Association of Anti-Müllerian Hormone, Inhibin A and B, with Genomic Prediction of Daughter Pregnancy Rate, Ovarian Responses, and Pregnancy per Artificial Insemination in Lactating Dairy Cows

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEDICATION

The pursuit of science is a lifelong calling—demanding, humbling, and transformative. As Nelson Mandela once said, "Education is the most powerful weapon which you can use to change the world." From Ancient Egypt and Greece to modern global society, civilizations have always risen on the shoulders of scientists and educators. Yet, the journey through graduate school is never easy. So, I owe immense gratitude to those who supported me in this pursuit.

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ABSTRACT

Anti-Müllerian hormone **(AMH)** has emerged as a potential biomarker for super-ovulatory response ovarian reserve and reproductive performance in dairy cattle, yet its association with fertility traits and reproductive outcomes remains unclear. Moreover, inhibin A and B are gonadal hormones involved in the regulation of FSH and folliculogenesis; however, their association with reproductive physiology and fertility traits in dairy cattle remains largely unexplored. Furthermore, the relationship between AMH and inhibin A & B, as potential biomarkers for reproductive performance, and its association with GDPR has yet to be investigated. The objectives of this study were to evaluate the association of plasma concentration of AMH and inhibin A and B at the first gonadotropin-releasing hormone **(G1)** and prostaglandin F2α **(PGF)** of breeding Ovsynch of a Double-Ovsynch (**DO**) protocol with genomic prediction of daughter pregnancy rate **(GDPR)**, ovulation at G1, and pregnancy per artificial insemination(P/AI). Lactating Holstein cows (n = 519) with data available for GDPR, ovarian dynamics at G1, PGF, and P/AI were used to investigate associations of AMH, GDPR, and pregnancy outcomes. At G1 and PGF, cows had their ovaries scanned by ultrasonography for characterization of ovarian structures, and blood samples were collected for assessment of AMH, inhibin-A, inhibin-B and progesterone (P4) concentrations. Univariate analyses were performed to explore the relationship among these hormones and parity, lactation number, body condition score (BCS) at G1 and PGF, GDPR at G1 and PGF, presence of corpus luteum (CL) >14mm, diameter of CL, presence of multiple CL, total luteal volume, presence of large antral follicles >10mm, diameter of the largest follicle, presence of multiple follicles, follicular volume, number of follicles, number CL, plasma P4 concentration, ovulation at G1, and P/AI. Multivariable logistic regression was used to assess the relationship of AMH with ovulation at G1, P/AI, and other variables associated with these responses. Also,

receiver operating characteristics (ROC) were performed to assess whether AMH concentrations could predict ovulation at G1 and P/AI. Concentrations of AMH at days -10 and -3 were moderately correlated (r = 0.76). Multiparous cows had greater AMH concentration than primiparous cows. Cows in their 2nd and 3rd lactations had greater AMH concentrations than those in their 1st lactation. The AMH concentrations were weakly correlated with GDPR at G1 (r = 0.05) and PGF (r = 0.12). Cows were categorized according to GDPR, and there was a quadratic relationship between AMH concentration and GDPR quartile. The presence of multiple antral follicles was negatively associated with AMH concentration at the PGF, but not at G1. The number of follicles > 10 mm tended to be associated with higher AMH concentration at both time points. Neither the size of the largest follicle nor the total follicular volume was associated with AMH at G1 and PGF. The presence of multiple CLs was not associated with AMH, and the total luteal volume was positively associated with high AMH concentrations at G1 and PGF. The AMH concentration was not associated with P4 concentration at G1 and PGF. The AMH concentration was greater in cows that ovulated than in cows that failed to ovulate, but AMH was not associated with ovulation and P/AI. The ROC analysis indicates that AMH concentrations could not predict ovulation at G1 or P/AI. For the inhibin A and B results, the concentration of inhibin A and inhibin B were moderately correlated between the two time points of the DO, the G1, and PGF (r = 0.64 and 0.73, respectively), while correlations between the two hormones at any time point remained low (r = 0.14 and 0.19, respectively). For luteal parameters, there were correlations only between Inhibin A and the largest CL at PGF (r = -0.11) and total volume at PGF (r = -0.11). In contrast, luteal parameters at G1 were not correlated with inhibin B had no correlations. For follicular parameters, there were no correlations for either Inhibin A or Inhibin B. For hormonal concentrations, there was a tendency for a correlation (r = -0.08) between P4 and Inhibin A at G1,

but Inhibin A at PGF and P4 and Inhibin B at G1 and PGF were not correlated. When Inhibin concentrations were categorized, cows in the High Inhibin A group had a greater number of follicles than cows in the low Inhibin A group at G1. Moreover, cows in the High Inhibin A group had a smaller size for the largest CL than cows in the low Inhibin A group at G1. Greater inhibin A concentrations at PGF were observed in multiparous cows. For GDPR, there was only a tendency for a correlation between inhibin A and GDPR at PGF. When categorized, cows in the High Inhibin A at PGF had greater GDPR than cows in the Low Inhibin A group. In a multivariable regression model for ovulation, cows in the High Inhibin A and Medium Inhibin A groups at G1 ovulate more than cows in the Low Inhibin A group. In our univariate analysis, Inhibin B at G1 was greater in non-pregnant cows than in pregnant cows, but the multivariable logistic regression models showed that neither inhibin A nor B were associated with P/AI. The ROC analysis indicates that inhibin A and B concentrations could predict ovulation at G1 or P/AI. In conclusion, AMH concentration was positively associated with lactation number and had a weak, quadratic relationship with GDPR quartiles, but was not associated with ovulation at G1 or pregnancy per timed AI. Inhibin A and B were modestly associated with reproductive outcomes, but their predictive utility for ovulation and pregnancy per AI was limited.

Keywords: Ovulatory response, fertility, reproductive performance, ovarian dynamics

CHAPTER I

Anti-Müllerian hormone (AMH):

At the early stages of fetal development in mammals, both sexes possess two pairs of ducts: the Wolffian and Müllerian ducts (Rey and Grinspon, 2024). In the 1940s, Alfred Jost demonstrated that a testicular product, distinct from testosterone, was responsible for the regression of Müllerian ducts in male fetuses (Jost, 1947). This hormone, a Müllerian-inhibiting substance (MIS), also referred to as anti-Müllerian hormone (AMH) (Rey and Grinspon, 2024). It is known as a member of the transforming growth factor β (TGF-β) family, and the highly conserved C-terminal domain shares marked homology with both human and bovine species (Cate et al., 1986). Structurally, AMH is a homodimeric disulfide-linked glycoprotein with a molecular weight of approximately 140 kDa, encoded by a gene located on the short arm of chromosome 19 (Pellatt et al., 2010).

