.3 (45.8-809.6)	17.8 (6-24)	92.9 (80.6-102.7)	14 (100%)	389.7 (372.0-423.0)
16	21 (3.0%)	12/5/22	12.3 (12.2-12.4)	12/16/22	12.6 (12.5-12.7)	3.7 (3.5-4.0)	358.0 (319.3-392.7)	422.2 (101.5-978.3)	18.9 (10-29)	98.5 (79.5-111.7)	20 (95.2%)	396.4 (372.0-455.0)
17	24 (3.4%)	12/12/22	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.7 (3.25-4.0)	370.9 (325.6-444.0)	329.6 (108.3-829.5)	16.8 (8-30)	92.3 (82.4-104.3)	24 (100%)	388.0 (367.0-417.0)
18	13 (1.9%)	12/19/22	12.3 (12.2-12.4)	1/6/23	12.9 (12.8-13.0)	3.6 (3.25-4.0)	363.1 (337.4-411.3)	295.6 (13.4-569.9)	13.9 (7-22)	100.2 (89.0-115.9)	13 (100%)	400.1 (373.0-468.0)
19	15 (2.1%)	1/2/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.6 (3.0-4.0)	371.3 (314.3-403.2)	288.3 (96.3-579.0)	14.7 (7-21)	92.3 (82-102.4)	15 (100%)	402.6 (373.0-451.0)
20	20 (2.9%)	1/9/23	12.3 (12.2-12.4)	1/20/23	12.6 (12.5-12.7)	3.6 (3.0-4.0)	379.0 (338.8-432.2)	311.8 (104.4-843.6)	14.6 (5-23)	95.7 (83.8-110.3)	19 (95.0%)	395.2 (373.0-445.0)
21	15 (2.1%)	1/16/23	12.2 (12.2-12.3)		12.4 (12.3-12.4)	3.4 (3.0-3.75)	373.9 (321.5-443.5)	228.1 (73.4-612.3)	11.8 (7-17)	93.7 (82-103.3)	15 (100%)	398.5 (369.0-437.0)
22	5 (0.7%)	1/23/23	12.3 (12.2-12.5)	2/3/23	12.7 (12.6-12.9)	3.5 (3.0-3.75)	368.9 (341.5-385.0)	237.8 (101.6-371.6)	12.3 (9-17)	102.1 (93.9-110.7)	5.0 (100%)	407.4 (374.0-506.0)
23	21 (3.0%)	1/30/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.6 (3.25-3.75)	367.1 (334.7-411.3)	412.7 (14.2-1001.1)	15.5 (5-28)	95.0 (85-101.8)	21 (100%)	401.5 (376.0-486.0)
24	14 (2.0%)	2/6/23	12.3 (12.2-12.4)	2/17/23	12.6 (12.5-12.7)	3.6 (3.25-4.0)	358.8 (337.9-399.5)	343.0 (149.7-583.7)	18.4 (8-29)	96.4 (82.8-112.1)	14 (100%)	387.8 (372.0-429.0)
25	33 (4.7%)	2/13/23	12.2 (12.2-12.4)		12.4 (12.3-12.5)	3.5 (3.25-4.0)	359.9 (313.4-430.4)	400.5 (91.4-1103.3)	17.2 (4-33)	99.0 (86.1-111.2)	33 (100%)	396.2 (372.0-474.0)
26	19 (2.7%	2/20/23	12.3 (12.2-12.4)	3/3/23	12.7 (12.6-12.7)	3.5 (3.0-3.75)	371.5 (336.1-423.1)	347.3 (112.7-782.6)	19.6 (9-38)	96.9 (84.8-105.4)	19 (100%)	394.4 (375.0-447.0)
27	22 (3.1%)	2/27/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.4 (3.0-3.75)	373.3 (345.1-406.3)	331.0 (21.3-732.7)	15.9 (8-28)	94.8 (79.3-117.7)	20 (90.9%)	394.4 (369.0-441.0)
28	12 (1.7%)	3/6/23	12.2 (12.2-12.4)	3/17/23	12.6 (12.5-12.7)	3.5 (3.0-3.75)	379.8 (327.0-449.0)	554.3 (127.2-1390.6)	20.6 (3-45)	95.7 (89.1-111.9)	12 (100%)	386.1 (369.0-411.0)

29	18 (2.6%)	3/13/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.4 (3.0-3.75)	368.7 (319.3-422.2)	359.0 (49.7-869.3)	15.8 (7-34)	90.6 (73.9-107.3)	16 (88.9%)	397.4 (369.0-449.0)
30	20 (2.9%)	3/20/23	12.3 (12.2-12.4)	3/31/23	12.6 (12.5-12.7)	3.5 (3.0-3.75)	375.5 (331.1-441.3)	440.1 (129.4-1099.4)	17.0 (9-48)	95.0 (83.1-106.6)	18 (90.0%)	394.2 (368.0-447.0)
31	14 (2.0%)	3/27/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.25 (2.75-3.5)	378.6 (324.3-425.9)	286.4 (87.9-665.3)	16.6 (10-36)	91.6 (74.7-105.5)	12 (85.7%)	399.5 (381.0-425.0)
32	14 (2.0%)	4/3/23	12.3 (12.2-12.4)	4/14/23	12.6 (12.5-12.7)	3.3 (2.75-3.5)	370.3 (325.2-427.7)	405.5 (159.6-889.4)	18.2 (10-30)	93.7 (80-108.2)	12 (85.7%)	394.0 (368.0-427.0)
33	27 (3.9%)	4/10/23	12.3 (12.2-12.4)		12.4 (12.3-12.5)	3.3 (2.75-3.75)	366.3 (308.8-443.5)	374.7 (121.8-913.4)	17.4 (5-43)	94.2 (86-105.4)	21 (77.8%)	398.0 (370.0-436.0)
34	23 (3.3%)	4/17/23	12.3 (12.2-12.4)	4/28/23	12.6 (12.5-12.7)	3.4 (3.25-3.5)	362.7 (311.1-405.9)	344.9 (29.1-828.3)	12.4 (8-25)	95.6 (84.9-107.4)	18 (78.3%)	391.7 (371.0-417.0)
TOTAL	698 (100%)	-	12.3 (12.0-12.5)	-	12.5 (12.1-13.0)	3.5 (2.0-4.5)	374.5 (308.8-488.4)	376.1 (13.4-1711.4)	16.1 (3-48)	94.5 (70.4-125.0)	663 (95.0%)	396.8 (367.0-514.0)

[1]BCS = body condition score; [2]AMH = Anti-Müllerin hormone in pg/mL; [3]AFC = Antral follicle count; [4]AGD = anogenital distance in mm.


1A. Reproductive Management

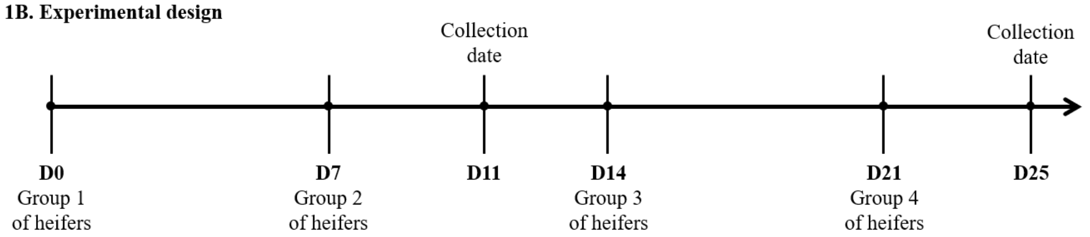
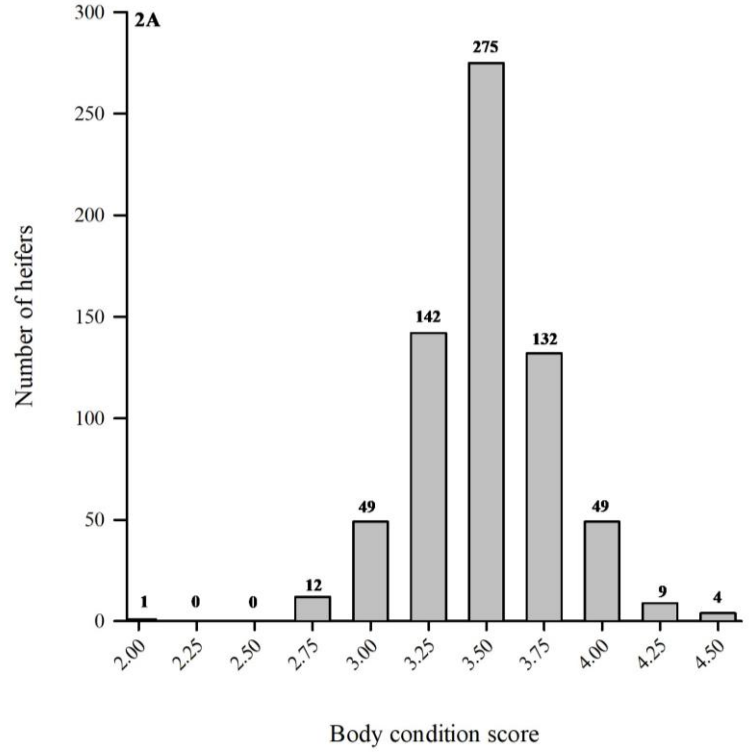
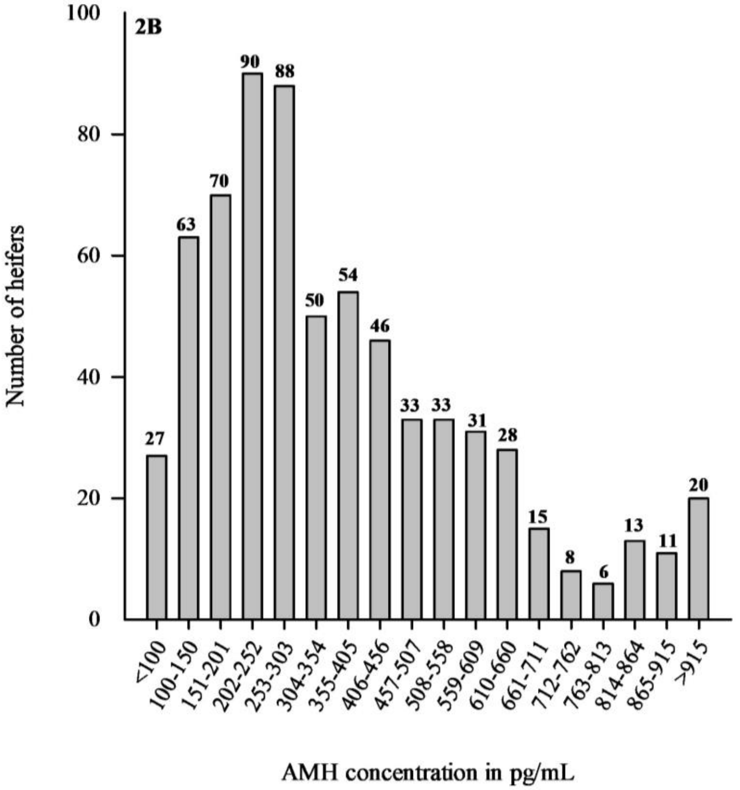

1B. Experimental design

Figure 1. Reproductive management of the heifers (**1A):** Heifers were moved to the breeding pen three days before they started AI, when they were weighted. During seven days heifers were AI to estrus. The not-bred heifers received an IM shot of prostaglandin $F_{2\alpha}$ (PGF1) and were AI at estrus, still, not-bred heifers received a second prostaglandin $F_{2\alpha}$ shot (PGF2) seven days later. After these managements not-bred heifers were submitted to a timed AI, being able to be submitted to 3 timed AI protocols. Heifers that had estrous detected during the breeding period were available to be bred. The breeding period was 150 days until each heifer reached 520 days of age or five inseminations. Pregnancy checks were performed every week at 36 ± 7 days after AI.

Experimental design and farm reproductive management (**1B):** The enrollment of heifers in the study happened every week, when they became available to be bred. Collections were accomplished every other week at 4 and 11 days away from the enrollment.


2A
Number of heifers
300
250
200
150
100
50
0
1
0
0
12
49
142
275
132
49
9
4
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75
4.00
4.25
4.50
Body condition score


2B
Number of heifers
100
80
60
40
20
0
27
63
70
90
88
50
54
46
33
33
31
28
15
8
6
13
11
20
<100
100-150
151-201
202-252
253-303
304-354
355-405
406-456
457-507
508-558
559-609
610-660
661-711
712-762
763-813
814-864
865-915
>915
AMH concentration in pg/mL