In the bovine species, a study showed that decreasing AMH mRNA and protein expression in granulosa cells as follicles matured toward dominance in the ovaries (Rico et al., 2009). However, in pathophysiological conditions such as spontaneously persistent follicles, granulosa cells exhibit altered endocrine activity, and AMH concentrations in follicular fluid were reported to be higher in persistent follicles at 10 and 15 days compared with normal follicles (Diaz et al., 2018). These findings suggest that while AMH normally declines with follicle dominance, its regulation may be disrupted in persistent follicles (Diaz et al., 2018). Furthermore, cows with cystic ovarian disease (COD), showed that the expression of inhibin and activin subunits is dysregulated in cystic follicles, and that the inhibin–activin–follistatin system is altered in granulosa and theca cells. Follicular fluid AMH concentrations increased in persistent follicles when compared to normal follicles (Diaz et al., 2018). Moreover, in bovine, AMH concentrations

were shown to decline in antral follicles ≥ 5 mm, while larger antral follicles ≥10 mm show a negative correlation between AMH concentration and progesterone (Monniaux et al., 2008).

It is believed that anti-Müllerian hormone plays a role in ovarian follicular dynamics. First, AMH limits the premature activation of primordial follicles, thereby preserving the ovarian reserve; as demonstrated in knockout mice, thus showing accelerated follicle depletion due to unchecked recruitment (Durlinger et al., 2002). Second, in human granulosa cells, AMH did not alter basal aromatase expression, but it significantly suppressed the gonadotropin-induced upregulation of CYP19A1 and P450scc mRNA, thereby attenuating estradiol synthesis (Sacchi et al., 2015). In mice, granulosa cell AMH expression is upregulated by oocyte-derived factors such as GDF9 and BMP15, whereas FSH exerts stage-specific effects—enhancing AMH expression in early developing follicles but reducing it as follicles advance toward dominance (Salmon et al., 2004). Third, AMH serves as a reliable endocrine marker of ovarian reserve, with circulating concentrations strongly correlating with antral follicle count and a predictive of super-ovulatory response in cattle (Ireland et al., 2008). Finally, as follicles were seen to mature toward dominance, AMH expression declined, suggesting to the study authors that there might be a physiological role in dominant follicle selection and ovulatory competence (Rico et al., 2009).

The AMH declines after puberty perhaps due to testosterone-mediated repression of Sertoli expression yet remains detectable in adult mice testis (Münsterberg and Lovell-Badge, 1991). In females, AMH modulates the size of the ovarian reserve by inhibiting the initial recruitment of primordial follicles, as shown in AMH-knockout mice which exhibited accelerated follicular depletion (Durlinger et al., 2002). Additionally, AMH reduces FSH responsiveness in growing follicles by suppressing aromatase and FSH receptor expression, thereby influencing estradiol synthesis and follicular selection (Durlinger et al., 2001, Chang et al., 2013).

Across farm animals, comparative studies indicated that AMH is detectable in the blood and ovaries of multiple species including bovine, equine, ovine, and swine, with conserved patterns of granulosa cell expression (Monniaux et al., 2011, Claes et al., 2015, Almeida et al., 2018). However, species-specific differences exist in concentration profiles, follicular dynamics, and correlations with reproductive outcomes, particularly in seasonal or litter-bearing species. Also, AMH has been successfully used as a predictor of embryo yield in super-ovulated heifers, with higher plasma concentrations associated with more transferable embryos and better *in vitro* oocyte competence (Monniaux et al., 2011). This predictive value has led to its incorporation into selection pipelines for donor cows in embryo transfer programs, improving efficiency and genetic gain (Batista et al., 2014). Recombinant AMH (rAMH) administration in mice has been shown to suppress primordial follicle recruitment and prevent gonadal-toxic follicular depletion during chemotherapy, highlighting its therapeutic potential (Kano et al., 2017).

While clinical use in livestock remains exploratory, the endocrine modulation achieved with rAMH may offer strategies for fertility preservation and cycle control in high-value females (Kano et al., 2017). Future AMH studies may investigate its follicular fluid concentrations and cellular signaling *in vivo* to refine its functional role beyond circulating levels. Additionally, expanding genomic analyses (Gobikrushanth et al., 2020) and including endocrine profiling may unlock novel applications of AMH in herd fertility management, reproductive lifespan prediction, and ovarian disorder diagnostics.

Interestingly, in cattle, the circulating AMH concentrations have minor variations throughout the estrous cycle (Rico et al., 2011, El-Sheikh Ali et al., 2013) but a wide variation across animals (Ribeiro et al., 2014, Gobikrushanth et al., 2019). This pattern of low variation of the AMH concentration during the estrous cycle, combined with the wide range of circulating

concentrations among cows, indicates that AMH may be a robust marker for phenotypical characterization and associations with reproductive responses. Indeed, in cattle, AMH concentrations were associated with antral follicle count (AFC) (Ireland et al., 2008, Mossa and Ireland, 2019), the number of follicles across development stages (Baruselli et al., 2018), and superovulation response (Souza et al., 2015). Studies also reported that AMH plasma concentrations decrease with parity and age (Ribeiro et al., 2014, Gobikrushanth et al., 2019), likely due to the depletion of the follicular reserve (Baruselli et al., 2018).

However, the association between AMH and pregnancy-related responses in cattle has been inconsistent (Ribeiro et al., 2014, Gobikrushanth et al., 2019, Sabuncu et al., 2019, Akbarinejad et al., 2020, Alward and Bohlen, 2020). A study performed with Holstein and crossbred grazing cows revealed that cows with low AMH concentration had greater pregnancy loss and a lower pregnancy rate following spontaneous estrus either by AI or natural service (Ribeiro et al., 2014). However, pregnancy per AI of cows receiving timed AI was not associated with AMH (Ribeiro et al., 2014). A negative association between AMH and estrous detection at timed AI was noted, leading the authors to suggest that differences in steroidogenesis in dominant follicles of cows may be associated with AMH concentrations in the circulation (Ribeiro et al., 2014). The authors suggested that synchronizing follicle development and ovulation seemed to override positive associations between AMH and fertility (Ribeiro et al., 2014). However, another study, including heifers and cows receiving timed AI, found a positive relationship between pregnancy per AI and AMH (Sabuncu et al., 2019).

In contrast, a study with dairy heifers bred after estrous detection revealed no association between AMH and pregnancy per AI to first service (Alward et al., 2021). Another recent study showed a quadratic relationship between AMH and pregnancy, with intermediate AMH

concentrations having the highest reproductive performance, especially in first-service insemination after estrus detection (Akbarinejad et al., 2020). The inconsistencies between AMH concentrations and pregnancy outcomes suggested that further studies are needed to help elucidate this relationship.