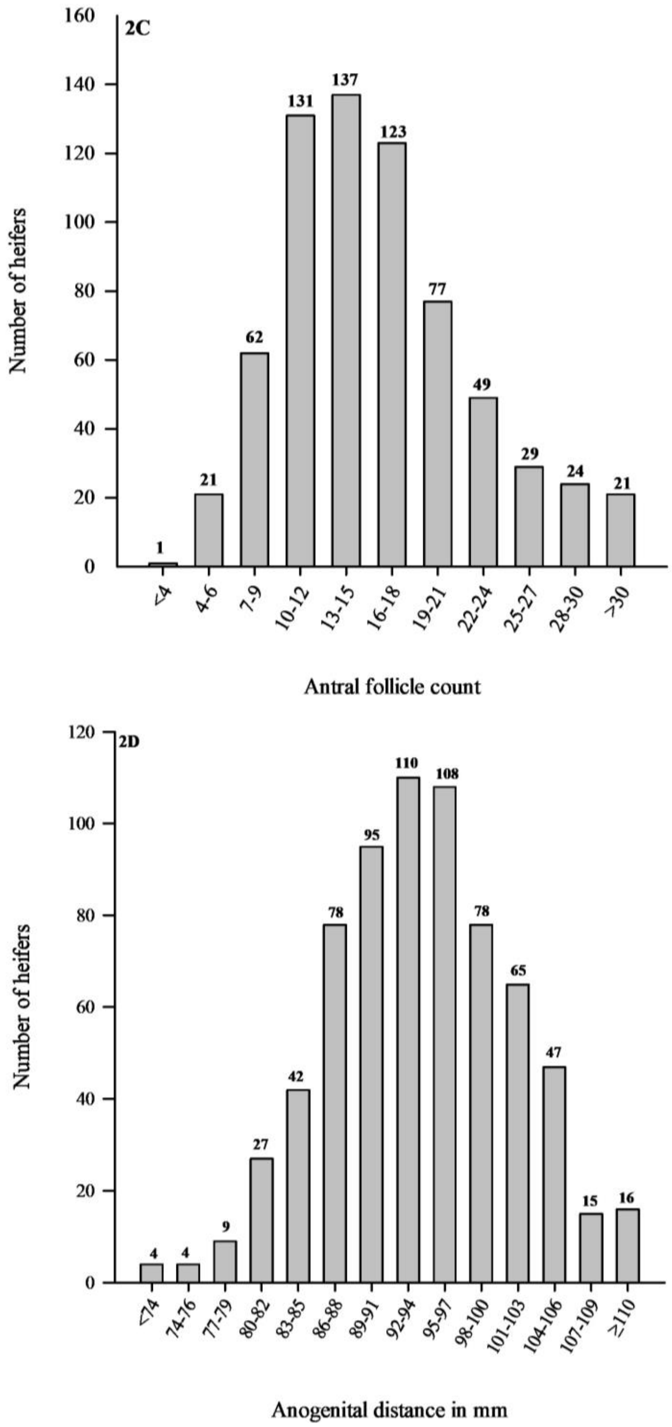


Figure 2. Distribution of body condition score (BCS), Anti-Müllerian hormone (AMH), antral follicle count (AFC), and anogenital distance (AGD) in Holstein nulliparous heifers

4. CHAPTER 3

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Genetic parameters for oocytes and embryo production and their association with linear type traits in dairy Gyr Cattle

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Running head: Relationship between IVEP and type traits

Interpretative Summary: Herein, we aimed to evaluate the phenotypic and genetic associations between aspirated oocytes, in vitro-produced embryos, and linear type traits in *Bos indicus* Gyr dairy cattle. The moderate aspirated oocytes (total and viable) heritability and repeatability, associated with a high correlation between them and embryos, would allow an indirect embryo selection based on oocytes starting early in the reproductive life. Additionally, a moderate correlation between the number of aspirated oocytes and ilium width supports progress on in vitro results by selecting dairy Gyr donors based on linear type measurements. Thus, our results support the advancement of in vitro production on animal breeding programs based on the number of aspirated oocytes and linear type traits in dairy Gyr cattle.

ABSTRACT

In vitro embryo production is one of the main reproductive techniques used in dairy Gyr cattle. In addition, linear type measures are well characterized and have been used in dairy Gyr breed selection for the last four decades. The estimation of genetic parameters for the number of aspirated oocytes and in vitro-produced embryos associated with the linear type measures would support genetic progress for animal breeding programs toward embryo production. This study aimed to obtain genetic parameters for aspirated oocytes, embryo in vitro production, and linear type traits, exploring the association between them. The repeatability model was applied to 14,251 ovum pick-up events from 1,916 Gyr donors. A subset of 604 donors from the same group had their body measurements taken. Single- and two-trait analyses were carried out using the BLUPF90 family programs. Heritability estimates of 0.38, 0.34, and 0.20 were obtained for total oocytes, viable oocytes, and embryos, respectively, as the heritability from the linear type traits ranged from 0.22 to 0.40. High genetic correlations between total oocytes and viable oocytes (0.99), and between oocytes (total and viable) and embryos (0.83) were obtained. Low to high genetic (-0.07 to 0.92) and phenotypic (0.32 to 0.86) correlations were obtained between the linear type traits. Moreover, low phenotypic correlations (0.01 to 0.13) were observed for oocytes (total and viable) and embryos with the linear type traits, whereas low to moderate genetic correlations (0.07 to 0.42) were observed between the same traits, especially for ilium width (0.42), rump area (0.38), and hip height (0.33). Thus, selection for in vitro production is achievable in Gyr dairy cattle, and superior genetic progress is associated with the selection of oocytes (total and viable). Furthermore, the moderate genetic association between oocytes and embryos with linear type traits, especially ilium width suggests that progress on in vitro embryo production may be achieved by accessing these measurements.

Key words: genetic correlation, phenotypic correlation, ovum pick-up, body measurements, zebu cattle, dairy Gyr

INTRODUCTION

Gyr cattle, the most important *Bos indicus* dairy breed in Brazil, is a main source of genetic material for replacement Gyr and crossbred Holstein x Gyr heifers in tropical dairy systems (Viana et al., 2017; Silva et al., 2018). Gyr animals are naturally resistant to heat and parasites, and the selection of superior animals for milk production, conformation, and management started in 1985, with the advent of the National Dairy Gyr Breeding Program (Programa Nacional de Melhoramento do Gir Leiteiro, Panetto et al., 2023). The progressive use of assisted reproductive techniques also allowed high genetic gains in purebred herds and heterosis in commercial crossbred herds, especially at F1 generations (Madalena et al., 2012). The 39 years of genetic advancement and the use of reproductive techniques, mostly in vitro embryo production **(IVEP)**, provided crucial progress to the breed, leading to the constantly growing exportations of semen and embryos (Fernandes, 2022). Therefore, the expanding market interest in the Gyr dairy cattle, especially regarding IVEP, makes the efficient multiplication of high genetic merit animals an important source of study.

Brazil, the second largest country in embryo production, transferred 252,721 IVEP embryos from dairy breeds in 2022, representing 16% of the world's IVEP embryos (1,616,971) in the same period (Viana, 2023). *Bos indicus* have on average a greater number of follicles growing per follicular wave, more recovered oocytes per ovum pick-up **(OPU)** (Pontes et al., 2010; Batista et al., 2014), and superior IVEP efficiency (Guerreiro et al., 2014) than *Bos taurus*. Published data on IVEP genetic parameters in Gyr cattle have indicated a moderate heritability (0.24) and repeatability (0.39) for the number of total and viable oocytes (Rocha et al., 2022), proposing that few OPU events would be a good indicator of the animal's lifetime potential (Vizoná et al., 2020; Feltes et al., 2022; Rocha et al., 2022). These results are similar to the Holstein cattle (Parker Gaddis et al., 2017), suggesting that adding IVEP traits to the selection indexes allows the identification of the best dams and bulls early in life to generate the most productive offspring and avoid IVEP efficiency deterioration (Vizoná et al., 2020).