Inhibin A and B:

Another hormone of interest that plays a role in ovarian follicular dynamics has in bovine species is inhibin (Robertson et al., 1986a; Hutchinson et al., 1987; Shukovski et al., 1993; Wrathall and Knight, 1993). At least six different inhibin forms have been identified in bovine follicular fluid (Miyamoto et al., 1986), and later, nine different forms of inhibin have been reported (Good et al., 1995). Two of these are known as inhibin β-subunits, which can be either β_A or β_B (Juengel et al., 1993; Walentowicz et al., 2014) and will be mentioned hereafter as inhibin A and B, respectively. Inhibin A & B are members of the transforming growth factor β (TGF-β) family and are produced by the growing preantral and antral follicles (Knight and Glister, 2006). Gene expression analyses in bovine granulosa cells confirmed transcription of INHA, INHBA, and INHBB, supporting the ovarian origin of inhibin subunits and their local production during folliculogenesis (Laird et al., 2019). Importantly, the β-subunits of inhibin are structurally identical to those of activins, allowing for the context-dependent synthesis of either inhibitory (inhibin) or stimulatory (activin) dimers depending on the relative availability of partner subunits (Knight and Glister, 2006).

Inhibin A is a glycoprotein that is hypothesized to be produced exclusively by the granulosa cells of preantral follicles, and it works to inhibit the synthesis and release of the follicle-

stimulating hormone (FSH) (Wrathall and Knight, 1993; Lu et al., 2009). The synthesis of inhibin subunits in bovine granulosa cells is tightly regulated by gonadotropins and steroid hormones. Follicle-stimulating hormone (FSH) has consistently been shown to stimulate the expression of INHA and INHBA in granulosa cells from small and growing follicles, with dose-dependent increases in both immunoreactive and bioactive inhibin secretion (Wrathall and Knight, 1993). This effect is enhanced in serum-free culture systems, where granulosa cells retain functional FSH receptors and maintain their responsiveness to endocrine cues (Wrathall and Knight, 1995, Laird et al., 2019). In women, serum inhibin B concentrations decline in the early follicular phase while FSH rises, demonstrating an inverse relationship between the two hormones (Welt et al., 2003). Moreover, serum inhibin B levels have been shown to decline with advancing age, supporting its role as a biomarker of ovarian reserve (Seifer et al., 1997).

In horses, the concentrations of inhibin have emerged as significant biomarkers for diagnosing granulosa cell tumors (GCTs) and assessing reproductive cyclicity. Research indicates a notable increase in serum inhibin levels in mares affected by GCTs, which occurs in approximately 87% to 90% of cases (Bailey et al., 2002), and this high diagnostic sensitivity has been consistently emphasized in subsequent reviews (Sherlock et al., 2016). However, it is important to note that although earlier reports indicated elevated serum inhibin concentrations in most mares with histologically confirmed GCTs (McCue et al., 2006). A later review of 52 cases demonstrated that only about 60% of affected horses showed such elevations (Sherlock et al., 2016). A more recent study has shown that isoform-specific assays revealed that inhibin B, rather than inhibin A or total inhibin, is a far more consistent biomarker of equine GCTs (Conley et al., 2018). Inhibin B was significantly elevated in all mares with GCTs compared with cyclic and pregnant mares, whereas inhibin A and total inhibin overlapped across groups, limiting their

diagnostic utility (Conley et al., 2018). Additionally, the role of inhibin in regulating reproductive cyclicity has been documented. In prepubescent equine females, circulating levels of immunoreactive inhibin (ir-inhibin) exhibit significant fluctuations, characterized by initial high levels at birth which decline as the horse matures (Dhakal et al., 2011). This decline aligns with the physiological changes associated with puberty and reflects the changing dynamics of pituitary-gonadal axis activity. Inhibin immunization in goats and buffalo has been explored as a strategy to increase super-ovulatory responses by enhancing endogenous FSH secretion (Geng et al., 2008a). Collectively, inhibin A and B serve as both regulators of gonadotropin secretion and potential biomarkers of ovarian function, with cross-species evidence supporting their physiological utility and emerging potential for reproductive manipulation in domestic livestock.

In dogs, immunohistochemical detection of inhibin-α in ovarian tissue has facilitated accurate diagnosis and differentiation of granulosa cell tumors (Riccardi et al., 2007). A recent study in feline species has reported that AMH and inhibin-B fluctuated in a highly correlated and cyclic pattern in cats who failed to ovulate, whereas AMH declined as progesterone increased in ovulated cats (Johnson et al., 2023). Since this study reports a large number of cats (intact ovary n=205, and ovariohysterectomized n=49), it established the reference ranges for AMH and inhibin B for both reproductive statuses (fertile and infertile) (Johnson et al., 2023). These studies affirm the physiological relevance and diagnostic utility of inhibin A and B beyond farm animals, highlighting their broad value across mammalian species for reproductive health and disease detection.

In bovine species, research has shown that inhibin A has low plasma concentration in the early follicular phase but rises during ovulation and the luteal phase (Bleach et al., 2001). The opposite takes place with inhibin B levels, which increase during the follicular phase and peak

during the mid-follicular (Beg et al., 2002). Research has shown that inhibin has a key role in follicles' growth and differentiation (Hutchinson et al., 1987; Stangaferro et al., 2014). Human recombinant inhibin A has been used to improve fertility either as supplementation of *in vitro* maturation medium (Stock et al., 1997) or, even more recently, as whereas more recent work has explored immunization against inhibin, in combination with progesterone supplementation, as a strategy to improve fertility under heat stress (Chen et al., 2022).

Bovine inhibin A was shown to regulate the apoptosis and cellular progression of granulosa cells (Xu et al., 2020) and may work to inhibit the synthesis of FSH (Taya et al., 1996, Kaneko et al., 1997). Inhibin B follows a different trend, marked by an increase during the follicular phase and peaks during the mid-follicular phase (Walton et al., 2011), and it works to inhibit the synthesis and release of FSH during the luteal phase (Henderson and Franchimont, 1981; Kaneko et al., 1997). Inhibin B is the predominant isoform in bovine follicular fluid, with immune-active concentrations several-fold higher than inhibin A, as shown by early biochemical characterizations of inhibin subunits (Miyamoto et al., 1986, Good et al., 1995,Wrathall and Knight, 1993). Reports have shown that eight out of nine identified inhibin isoforms suppress basal FSH secretion in pituitary cell cultures and GnRH release from hypothalamic explants of ovariectomized ewes, with secretion quantified by radioimmunoassay (Good et al., 1995b). Moreover, inhibin A has been associated with follicle diameter, especially in small and medium-sized follicles (Geng et al., 2008b). Also, research has shown that inhibin A negatively correlates with FSH but positively correlates with estradiol through the estrous cycle (Bleach et al., 2001). However, the relationship between plasma inhibin and ovarian responses to estrous synchronization programs and pregnancy outcomes has not been established in dairy cows. Still, the relationship between inhibin A and B and GDPR in dairy cows remains unexplored.