The identification of easily measured and low-cost phenotypes associated with IVEP would make possible their application in genetic programs to support donor selection. Linear type traits, measured in almost all international breeding programs, are predominantly related to body dimensions (Price et al., 2000). In the past, unfavorable genetic correlations were obtained between linear type traits and fertility (Haile-Mariam et al., 2004; Wall et al., 2005). More recently, a low genetic correlation (0.08) between anogenital distance and viable oocytes (Machado et al., 2023), as a low phenotypic correlation (0.07) between vulva width and antral follicle count (Mesquita et al., 2016; Maculan et al., 2018) was suggested in dairy Gyr donors and Tabapuã cows, respectively. Vulva width and antral follicle count were also phenotypically associated with oocyte viability and in vitro embryo production efficiency in *Bos indicus* and *Bos taurus* breeds (De Vasconcelos et al., 2020). In addition, a positive phenotypic correlation between body length (0.09) and thoracic depth (0.10) with the antral follicle count has been observed (Maculan et al., 2018). Considering that antral follicle count is highly repeatable within the same animal (Ireland et al., 2007), and related to the number of viable oocytes (Silva-Santos et al., 2014; Santos et al., 2016), the selection for antral follicle count based on linear type traits could be a useful phenotype to select oocyte donors. Thus, considering the many years of characterization and selection of type traits in the Gyr dairy breed our hypothesis was based on the possibility of a positive genetic and phenotypic correlation between type traits and IVEP. Our aim with this study was to obtain genetic parameters for aspirated oocytes, embryo in vitro production, and linear type traits, exploring the association between them.

MATERIAL AND METHODS

All animal handling was performed according to the Animal Care and Use Committee of the Universidade Federal de Viçosa (Viçosa, MG, Brazil) under protocol # 010/2022.

Data description

The analyzed dataset contained 14,251 records of follicular aspirations from 1,916 dairy Gyr donors. A subset of 604 Gyr donors of the same group of aspiration data had their body measurements taken. The pedigree file included 4,260 individuals, considering up to 7 generations.

Donors were raised and maintained in the states of Minas Gerais (five herds) and Rio de Janeiro (one herd), Brazil. Donors were housed in grazing systems with ad libitum access to water and mineral salt and were supplemented with corn silage and concentrate.

The OPU sessions occurred between May 2010 and March 2023 in the six herds. The traits evaluated per OPU section were: number of total aspirated oocytes **(TO)**, viable aspirated oocytes **(VO)**, and embryos produced in vitro **(EMB)**. The EMB trait was considered as the total number of recovered embryos, regardless of the quality or the stage of development. Contemporary groups **(CG)** for IVEP traits were formed based on the OPU date and farm, while CG for linear type traits was determined by the date of collection of body measurements and farm. Only CG with more than one animal were retained for analysis.

Linear type traits measurements

A single external measurement (collected between July to December of 2021) of rump length (cm, **RL**), ilium width (cm, **ILW**), ischium width (cm, **ISW**), hip height (cm, **HH**), body length (cm, **BL**), and thoracic perimeter (cm, **TP**) was collected from the 604 Gyr oocyte donors **(Figure 1)**. Briefly, each donor was restrained in a chute, allowing two technicians to conduct the measurements. Using a livestock measuring stick, the first technician measured the (1) rump length, the distance between the ilium and ischium tuberosity, (2) ilium width, the distance between the left and the right iliac tuberosity, and (3) ischium width, the distance between the left and right ischial tuberosity. The second technician also using a livestock measuring stick measured the (4) hip height, the distance between the sacral tuberosity and the ground, and (5) body length, the distance between the scapulohumeral joint and the ilium tuberosity. The second technician accessed the (6) thoracic perimeter, the perimeter immediately caudal to the scapula passing through the sternal processes and the thoracic vertebrae, using cattle weighing tape. It is worth mentioning that all the measurements in this study were assessed by the same two technicians.

The rump length, ilium width, and ischium width measures were used to calculate the rump area. Considering that rump has an isosceles trapeze shape, rump area was estimated in cm², and the following formula was used:

$$A = \frac{(B + b) \times h}{2} \quad (1)$$

where A is the area of the trapezium (rump area), B is the major base (ilium width); b is the smallest base (ischium width), and h is the height of the trapezium. The h was obtained using the following formula:

$$h = \sqrt{L^2} - \left(\frac{B - b}{2}\right)^2 \quad (2)$$

where L is the rump length.

Statistical analysis

The IVEP traits were transformed using the natural logarithmic scale (ln (X + 1)) to obtain a normal distribution of the residuals. The normal distribution was verified by the

Anderson-Darling test (Stephens MA, 1986; Thode, 2002). Then, we performed analysis of variance to test hypothesis associated with the effects of some independent variables, like CG, OPU age, and age at body measurement. Different models were tested and the model with the best (lowest) Akaike Information Criterion **(AIC)** value was chosen for each trait. The final statistical model included CG as a systematic effect for all traits, OPU age as covariable (linear and quadratic effect) for the IVEP traits, and age at body measurement as covariable (linear and quadratic effect) for the linear type traits.

Single and two-trait analyses were carried out for three IVEP traits (TO, VO, and EMB), and seven linear type traits measurements (RL, ILW, ISW, RA, HH, BL, and TP).

The general statistical model for IVEP traits was:

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Z\alpha} + \mathbf{Wpe} + \mathbf{e} \text{ (3)},$$

and the general statistical model for linear type traits was:

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Z\alpha} + \mathbf{e}, \text{ (4)}$$

where $\mathbf{y}$, $\boldsymbol{\beta}$, $\boldsymbol{\alpha}$, $\mathbf{pe}$, and $\mathbf{e}$ are the vectors of observations; systematic effects (CG and covariables); animal additive genetic effect; animal permanent environment effect, and residual effect, respectively; **X**, **Z**, and **W** are the incidence matrices of described effects, respectively. The variance components were obtained by Bayesian approach. In the single-trait models, it was assumed uniform *a priori* distribution for β $(\beta \sim \text{constant})$; normal distributions for $\boldsymbol{\alpha}$ $\left(\alpha|\mathbf{A},\sigma^2_\alpha \sim \text{N } (0, \mathbf{A}\sigma^2_\alpha)|\right)$, $\mathbf{pe}$ $\left(\text{pe}|\mathbf{I},\sigma^2_{pe} \sim \text{N } \left(0, \mathbf{I}\sigma^2_{pe}\right)|\right)$ and $\mathbf{e}$ $\left(\text{e}|\mathbf{I},\sigma^2_e \sim \text{N } (0, \mathbf{I}\ \sigma^2_e)|\right)$; inverse chi-square for σ^2_α $\left(\sigma^2_\alpha \sim \chi^{-2}\left(\text{V}_\alpha, \text{S}^2_\alpha\right)\right)$, σ^2_{pe} $\left(\sigma^2_{pe} \sim \chi^{-2}\left(\text{V}_{pe}, \text{S}^2_{pe}\right)\right)$ and σ^2_e $\left(\sigma^2_e \sim \chi^{-2}\left(\text{V}_e, \text{S}^2_e\right)\right)$. The terms **A** and **I** represent the relationship and identity matrices, respectively; σ^2_α, σ^2_{pe} and σ^2_e represent the additive genetic, permanent environmental (for IVEP traits) and residual variances, respectively; and V_α, V_{pe}, V_e, S^2_α, S^2_{pe} and S^2_e represent the hyperparameters of prior distributions.

In the two-trait model, it was assumed uniform *a priori* distributions for the systematic effects in $[\beta_1\beta_2]'$; normal distributions for $[\alpha_1\alpha_2]'$ $\left([\alpha_1\alpha_2]'|\mathbf{A}, \boldsymbol{G}_0 \sim N(0, \boldsymbol{G}_0 \otimes \boldsymbol{A})\right)$, $[pe_1pe_2]'$ $\left([pe_1pe_2]'|\mathbf{I}, \boldsymbol{W}_0 \sim N(0, \boldsymbol{W}_0 \otimes \boldsymbol{I})\right)$ and $[e_1e_2]'$ $\left([e_1e_2]'|\mathbf{I}, \boldsymbol{R}_0 \sim N(0, \boldsymbol{R}_0 \otimes \boldsymbol{I})\right)$; inverse Wishart distributions for G_0 $\left(\boldsymbol{G}_0 \sim W^{-1}(\boldsymbol{\Sigma}_\alpha, \upsilon_\alpha)\right)$, W_0 $\left(\boldsymbol{W}_0 \sim W^{-1}(\boldsymbol{\Sigma}_{pe}, \upsilon_{pe})\right)$ and R_0 $\left(\boldsymbol{R}_0 \sim W^{-1}(\boldsymbol{\Sigma}_e, \upsilon_e)\right)$, which represent 2 x 2 additive genetic, permanent environment, and residual matrices of covariance between traits 1 and 2, respectively. The terms Σ_α, Σ_{pe}, Σ_e, υ_α, υ_{pe} and υ_e represent the hyperparameters of inverse Wishart distritutions.

Samples of full conditional posterior distributions of genetic parameters were generated by Gibbs sampling using the GIBBSF90+ module of the BLUPF90 software (Misztal et al., 2014). Markov Chain Monte Carlo (MCMC) chains of 2,200,000 iterations, with burn-in period of 200,000 iterations and a sampling interval of 10 iterations were generated in each analysis. Thus, 200,000 samples were effectively used for final inferences. Post-Gibbs analysis were realized using POSTGIBBSF90 (Misztal et al., 2014) to obtain posterior means and lower and upper limits for high-density intervals (90% of samples) for variance, covariance, heritability, repeatability, and correlations. Convergences of the MCMC chains were double-checked with graphic inspection of posterior distributions generated by the Coda R package (Plummer et al., 2006).

RESULTS

The descriptive statistics analysis for the studied traits, donor's age during OPU, and donor's age during body measurements are presented in **Table 1**. Means of donors' age during the OPU sessions (5.95 years) and body measurements (5.69 years) were similar, with a wide superposition of range (0.40 to 18.95 years, and 1.38 to 15.72 years, respectively). The number of TO collected in the described period reached almost 300,000 structures, with 21.09 oocytes recovered on average per OPU section per donor. VO ranged from 0 to 133, with a mean of 15.78 VO produced per OPU event, representing 75% of the mean number of TO. During the 13 years of OPU 54,000 embryos were produced, an average of 3.81 EMB per OPU section, representing 24% of the mean number of VO and 18% of the mean number of TO.

Posterior means of variances, heritability, and repeatability from the univariate analysis of IVEP and linear type traits are shown in **Table 2**. Moderate posterior mean heritability was observed for the IVEP traits, TO demonstrate the highest posterior mean heritability (0.38), followed by VO (0.34) and EMB (0.20). Repeatability displayed similar behavior, as TO had the greatest value of repeatability (0.54), followed by VO (0.52) and EMB (0.32). Moderate heritability was also identified for all the linear type traits, being rump length and body length the greatest (0.40) and ischium width the lowest (0.22).

The genetic and phenotypic correlations for IVEP and linear type traits are presented in **Table 3**. Genetic and phenotypic correlations between the IVEP traits were high, as TO and VO associations equal 0.99 and 0.78, respectively. The genetic correlations of EMB with TO and VO were also high (0.83), despite their moderate phenotypic correlations (0.45). The genetic correlations between the linear type traits ranged from low to high (-0.07 to 0.92). The rump area was highly correlated with rump length (0.92) and ilium width (0.90), whereas low genetic positive correlations were observed between rump length and hip height (0.20), and between ischium width and thoracic perimeter (0.23). The phenotypic correlations between linear type traits ranged from moderate to high (0.32 to 0.86), and the association of rump area and ilium width was identified as the measurement with the greatest (0.86) phenotypic correlation.

The genetic and phenotypic correlations between the IVEP and linear type traits were low to moderate, ranging from 0.07 to 0.42 and 0.01 to 0.13, respectively. The highest genetic correlations between traits from these two groups (one linear type trait and the other IVEP result) were obtained between ilium width and the three IVEP measures (0.41, 0.42, and 0.29 with TO, VO, and EMB, respectively). The posterior means of genetic correlations between rump area and hip height with TO and VO were positive, and their high-density intervals did not include zero, which suggests they are statistically significant. Posterior means of genetic correlations between rump length, ischium width, body length, and thoracic perimeter with IVEP traits were of low magnitude or their high-density intervals included zero, which suggests most of these genetic correlations are not different from zero.

DISCUSSION

In summary, our main results were the identification of additive genetic variance in oocytes and embryo production, associated with moderate heritability (0.20 to 0.38) and repeatability (0.32 to 0.54) for these same traits. Testing the genetic and phenotypic correlations between TO, VO, and EMB, a high genetic correlation was demonstrated (0.83), whereas a moderate phenotypic correlation (0.45) was detected for the association of EMB with TO and VO. Regarding linear type traits, most genetic and phenotypic correlations ranged from moderate to high, associated with a moderate heritability (0.22 to 0.40). Finally, evaluating the genetic and phenotypic correlations between the IVEP (TO, VO, EMB) and linear type traits, values of correlations indicate the possibility of conducting selection to obtain responses for IVEP traits, especially considering ilium width.

Bos indicus superiority in the number of growing ovarian follicles and the number of total and viable oocytes allows a greater performance of the Zebu breeds under the IVEP technology compared to *Bos taurus* (Batista et al., 2014; Gimenes et al., 2015). Based on that, we focus on comparing our IVEP results to other *Bos indicus* information in the literature. For TO, similar numbers such as 21.66 (Rocha et al., 2022) and 21.72 (Feltes et al., 2022) were reported for Gyr cattle, whereas a superior number of 35.2 TO was identified for Nellore cows (Gimenes et al., 2015). For VO, our results are similar to the reports of 15.82 (Rocha et al., 2022) and 15.19 (Perez et al., 2016) for Gyr and Guzerat donors, respectively, but lower than 24.7 VO reported for the Nellore breed (Gimenes et al., 2015). Average EMB in this study is lower compared to 4.59 embryos identified in the Gyr cattle (Vizoná et al., 2020). In general, among *Bos indicus* breeds there is a similarity of our results with other Gyr and Guzerat donors, as well as numerical superiority for Nellore cattle regarding aspirated oocytes.