An interesting study (Morris et al., 1997) demonstrated that immunization against the inhibin-subunits significantly disrupted the ovulation rate in cattle. Moreover, a recent study showed that the concentrations of interferon-tau in blood at pregnancy recognition have doubled in cows immunized against the inhibin α-subunit (Chen et al., 2022). Juengel et al. (1993) reported that inhibin α- and βA-subunit mRNA in bovine follicles declined slightly after PG but rose transiently following GnRH, whereas Bleach et al. (2001) measured plasma inhibin A protein, which peaked with dominant follicle growth and fell sharply after the LH surge—differences that reflect measurement of follicular mRNA versus circulating protein. Moreover, inhibin A has been associated with follicle diameter, especially in small and medium-sized follicles (Geng et al., 2008). The abundance of inhibin α- and β-subunits determines the synthesis of bioactive inhibin, which suppress pituitary FSH (Robertson et al., 1986) and influence follicle development, ovulation rate, and pregnancy recognition in cattle (Morris et al., 1997) (Morris et al., 1997; Chen et al., 2022). Also, inhibin A was negatively correlated with FSH but positively correlated with estradiol through the estrous cycle (Bleach et al., 2001), but further research is needed to examine the relationship between inhibin A & B and reproductive response parameters

Building upon the original Ovsynch synchronization protocol, the Double-Ovsynch is an advanced fertility program designed for better reproductive efficiency in lactating dairy cows (Souza et al., 2008). It works by enhancing pregnancies per artificial insemination (P/AI) and reducing pregnancy losses by incorporating a pre-synchronization phase that ensures cows begin the final synchronization at the optimal stage of the estrous cycle to start the Ovsynch program (Souza et al., 2008). The Double-Ovsynch effectiveness is supported by several improved physiological responses, including increased ovulatory response, optimized progesterone

concentrations during follicular development, and improved luteolysis before induction of ovulation for timed AI (Souza et al., 2008).

The DO protocol offers controlled physiological conditions for dairy cows (Souza et al., 2008). It offers a tightly synchronized hormonal environment, allowing for the assessment of hormones like AMH and inhibin A and B concentrations at standardized time points relative to the estrous cycle and follicular wave emergence. By aligning cows to a predictable ovulatory pattern, this protocol minimizes variability in endocrine profiles due to stage-of-cycle differences (Souza et al., 2008), which is helpful in reducing variability, a desirable condition when comparing hormonal biomarkers like AMH and inhibin isoforms. Moreover, the DO enables precise sampling at the first GnRH (G1) and PGF stages—two time points that place cows approximately at 7 or 8 of the estrous cycle (Giordano et al., 2013, Fricke and Wiltbank, 2022). The DO has been shown to improve ovarian response of the Ovsynch for breeding and pregnancy per AI (P/AI) rates compared to traditional timed AI programs, especially in high-producing dairy cows (Souza et al., 2008), so DO offers a sound platform to assess association with ovarian responses and pregnancy per AI.

The Double-Ovsynch protocol establishes a favorable endocrine environment, with elevated progesterone during follicle development and increased estradiol coupled with reduced progesterone at the time of AI (Giordano et al., 2013). These hormonal dynamics are associated with improved uterine receptivity and fertilization success, and several studies have consistently reported a 5–10% increase in pregnancy per AI in cows subjected to Double-Ovsynch compared with Presynch-Ovsynch or AI following estrus detection (Giordano et al., 2013, Pursley et al., 1995). In addition, the protocol has been linked to reduced double ovulation rates, lower incidence of twin pregnancies, and decreased pregnancy loss. Studies has shown that the DO enhances

progesterone profiles during follicular development by increasing the proportion of cows with functional CL at GnRH treatment (Ayres et al., 2013). A recent study indicated that cows with lower progesterone levels during this period of the DO are more prone to multiple ovulations (Valdés-Arciniega et al., 2025). As a result, the DO has become a key tool for maximizing reproductive performance in lactating dairy cows. It is reasonable to surmise that using cows undergoing the Double-Ovsynch might be the best option to explore the relationship of AMH, inhibin A, and B with fertility traits and pregnancy-associated parameters.

Genomic Daughter Pregnancy rate:

Another factor that might contribute to variable pregnancy outcomes is genetics. In early 2010, with the exponential increase in genotyping in dairy cows (Weller et al., 2017), dairy farms started to utilize genomic prediction index value such as genomic daughter pregnancy rate (GDPR) to improve the selection of dairy cows (Norman et al., 2009) for improved fertility. Indeed, GDPR is a genomic evaluation metric used to estimate a dairy cow's genetic merit for fertility, specifically the association with reproductive outcomes within a given 21-day estrous cycle (Norman et al., 2009). It is SNP-based genomic data combined with large phenotypic datasets, GDPR is expressed as a percentile rank or standardized value predicting a bull's expected contribution to female fertility in his daughters. Originally developed to counter declining fertility trends in high-producing dairy cows, GDPR has been incorporated into national selection indices such as net merit (NM$) and total performance index (TPI) (Lima et al., 2020). The metric is derived from days open, number of inseminations per conception, and pregnancy per AI, and has shown low heritability (h^2 = ~0.03–0.07) and genetic correlations with genomic prediction values (Gobikrushanth et al., 2020, Neupane et al., 2021).

A series of studies confirmed that GDPR is positively associated with estrus characteristics, estradiol, and pregnancy outcomes in heifer and parous cows (Veronese et al., 2019a, Lima et al., 2020, Madureira et al., 2022) independently of the breeding program females were exposed to estrus detection or timed AI. The exact mechanisms by which females with greater GDPR express improved fertility remain unclear. Recently, AMH was shown to have a relatively high heritability (h^2 = 0.36 to 0.40) in Holstein heifers (Nawaz et al., 2018), and grazing Irish dairy cows (h^2 = 0.40) (Gobikrushanth et al., 2019). Conversely, fertility as predicted by GDPR has low heritability (Cole et al., 2011) and could benefit from co-selection with other biologically related measures. Future advancements may include the integration of GDPR with endocrine biomarkers and cow-specific response profiles to support precision reproductive strategies and the selection of elite sires and females with high reproductive performance.

CHAPTER II

Interpretative Summary

Association of anti-müllerian hormone with genomic prediction of daughter pregnancy rate, ovarian responses, and pregnancy per AI in lactating dairy cows *By Salman et al.,* Anti-Müllerian hormone have been suggested as a predictor of the antral follicle count, while genomic prediction of daughter pregnancy rate (GDPR) and ovulation at the beginning of Ovsynch (G1) are associated with improved pregnancy outcomes in dairy cows. Herein, we performed a study to test the hypothesis that AMH is positively associated with GDPR, ovulation at G1, and pregnancy outcomes. The results of the current study indicated that AMH is positively associated with GDPR but not associated with ovulation at G1 nor with pregnancy outcomes.