Genetic parameters of IVEP traits have been described for Gyr (Vizoná et al., 2020; Rocha et al., 2022), Guzerat (Perez et al., 2016), and Nellore (Pinheiro, 2019) cattle. Rocha et al. (2022) evaluating 17,526 follicular aspirations from 1,641 donors observed a genetic variance of 0.13, 0.12, and 0.06, an environment variance of 0.08, 0.07, and 0.05, and a residual variance of 0.31, 0.30, and 0.45 for TO, VO, and EMB, respectively. In our study, the genetic additive, environment, and residual variance **(Table 2)** were alongside the results presented by Rocha et al. (2022). The population used in our study was similar to the one studied by Rocha et al. (2022) which makes reasonable the similarity of additive, environment, and residual variance observed in both studies. Also, these herds are in different environments and submitted to distinct managements contributing to high residual variance in both studies.

Merton et al. (2009) reported a moderate heritability of 0.25 for the number of viable oocytes in Holstein donors following a univariate sire model. Rocha et al. (2022) demonstrated a similar result of heritability for VO (0.25) and TO (0.24) using repeatability models. In our results, we identified moderate heritability for TO (0.38) and VO (0.34). Our data is similar to the heritability (0.31) described for antral follicle count in Irish Holstein dairy cows (Walsh et al., 2014). Jaton et al. (2016) looking into superovulatory embryo heritability identified a low heritability of 0.15 for the donor side, similar to the observed in this study. The low heritability observed for EMB suggests that this trait is more affected by non-genetic factors, such as climate (Silva et al., 2013), nutrition (Armstrong et al., 2001; Sales et al., 2015), hormonal stimulation protocols (Elliff et al., 2019; Garcia et al., 2020), semen type (Palma et al., 2008; Magata et al., 2021) and laboratory procedures (Ashry et al., 2018; Gonzalez Andueza et al., 2022).

The repeatability of a trait indicates the extent to which an individual's performance, for that particular trait, remains consistent over time (Feltes et al., 2022). Repeatability related to IVEP ranged from 0.20 for embryos in Gyr and Guzerat cattle (Perez et al., 2016; Rocha et al., 2022) to 0.63 for viable oocytes in Gyr donors (Vizoná et al., 2020). The moderate repeatability for the oocyte and embryo counts suggests that there is no need for a high number of OPUs to estimate the potential donor performance, making it a tool to select young animals after the first OPU/IVEP events (Vizoná et al., 2020). Our results of repeatability ranged from 0.32 for EMB to 0.54 for TO. High repeatability (0.89) was observed before for the average number of ovarian follicles per follicular wave within the same animal (Ireland et al., 2007), it indicates the association of TO and VO with the antral follicle population and helps to explain the moderate repeatability of oocytes.

Genetic correlation between IVEP traits was higher than phenotypic correlation in the present study, since phenotypic correlation is dependent on additive genetic and not additive genetic effect; differences in phenotypic and genetic correlation must be explained by the relationship between genetic and environment (Hadfield et al., 2007). As the number of viable oocytes is a subset of the number of total oocytes, a high correlation between TO and VO (0.99 and 0.78) was expected (Parker Gaddis et al., 2017; Rocha et al., 2022). A high genetic correlation was also observed for oocytes (total and viable) and embryos (0.83). Indicating that the genes contributing to these traits are probably co-inherited (Sodini et al., 2018), additionally to an effect of the number and quality of cumulus-oocyte complexes on the production of blastocysts (Souza-Fabjan et al., 2016). Considering that the main objective of IVEP is to produce embryos, and the moderate (0.20) heritability for this trait, response to direct selection would be achieved. However, as shown here and in other studies, greater values of heritability and repeatability for oocytes (total and viable), and the high genetic correlation between them and embryos would enable an indirect selection for embryos starting early in the reproductive life (Merton et al., 2009; Rocha et al., 2022; Vizoná et al., 2020). Moreover, considering the development of animal breeding programs the collection of information on OPU events is easier and faster compared with the number of produced embryos, dependent on OPU and in vitro procedures (Vizoná et al., 2020). Thus, the selection of a trait with greater heritability, repeatability, and correlation with the others would bring superior genetic progress for the oocyte donors.

Heritability for linear type traits has been described as moderate in the literature (Haile-Mariam et al., 2004; Eaglen et al., 2013). In our data, linear type traits heritability was moderate, and moderate to high genetic correlation was identified between most traits. Linear type traits measures, especially related to the rump, have been associated with calving ease (Cue et al., 1990), but also other measures such as stature, body depth, and chest width were unfavorably related to fertility before (Pryce et al., 2000; Royal et al., 2002). Wall et al. (2005) examining phenotypic and genetic correlations for rump (angle and width) and fertility were not able to identify a significant phenotypic association, however, a negative genetic correlation (-0.16) was observed between the rump angle and calving interval. In addition, Vizoná et al. (2020) evaluating the Pearson's correlation of estimated breeding values for IVEP and conformation traits observed negative correlations of low to moderate magnitude for rump width and angle. In our data, the phenotypic and genetic correlations between linear type traits and IVEP were positive, while all phenotypic correlations were low (≤ 0.13), the genetic correlations ranged from 0.07 to 0.42. Despite most of the associations observed for linear type measures and reproduction being low in the literature, our results suggest a moderate genetic correlation between these measures.

A moderate correlation that could be explored to attain improvements in donor’s embryo production, particularly between ilium width and TO and VO was identified. Over the years the selection of Gyr breed generated cattle of greater rump length, ilium width, body length, and thoracic perimeter, although hip height remained similar (Verneque et al., 2011; Panetto et al., 2023). In that sense, Gyr type selection focused on increasing the animal’s depth and length but not the height. Considering our results, ilium width, rump area, and hip height would be the best type traits to indirectly select oocyte donors for embryo production. According to the directions of genetic advances in the dairy Gyr cattle, the more applicable trait to develop the selection of IVEP would be the ilium width, since rump area is an indirect trait calculated from other measures and there is no interest in selecting taller animals in the Gyr breed. Taking into consideration the results described here, the 39 years of genetic progress associated with the dairy Gyr cattle, and the selection of type traits being able to indirectly affect IVEP, more studies should be done to better describe the association between rump measures and fertility, especially in heifers at early stages of life, such as pre-pubertal measurements.

Part of our objective in this study was to characterize the association of IVEP and linear type traits. The linear type measures are easy to collect and are usually taken in the Gyr breeding programs, which have a long period of characterization and selection. Literature indicated a breed-dependent relationship between the vulva width, oocyte viability, and in vitro embryo production efficiency (De Vasconcelos et al., 2020), demonstrating a possible association of the animal body size with the follicular reserve. In addition, a prospective study in our group evaluating puberty in crossbred Holstein x Gyr heifers indicated an association between this measure and rump area (Schmidt, 2021), proposing a relationship between rump measures and puberty in crossbred Holstein x Gyr heifers.