Running Head: ASSOCIATION OF AMH and GDPR IN DAIRY COWS

Association of anti-Müllerian hormone with genomic prediction of daughter pregnancy rate, ovarian responses, and pregnancy per AI in lactating dairy cows

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INTRODUCTION

The anti-Müllerian hormone (AMH) is a dimeric glycoprotein produced mainly by granulosa cells of growing preantral and antral follicles and is known as a member of the transforming growth factor-β family (Cate et al., 1986). It has been suggested that AMH plays a role in follicle growth and differentiation by inhibiting the recruitment of primordial bovine follicles (Yang et al., 2017). Notably, circulating AMH concentrations in cattle exhibit minimal fluctuation throughout the estrous cycle (Rico et al., 2011, El-Sheikh Ali et al., 2013) yet show considerable variation among individual animals (Ribeiro et al., 2014, Gobikrushanth et al., 2019). This inter-animal variability suggests that AMH may serve as a reliable biomarker for phenotypic characterization and associations with reproductive outcomes. Indeed, in cattle, AMH concentrations were associated with antral follicle count AFC (Ireland et al., 2008, Mossa and Ireland, 2019), the number of follicles across development stages (Baruselli et al., 2018), and superovulation response (Souza et al., 2015). Studies also reported that AMH plasma concentrations decrease with age (Ribeiro et al., 2014, Gobikrushanth et al., 2019), likely due to the depletion of the follicular reserve (Baruselli et al., 2018).

Conversely, the association between AMH and pregnancy-related responses in cattle has been inconsistent (Ribeiro et al., 2014, Gobikrushanth et al., 2019, Sabuncu et al., 2019, Akbarinejad et al., 2020, Alward and Bohlen, 2020). A study performed with Holstein and crossbred grazing cows revealed that cows with low AMH concentration had greater pregnancy loss and a lower pregnancy rate following spontaneous estrus, bred either by AI or natural service (Ribeiro et al., 2014). However, the P/AI of cows receiving timed AI was not associated with AMH (Ribeiro et al., 2014). A negative association between AMH and estrus detection at timed AI was

noted, suggesting differences in steroidogenesis in dominant follicles of cows with distinct AMH concentrations (Ribeiro et al., 2014). The authors suggested that synchronizing follicle development and ovulation seemed to override positive associations between AMH and fertility (Ribeiro et al., 2014). However, another study, including heifers and cows receiving timed AI, found a positive relationship between P/AI and AMH (Sabuncu et al., 2019). In contrast, a study with dairy heifers bred after estrus detection revealed no association between AMH and conception risk to first service (Alward and Bohlen, 2020). Another recent study showed a quadratic relationship between AMH and pregnancy, with intermediate AMH concentrations having the highest reproductive performance, especially in first-service insemination after estrus detection (Akbarinejad et al., 2020). The inconsistencies between AMH concentrations and pregnancy responses suggested that further studies are needed to help elucidate the nuances of this relationship.

Another factor that might contribute to variable pregnancy outcomes is genetics. In early 2010, with the exponential increase in genotyping in dairy cows (Weller et al., 2017), dairy farms started to utilize traits such as genomic DPR (GDPR) to improve selection towards improved fertility for dairy cows (Norman et al., 2009). A series of studies confirmed that GDPR is positively associated with estrus characteristics, estradiol, and pregnancy outcomes in heifers and parous cows (Veronese et al., 2019b, Lima et al., 2020, Madureira et al., 2022, Melo et al., 2025), independent of the breeding program, estrus detection, or timed AI. Still, potential mechanisms by which GDPR impacts fertility remain unclear. Recently, AMH was shown to have a relatively high heritability, $h^2 = 0.36$, in Holstein heifers (Nawaz et al., 2018) and grazing Irish dairy cows, $h^2 = 0.40$ (Gobikrushanth et al., 2019). Conversely, fertility traits such as GDPR are lowly heritable (Cole et al., 2011) and could benefit from co-selection of other biologically related traits. However,

the relationship between AMH, a reproductive hormone with high heritability, and GDPR has yet to be investigated.

The plasma concentrations of AMH have been associated with various herd parameters in dairy cattle. Breed, body condition score BCS, number of lactations, and parity are among the key traits for identifying cows in most herds. Several studies have reported that AMH plasma concentrations decrease with age, likely due to the depletion of the antral follicle reserve (Baruselli et al., 2018, Haldar et al., 2019). The number of lactations (Ribeiro et al., 2014) and parity (Gobikrushanth et al., 2018) were shown to be negatively associated with AMH concentrations in lactating dairy cows. When AMH concentrations of daughters of multiparous cows were compared to those of daughters of nulliparous cows, the AMH concentrations were greater in the daughters of multiparous cows than in primiparous and associated with better fertility outcomes (Akbarinejad et al., 2018). This suggests that plasma concentrations of AMH can be used as a marker of ovarian reserve in dairy cows (Garcia-Guerra et al., 2017, Alward and Bohlen, 2020), yet the relationship between AMH and parity needs further investigation.

Several ovarian response parameters were investigated in association with AMH concentration in cattle (Ribeiro et al., 2014, Jimenez-Krassel et al., 2015a, Alward and Bohlen, 2020). Research has shown that AMH plasma concentrations were positively correlated with ovary size (Ireland et al., 2008), the number of antral follicles growing during follicular waves (Ireland et al., 2008), the size of the ovarian reserve, and progesterone (**P4**) from the ovaries (Ireland et al., 2009b). Also, AMH was shown to be positively associated with superovulation response, total structures collected, and total transferable embryos (Souza et al., 2015). Previous studies have reported that AMH can predict multiple ovulations (Singh et al., 2004) and the number of oocytes recovered by OPU (Mossa et al., 2017). Contrarily, another study showed that no association was

found between AMH concentration and AFC or ovulation rate in cows with Trio high-fecundity allele (Garcia-Guerra et al., 2017). Thus, further investigation is needed to characterize the association between AMH concentrations and ovarian parameters and responses.

We hypothesize that AMH concentration at the first GnRH (G1) and $PGF_{2\alpha}$ of breeding Ovsynch of a Double-Ovsynch (**DO**) protocol is positively associated with GDPR, ovulation at G1, and P/AI; and AMH can be used to predict ovulation at G1 and P/AI of cows undergoing a DO protocol. Therefore, the objectives of this study were to evaluate the association of plasma concentration of AMH at G1 and PGF of breeding Ovsynch of a DO protocol with GDPR, ovulation at G1, and P/AI. We also aimed to assess the association of AMH with GnRH dose at G1, ovarian structures at G1, P4 at G1, PGF, parity, BCS, and lactation.

MATERIALS AND METHODS

The procedures in this experiment were revised in accordance with protocol submission #22792 by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis. It received an ethical review because it was deemed a "non-university client-owned" study that is exempt from protocol according to the University of California, Davis IACUC policies. All animal procedures followed the recommendations of the Guide for the Care and Use of Agricultural Animals in Research and Teaching FASS, 2010.

Animals, housing, and diet

For this experiment, a subset of 523 Holstein genotyped cows (Primiparous = 358 and Multiparous = 165), from a commercial dairy farm located in Hanford, CA, was used. All animals were genotyped using SNP platforms commercially available in the United States, Clarifide,

Zoetis, Parsippany, NJ before first breeding. The GDPR values were obtained from the US national evaluation provided by the Animal Improvement Programs Laboratory of the USDA when the animals were heifers. Cows were housed in free-stall barns equipped with sprinklers and fans for cooling. Cows were fed a TMR ration to meet or exceed their nutrient requirements for a lactating Holstein producing 48 kg/d with 3.5% fat and 3.2% true protein when DM intake is 25 kg/d NRC, 2001. The cows were fed twice a day and milked three times a day. The diet consisted of alfalfa and wheat hay, whole cottonseed, almond hulls, ground corn, ground wheat, dried-distilled grains, beet pulp, solvent-extracted soybean meal, and minerals and vitamins premixes.

Reproductive program and experimental design

Cows used in the study were enrolled in a DO as previously described (Souza et al., 2008). Briefly, on Day -27 of the study (61 ± 3 DIM), cows received an i.m. injection of 2 ml Factrel, 100 μg of gonadorelin hydrochloride, Zoetis Inc., Kalamazoo. Then, 7 days later, on Day -20 (68 ± 3 DIM), cows received i.m. injection of 2 ml $PGF_{2\alpha}$ Lutalyse HighCon, 25 mg of Dinoprost tromethamine. Zoetis Inc., Kalamazoo, MI. Three days later, on Day -17 (71 ± 3 DIM), a 2nd dose of GnRH was administered. At Day -10 (71 ± 3 DIM), a 3rd dose of GnRH i.m. was given at initiation of the breeding Ovsynch component of the DO. Seven days later Day -3 (78 ± 3 DIM), the 2nd dose of $PGF_{2\alpha}$ was administered. Then, 56 hours later, at Day -1 (80 ± 3 DIM), a fourth GnRH dose was administered, followed by a timed AI 16 hours later at Day 0 of the study (Day 81 ± 3 DIM) (Figure 1). Cows enrolled in the study were blocked by parity (primiparous vs. multiparous), and then within parity, cows were ranked by ascending GDPR and randomly allocated to receive either 100 μg or 200 μg of gonadorelin hydrochloride

Ovarian ultrasonography and pregnancy diagnosis

The subset of 523 Holstein genotyped cows received ultrasonography examination at Day -10 and -3 of the study to characterize ovarian characteristics and responses to the first GnRH of breeding Ovsynch. Briefly, the transrectal ultrasonography of the ovaries was performed using a portable ultrasound device with a 7.5-MHz transrectal probe (Easi-Scan: Go, BCF Technologies USA Ltd. LLC, Rochester, MN). The presence of CL larger than 14 mm, follicles equal to or greater than 10 mm in diameter, presence of multiple follicles and/or corpus lutea was documented. Ovulation at the first GnRH of the breeding Ovsynch was assumed if a cow had a follicle equal to or greater than 10 mm on Day -10, and a newly formed CL was observed in the same ovary on Day -3 of the study. Cows with follicles smaller than 10 mm on Day -10, but with a new CL on study Day -3, were considered to have a new CL, though they ovulated before GnRH treatment. The diameter of the follicles and CL cavity was measured on Days -10 and -3. Then, follicular and luteal volumes were calculated by using the following equation $4.1888 \text{ X} \left(\frac{\text{Diameter of follicle or CL}}{2}\right)^3$, as previously described (Merce et al., 2006).

For pregnancy diagnosis, transrectal ultrasonography of the ovaries was performed using a portable ultrasound device with a 7.5-MHz transrectal probe (Easi-Scan, BCF Technologies USA Ltd., Rochester, MN) at 32 days post-timed artificial insemination (AI) by the herd veterinarian. The presence of an amniotic vesicle with a visible embryo heartbeat was used as a criterion to determine pregnancy. Cows diagnosed pregnant on day 32 received another transrectal palpation examination of uterine contents at 60 days post-AI to confirm pregnancy status. The pregnancy rate per AI was determined by dividing the number of pregnant cows on day 32 by the total number of AI recipients. Pregnancy loss was defined for cows diagnosed pregnant on d 32 and non-pregnant on d 60 after timed AI. Pregnancy loss between 32 and d 60 of gestation was calculated

by dividing the number of cows identified as non-pregnant on d 60 after timed AI by the number of cows pregnant on d 32 after timed AI.

Blood samples and hormonal essays

Blood samples were collected to measure the concentration of P4 and AMH at (Days -10) 1st GnRH of breeding Ovsynch and (Days -3) $PGF_{2\alpha}$ treatments of the breeding Ovsynch of the study by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K2 EDTA. Immediately after collection, samples were placed on ice and refrigerated until transported to the laboratory. Upon arrival at the laboratory, blood tubes were centrifuged at 2,000 × g for 15 minutes at 5°C, and a 2 mL plasma aliquot was frozen at -80°C for later analysis. The P4 was analyzed using a competitive enzyme immunoassay assay (Munro and Stabenfeldt, 1984), as described previously (Gomez et al., 2018). The concentration of AMH was measured using a commercial Bovine AMH ELISA as previously validated for use in cattle, as previously described (Fushimi et al., 2019). The measurement of plasma concentrations of AMH was performed using a commercial chemiluminescence immunoassay as previously validated for use in cattle, as previously described (Ribeiro et al., 2014) by Ansh Labs LLC, Bovine AMH CLIA, Ansh Labs, Webster, TX. Briefly, the concentration of AMH was analyzed using a commercial chemiluminescence immunoassay developed for specific binding and accurate quantitation of bovine AMH. The monoclonal antibody pair used in this assay detects pro-mature covalent and nonconvex-lent bovine AMH complexes and does not have cross-reactivity to other proteins of the transforming growth factor family, such as inhibin A and B and activins A, B, and AB.

Statistical analysis

All data were analyzed using JMP version 17.2 (SAS/STAT; SAS Institute Inc., Cary, NC). A 3-step approach was used to assess the association of AMH concentration in plasma and responses of interest. First, descriptive statistics and distributions were performed for all continuous variables, and normality of residuals was evaluated using histograms, Q-Q plots, and the Shapiro-Wilk test. The concentrations of AMH were shown to be normally distributed. A total of four cows were not used for statistical analyses because we did not obtain hormonal results for them. Cows were classified into quartiles based on AMH concentration as Q1: range = 10.8-134.0 pg/mL, average = 96.6 pg/mL (n = 128); Q2: range = 134.3-214.5 pg/mL, average = 172.5 pg/mL (n = 128); Q3: range 215.8-323.2 pg/mL, average = 264.7 pg/mL, (n = 129); Q4: range = 324.5-1,346.6 pg/mL; average = 495.0 pg/mL (n = 134). The AMH concentrations at G1 were also categorized as: low: (10.8-156.4; $\bar{x}$ = 108.1, n = 171); moderate: (159-283.5; $\bar{x}$ = 219.0, n = 176); high: (285-1346.6; $\bar{x}$ = 450.2; n = 172) pg/mL for further investigation into its associations with ovulation and P/AI. We also categorized cows according to GDPR into quartiles as follows: Q1: (-3.3 to -0.6; $\bar{x}$ = -1.4; n = 142); Q2: (-0.5 to 0.0; $\bar{x}$ = -0.14; n = 137), Q3: (0.1 to 0.9; $\bar{x}$ = 0.48; n = 122), Q4: (1.0 to 3.9; $\bar{x}$ = 1.68; n = 118). The P4 concentrations at G1 were also categorized as: low: (0.09 to 2.85; $\bar{x}$ = 1.85, n = 171); moderate: (2.87 to 4.82; $\bar{x}$ = 3.83, n = 189); high: (4.83 to 20.3; $\bar{x}$ = 7.41; n = 160) ng/mL for further investigation.