CONCLUSION

Selection for IVEP traits in Gyr donors is feasible, due to the existence of genetic variance. Genetic programs based on the number of aspirated oocytes (total or viable) might support IVEP progress starting early in reproductive life, since these traits show moderate heritability and repeatability, and a high correlation with embryo production. Furthermore, the moderate heritability of linear type traits and mainly the moderate genetic correlation observed between linear type and IVEP results, especially for ilium width, rump area, and hip height may contribute to an indirect selection of Gyr donors with superior IVEP outcomes.

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Data availability: The data used in this study is from commercial farms and they might be available under request.

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Table 1. Descriptive statistics[A] on donors' age, IVEP structures, and linear type measurements in dairy Gyr donors

Item[1]	n	Total	Mean	Sd	Min	Max	CV
Ovum pick-up age (yrs)	14,251	--	5.95	2.97	0.40	18.95	49.91
TO	13,375	282,018	21.09	15.28	0.00	161.00	72.45
VO	14,227	224,433	15.78	12.91	0.00	133.00	81.81
EMB	14,207	54,134	3.81	4.28	0.00	46.00	112.34
Body measure age (yrs)	604	--	5.69	2.70	1.38	15.72	47.45
RL (cm)	604	--	37.85	3.06	27.10	53.80	8.08
ILW (cm)	604	--	41.50	2.78	31.40	49.90	6.70
ISW (cm)	604	--	25.93	3.18	15.80	38.20	12.26
RA (cm^2)	604	--	1,314.04	183.82	818.78	2,039.47	13.99
HH (cm)	604	--	137.80	4.80	120.00	158.50	3.48
BL (cm)	604	--	140.59	9.01	110.00	172.00	6.41
TP (cm)	604	--	177.89	10.63	142.00	218.00	5.98

[A] n= number of data samples; Total = total sum of structures (oocytes and embryos); Sd = standard deviation; Min = minimum; Max = maximum; CV = coefficient of variation; (--) = variable not log transformed.

[1] TO = total oocytes; VO = viable oocytes; EMB = embryo; RL = rump length; ILW = ilium width; ISW = ischium width; RA = rump area; HH = hip height; BL = body length; TP = thoracic perimeter.

Table 2. Posterior means and lower and upper limits (in parentheses) for high-density intervals with 90% of samples for variance components and genetic parameters[A] for in vitro embryo production and linear type traits estimated by single-trait analysis in dairy Gyr donors

Item[1]	σ_a^2	σ_{pe}^2	σ_e^2	σ_p^2	h^2	r
TO	0.17	0.07	0.20	0.44	0.38	0.54
	(0.13; 0.20)	(0.05; 0.09)	(0.19; 0.20)	(0.41; 0.46)	(0.31; 0.45)	(0.52; 0.57)
VO	0.16	0.08	0.23	0.48	0.34	0.52
	(0.13; 0.20)	(0.06; 0.11)	(0.22; 0.23)	(0.45; 0.50)	(0.28; 0.41)	(0.50; 0.55)
EMB	0.13	0.08	0.43	0.64	0.20	0.32
	(0.09; 0.16)	(0.06; 0.11)	(0.42; 0.44)	(0.62; 0.66)	(0.14; 0.25)	(0.30; 0.35)
RL (cm)	3.29	--	4.83	8.12	0.40	--
	(1.34; 5.16)		(3.46; 6.24)	(7.13; 9.07)	(0.20; 0.60)	
ILW (cm)	2.27	--	3.48	5.76	0.39	--
	(0.86; 3.66)		(2.43; 4.49)	(5.03; 6.44)	(0.18; 0.60)	
ISW (cm)	1.99	--	7.08	9.07	0.22	--
	(0.39; 3.50)		(5.71; 8.41)	(8.07; 10.03)	(0.05; 0.37)	
RA (cm²)	809.46	--	1,845.06	2,654.52	0.30	--
	(197.70; 1,376.00)		(1,388.00; 2,302.00)	(2,341.00; 2,958.00)	(0.09; 0.49)	
HH (cm)	0.62	--	1.43	2.05	0.30	--
	(0.16; 1.06)		(1.08; 1.78)	1.81; 2.29)	(0.10; 0.49)	
BL (cm)	2.21	--	3.19	5.39	0.40	--
	(0.82; 3.59)		(2.16; 4.17)	(4.70; 6.04)	(0.18; 0.62)	
TP (cm)	1.98	--	5.67	7.65	0.25	--
	(0.23; 3.58)		(4.30; 7.05)	(6.74; 8.52)	(0.04; 0.45)	

[A] σ_a^2 = additive genetic variance; σ_{pe}^2= permanent environment variance; σ_e^2= residual variance; σ_p^2 = phenotypic variance; h^2= heritability; r = repeatability.

[1] TO = total oocytes; VO = viable oocytes; EMB = embryo; RL = rump length; ILW = ilium width; ISW = ischium width; RA = rump area; HH = hip height; BL = body length; TP = thoracic perimeter.

Table 3. Posterior means and lower and upper limits (in parentheses) for the high-density intervals with 90% of samples for genetic (above the diagonal) and phenotypic (below the diagonal) correlations for in vitro embryo production and linear type estimated by two-trait analysis in dairy Gyr donors

Item[1]	TO	VO	EMB	RL	ILW	ISW	RA	HH	BL	TP
TO	--	0.99 (0.99; 1.00)	0.83 (0.76; 0.91)	0.19 (-0.01; 0.39)	0.41 (0.18; 0.64)	0.07 (-0.19; 0.40)	0.34 (0.07; 0.61)	0.30 (0.08; 0.52)	0.21 (0.02; 0.40)	0.22 (-0.07; 0.50)
VO	0.78 (0.73; 0.83)	--	0.83 (0.75; 0.91)	0.22 (0.02; 0.42)	0.42 (0.19; 0.65)	0.12 (-0.14; 0.39)	0.38 (0.09; 0.67)	0.33 (0.11; 0.53)	0.25 (0.06; 0.44)	0.23 (-0.05; 0.51)
EMB	0.45 (0.40; 0.50)	0.45 (0.41; 0.50)	--	0.17 (-0.06; 0.40)	0.29 (0.05; 0.54)	0.11 (-0.20; 0.43)	0.31 (-0.02; 0.63)	0.23 (-0.02; 0.48)	0.22 (0.00; 0.45)	0.15 (-0.18; 0.49)
RL (cm)	0.07 (-0.01; 0.15)	0.08 (0.01; 0.16)	0.02 (-0.06; 0.09)	--	0.77 (0.59; 0.97)	0.47 (0.11; 0.84)	0.92 (0.84; 1.00)	0.20 (-0.25; 0.65)	0.88 (0.73; 1.00)	0.85 (0.70; 1.00)
ILW (cm)	0.12 (0.04; 0.20)	0.13 (0.05; 0.20)	0.03 (-0.05; 0.10)	0.65 (0.61; 0.70)	--	0.31 (-0.15; 0.75)	0.90 (0.82; 0.99)	0.60 (0.30; 0.90)	0.69 (0.42; 0.96)	0.86 (0.73; 1.00)
ISW (cm)	0.02 (-0.06; 0.10)	0.03 (-0.05; 0.11)	0.02 (-0.06; 0.09)	0.49 (0.43; 0.56)	0.43 (0.36; 0.50)	--	0.65 (0.36; 0.92)	-0.07 (-1.00; 0.53)	0.41 (-0.03; 0.85)	0.23 (-0.51; 0.85)
RA (cm^2)	0.09 (0.01; 0.17)	0.10 (0.02; 0.18)	0.03 (-0.04; 0.10)	0.83 (0.81; 0.86)	0.86 (0.84; 0.88)	0.78 (0.75; 0.81)	--	0.46 (0.07; 0.85)	0.87 (0.69; 1.00)	0.85 (0.71; 1.00)
HH (cm)	0.08 (0.00; 0.16)	0.09 (0.01; 0.17)	0.04 (-0.03; 0.11)	0.34 (0.26; 0.41)	0.46 (0.40; 0.53)	0.32 (0.24; 0.39)	0.46 (0.40; 0.53)	--	0.37 (-0.07; 0.81)	0.84 (0.63; 1.00)
BL (cm)	0.06 (-0.02; 0.14)	0.11 (0.03; 0.19)	0.01 (-0.06; 0.09)	0.52 (0.46; 0.58)	0.56 (0.50; 0.62)	0.32 (0.25; 0.40)	0.56 (0.51; 0.62)	0.39 (0.31; 0.46)	--	0.83 (0.64; 1.00)
TP (cm)	0.09 (0.01; 0.17)	0.10 (0.02; 0.18)	0.02 (-0.05; 0.09)	0.68 (0.64; 0.73)	0.67 (0.63; 0.72)	0.45 (0.38; 0.51)	0.72 (0.68; 0.76)	0.50 (0.44; 0.56)	0.54 (0.49; 0.60)	--