Second, we performed univariable analyses for each independent variable to assess its association and correlation with the concentration of AMH. The relationship of AMH with parity, number of lactations, BCS, GDPR, presence of CL, size of CL, multiple CL, total luteal volume, presence of follicles, size of the largest follicle, multiple follicles, follicular volume, ovulation, and plasma P4 concentration was analyzed. Third, multivariable logistic regression was used to

assess the relationship between AMH and ovulation at G1 and P/AI, as well as other covariates associated with these responses. Additionally, receiver operating characteristic (ROC) curves were performed to assess whether AMH could predict ovulation at G1 and P/AI. Multicollinearity was assessed using the variance inflation factor (VIF), and model selection was guided by the Akaike information criterion (AIC) and Bayesian information criterion (BIC). Significance was declared at $P < 0.05$, while tendency was declared when $0.05 \leq P < 0.10$.

RESULTS

Characterization of plasma AMH concentrations

The distribution of AMH concentrations in a subset of 519 cows (n = 1,038 samples) showed a wide range (10.8 to 1,495.1 pg/mL), with a mean ± SD of 259.1 ± 179.5 pg/mL (Figure 2A). The histogram help illustrates that approximately 90% of the samples were below the 500 pg/mL (Figure 2A). Pearson correlations showed that AMH concentrations measured at the 1st GnRH (d −10) and $PGF_{2\alpha}$ (d −3) were strongly correlated (r = 0.76; $P < 0.001$), indicating minor variation across the plasma concentration of AMH within individual cows (Figure 2B). This small variability within each animal corroborates the concept that plasma AMH concentrations have the potential to be a reliable predictor across the estrous cycle.

Characterization of AMH plasma concentration and its association with GDPR

Pearson correlations between GDPR and AMH concentrations showed a weak correlation but significant at both the 1st GnRH (r = 0.03; $P < 0.001$) and PGF (r = 0.1; $P < 0.001$) time points (Figure 3A and 3B). Moreover, when cows were categorized by GDPR quartiles, a quadratic

relationship emerged at both time points, with the highest mean AMH concentrations observed in cows within the third quartile (Q3) of GDPR indicating a nonlinear U-shaped relationship at both the 1st GnRH ($P = 0.004$) and PGF ($P = 0.009$) time points (Figure 3C).

Characterization of AMH association with parity, number of lactations, and BCS

Multiparous cows had higher ($P < 0.001$) circulating AMH concentrations compared with primiparous cows (Figure 4A). When we analyzed the data by lactation number, AMH concentrations increased ($P < 0.001$) as the number of lactations increased (Figure 4B). Cows in their 2nd and 3rd lactations were associated with higher AMH concentrations when compared to cows in their 1st lactation, which was consistent with the parity effect (Figure 4B). No significant differences were observed between the 2nd and 3rd lactations in this cohort of cows. There were no significant differences between the high, moderate, and low categories of BCS in the current study (Figure 4C).

Characterization of AMH concentrations and circulating P4

Associations between AMH and circulating P4 concentrations were evaluated at two points during the DO protocol (Figure 5). No significant correlation was detected between AMH and P4 at the G1 ($r = -0.02$; $P = 0.20$) (Figure 5A). A weak positive correlation was observed at PGF ($r = 0.09$; $P = 0.02$) (Figure 5B). When cows were categorized by P4 concentration (low, moderate, or high) at each time point, no differences in AMH concentration were detected at 1st GnRH ($P = 0.50$). In contrast, at PGF, cows in the low P4 had lower ($P = 0.02$) AMH concentrations compared with cows in the moderate and high P4 categories (Figure 5C).

Characterization of AMH concentration and ovarian responses

The results of the current study indicated that cows with multiple follicles had higher AMH concentrations than those with a single follicle at the PGF time point ($P = 0.04$), but not at the G1 (Figure 6A). Nevertheless, the AMH concentrations were not associated with the presence of multiple CLs (Figure 6B). The number of follicles tended to be associated with the categories of AMH concentrations at G1 ($P = 0.09$) and PGF ($P = 0.07$) (Figure 6C). However, no difference ($P > 0.10$) was observed between the categories of AMH concentrations and the number of CLs or the total follicular volume (Figures 6D and 6E). In contrast, cows with higher AMH concentrations had significantly higher total luteal volume at G1 ($P = 0.04$) and at PGF ($P = 0.006$), respectively (Figure 6F).

Characterization of AMH concentration and its association with ovulation

The AMH concentration was not associated ($P = 0.41$) with ovulation at the G1 (Figure 7A). A predictive model was constructed to determine whether AMH can be used to predict ovulation at the G1 of the DO protocol. The ROC analysis revealed that AMH concentrations cannot be used to predict ovulation (AUC = 0.52, $P = 0.45$) in lactating Holstein cows enrolled in the DO protocol (Figure 7B). Moreover, ovulation was not associated with the categories of AMH concentration ($P = 0.80$) (Figure 7C).

Characterization of AMH concentrations and their association with pregnancy per AI

Plasma AMH concentrations were not associated with P/AI on day 60 post-AI in cows enrolled in a DO protocol (Figure 8A). Moreover, AMH concentrations were unable to predict pregnancy ($P = 0.51$) in this study (Figure 8B). The P/AI also did not differ among cows stratified

by categories of AMH concentration at either time point ($P = 0.28$ at G1; $P = 0.60$ at PGF; Figure 8C).

DISCUSSION

This study was designed to advance our understanding of the relationship between the plasma concentration of AMH at G1 and PGF of breeding Ovsynch in a DO protocol with GDPR, ovulation at G1, and P/AI. Indeed, AMH has been associated with antral follicle count and researchers have suggested that might be a potential biomarker for ovarian reserve in cattle, with increasing interest in its potential utility for predicting reproductive performance (Mossa and Ireland, 2019). Concurrently, genomic prediction of daughter pregnancy rate (Lima et al., 2020, Melo et al., 2025) and ovulatory response to the 1st GnRH of the Ovsynch (Giordano et al., 2013, Fricke and Wiltbank, 2022) protocol has been associated with enhanced fertility outcomes in lactating dairy cows. In this study, we evaluated the hypothesis that circulating AMH concentrations were positively associated with GDPR, ovulatory response at G1, and subsequent P/AI. Our findings indicated a modest but significant association between AMH and GDPR, suggesting a potential biological linkage between endocrine and genomic indicators of fertility. However, this is likely insufficient to recommend that co-selection for these traits is promising. Furthermore, AMH was not associated with ovulation at G1 nor with outcomes, suggesting that while AMH reflects aspects of ovarian capacity, it may not reliably predict downstream reproductive success under timed artificial insemination protocols.