[1] TO = total oocytes; VO = viable oocytes; EMB = embryo; RL = rump length; ILW = ilium width; ISW = ischium width; RA = rump area; HH = hip height; BL = body length; TP = thoracic perimeter.

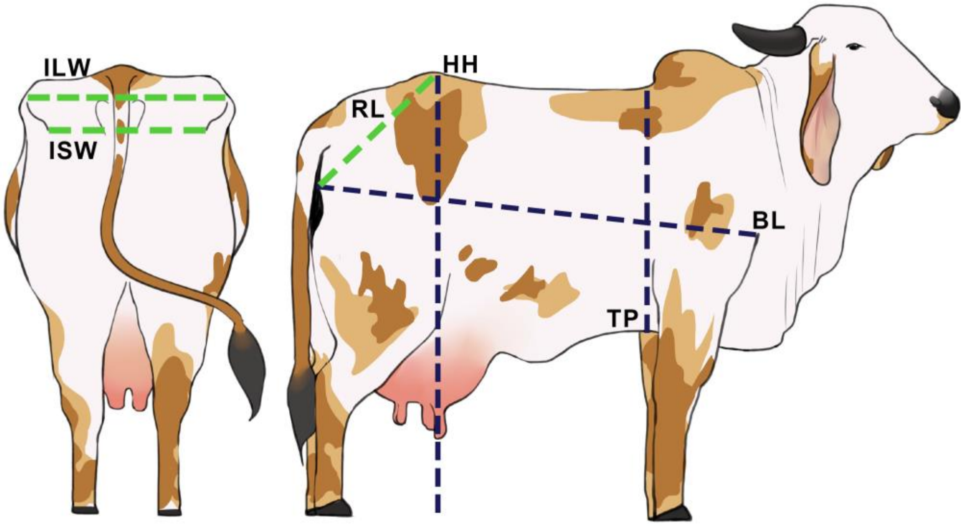

Figure 1. Schematic image of a Gyr donor on the lateral and posterior position. The measurements of rump length (RL): the distance between the ilium and ischium tuberosity, ilium width (ILW): the distance between the left and the right iliac tuberosity, and the ischium width (ISW): the distance between the left and right ischial tuberosity were taken by the first technician (green lines). The second technician (black lines) measured the hip height (HH): the distance between the sacral tuberosity and the ground, body length (BL): the distance between the scapulohumeral joint and the ilium tuberosity, and the thoracic perimeter (TP): the perimeter immediately caudal to the scapula passing through the sternal processes and the thoracic vertebrae

5. GENERAL CONCLUSIONS

The main objectives of this thesis were to explore the association of phenotypes and genetic traits with fertility in dairy cattle. In the first chapter we characterized for the first time the AGD distribution on the Gyr cattle, and the association of this trait to IVEP. In the second chapter we studied the correlation of body condition score, Anti-Mullerian hormone, antral follicle count, and anogenital distance and their association to fertility. Lately, in the third chapte, we assessed genetic parameters of IVEP and type traits and indicated type traits as a tool to indirectly select the best Gyr donors for IVEP.

According to our results in the first chapter, AGD might be used to select oocyte Gyr donors since it was inversely phenotypically associated with a greater number of produced embryos. In addition, a positive genetic association between AGD and viable oocytes and AGD and embryo rate was also identified. Although correlations were pointed out, these results should be considered cautiously, considering that the proportion of produced embryos explained by AGD is very low, in the same way that the genetic results are low and in a limited sample size. Our study was the first of our acknowledgement looking into the association of AGD and IVEP. Considering we worked with retrospective data, the evaluation of AGD and IVEP results obtained at the same period, as at young ages would bring contributions to the selection of oocyte donors and confirm the results obtained here.

In our second chapter, AGD was associated to AFC, and tended to be associated with AMH, however, no association of AGD with fertility in nulliparous Holstein heifers was observed for us. AGD is a new proxy for fertility in cattle explored widely in the last seven years. Until recently all the results indicated an inverse association between AGD and fertility, our data otherwise indicates it may be not true for pregnancy outcomes in Holstein heifers at the same age. The pregnancy results at first AI and all services for nulliparous Holstein heifers were greater on the Top BCS and Middle AMH categories. Similar results can be found in the literature, tmanipulating the diet to obtain adequate BCS , and select heifers with moderate concentration of AMH, although controversial, would bring benefits to the reproductive outcomes of nulliparous Holstein heifers.

Lastly, in the third chapter genetic and phenotypic parameters of IVEP have been explored in Gyr cattle. The heritability of IVEP traits was moderate, followed by moderate repeatability and high correlation between the traits. Considering the IVEP technique, the greater heritability and repeatability of oocyte traits, and the high genetic correlation with embryos, the use of oocytes as a tool to indirectly select for embryo production would bring benefits to the IVEP on *Bos indicus* Gyr cattle. Moreover, the moderate heritability of type traits and the moderate correlation between IVEP and ileum width, rump area, and hip height suggest that the selection of type, easily obtained and already measured in the Gyr selection program, would bring benefits to the selection of oocyte donors, especially considering the ileum width measurement.

Thus, our results present phenotypes that may contribute to the selection for fertility. Future studies should start assessing these phenotypes at early ages and correlate them with the future reproductive results of those animals, this way selection for reproduction would start earlier. Furthermore, the evaluation of genomic data and the identification of genes that can associate these traits to reproduction would guide new studies and selection.