The high correlation of AMH concentrations between 1st GnRH and PGF time points confirms its physiological stability within individuals, consistent with prior findings in cattle

(Jimenez-Krassel et al., 2015a, Mossa et al., 2017), and supports its utility as a potentially reliable endocrine marker. The broad distribution of AMH concentrations observed in this study aligns with reports in both dairy and beef cattle, where variation is attributed to inherent differences in antral follicle count AFC, granulosa cell function, and ovarian reserve (Rico et al., 2011, Gobikrushanth et al., 2017). Similar AMH ranges have been previously described in Holstein cows using validated ELISA platforms, and repeatability exceeding r = 0.79 has been reported, particularly when samples are collected at similar stages of the estrous cycle (Pfeiffer et al., 2014, Souza et al., 2015). The robustness of AMH measurement across sampling points provides a strong rationale for its incorporation into predictive fertility tools, particularly in large-scale reproductive programs such as DO (Rico et al., 2011, Gobikrushanth et al., 2019).

The weak but significant correlations observed between plasma AMH and GDPR suggest that AMH captures elements of intrinsic fertility potential not fully explained by genomic evaluations alone (Mossa et al., 2017, Gobikrushanth et al., 2019). Cows in the highest GDPR quartile did not exhibit the greatest AMH levels, which may reflect a decoupling of genomic merit and endocrine markers, especially in high-producing cows selected intensively for other traits (Gobikrushanth et al., 2019). These findings reinforce the concept that AMH serves as a physiological biomarker reflecting antral follicle count and granulosa cell function (Ireland et al., 2009a, Batista et al., 2014). However, its role in predicting fertility should be interpreted within the broader genomic and environmental context.

In this study, we observed an increase in AMH concentrations with advancing parity and lactation number. Ribeiro et al. reported higher AMH concentrations were greater for cows in 2nd and 3rd lactation than for cows in 1st and $\geq$4th lactation (Ribeiro et al., 2014). In the current study, we did not have data for cows in lactation 4 and above to see if our data had a similar quadratic

relationship to the previous study. In the previous study, authors suggested that follicular recruitment potential declines with reproductive aging. However, our results align with emerging data indicating that reproductive management protocols and environmental conditions may modulate this relationship. For instance, in cows subjected to intensive synchronization and selection, multiparous cows with inherently superior fertility and endocrine function may be retained longer in the herd, potentially biasing the parity–AMH association upward (Rico et al., 2009, Jimenez-Krassel et al., 2015b). Moreover, Uliani et al (2019) demonstrated that AMH concentration was higher in mares at 5-10 and 10-15 years of age than in younger mares 1-5 years of age. The same is true in women, thus suggesting that AMH is not inherently linked to follicle numbers or depletion with age or else it would only decline from the time of birth (Uliani et al., 2019).

The lack of correlation between AMH and P4 at the start of the synchronization protocol aligns with previous findings indicating that AMH reflects long-term granulosa cell activity and antral follicle count rather than short-term luteal function (Batista et al., 2014, Jimenez-Krassel et al., 2015b). The weak, yet positive association observed at the PGF time point may reflect the combined endocrine activity of the follicular-luteal axis, as this time point represents a transitional phase marked by luteolysis and follicle recruitment (Gobikrushanth et al., 2017). Prior studies have shown that cows with higher AFC or AMH concentrations tend to have more developed luteal tissue and greater luteal function (Rico et al., 2009), which could explain the elevated AMH in cows with higher P4 at PGF. However, because it is presumed that AMH is primarily produced by small antral follicles and is not directly influenced by luteal P4 synthesis, these associations are likely indirect (Monniaux et al., 2011). Thus, AMH has the potential to serve as a stable marker of antral follicle count but not as a reliable predictor of the success of synchronization protocols.

The observed association between higher AMH concentrations and the presence of multiple follicles supports the suggestion that AMH better reflects the size of the small antral follicle pool more than the total follicle numbers, which contributes directly to ovarian follicular activity (Rico et al., 2009). However, the lack of a linear relationship between AMH category and total follicle count or volume suggests that follicular recruitment dynamics may be temporally uncoupled from AMH at this stage in the synchronization protocol. Interestingly, although the number of CL was not associated with AMH, total luteal volume increased with higher AMH, consistent with studies reporting a positive relationship between AMH and the functional capacity of the corpus luteum (Monniaux et al., 2010). The AMH expression has been localized to luteal tissue and shown to be expressed in granulosa-derived luteal cells, implicating it in the process of luteal vascularization (Batista et al., 2014). These findings suggest that while AMH does not directly influence CL number, it may reflect a greater follicular contribution to luteogenesis and a more robust luteal phenotype in cows with superior ovarian reserves (Rico et al., 2011).

Although AMH concentrations have been positively associated with reproductive potential in donor cows undergoing superovulation (Rico et al., 2009, Souza et al., 2015), our findings confirm prior reports that AMH does not reliably predict ovulation or P/AI in synchronized lactating dairy cows (Jimenez-Krassel et al., 2015a, Gobikrushanth et al., 2017). The lack of association between AMH and ovulation response at the first GnRH administration is consistent with the fact that GnRH-induced ovulation relies primarily on dominant follicle responsiveness and LH receptor expression (Batista et al., 2014), processes that are not directly governed by AMH activity. Similarly, AMH was not associated with pregnancy outcomes in our population, which is supported by earlier studies. This lack of association between AMH and pregnancy suggests that although AMH reflects ovarian reserve, it is not consistently associated with conception that

follows timed AI in cyclic, non-superstimulated cows (Baruselli et al., 2018, Gobikrushanth et al., 2018). The ROC analysis reinforced the conclusion that AMH lacks discriminatory power in this context, despite its potential to predict embryo yield and follicle number in IVF settings (Rico et al., 2011, Mossa et al., 2012). Overall, these results underscore the context-specific nature of AMH as a biomarker; while useful for assessing ovarian capacity, it is not a robust predictor of ovulation or fertility outcomes in synchronized AI programs.

Contrary to our expectations, plasma AMH concentrations at the beginning of the synchronization protocol were not associated with pregnancy outcomes following timed AI. Although AMH has been proposed as a marker of fertility and ovarian reserve (Rico et al., 2009, Batista et al., 2014, Jimenez-Krassel et al., 2015b), our results demonstrate limited predictive utility for AMH in the context of PAI under a timed AI protocol. Similar to the findings of Souza et al. (2015) and Ribeiro et al. (2014), the lack of association may reflect the multifactorial nature of conception, where oocyte competence, uterine environment, and embryonic development are influenced by factors beyond follicular reserve alone. The poor diagnostic performance of AMH for predicting pregnancy aligns with observations in another study (Rico et al., 2011), suggesting that while AMH may correlate with ovarian response to stimulation, it does not reliably capture the downstream determinants of fertility success in lactating dairy cows. Therefore, caution is warranted when interpreting AMH as a sole biomarker for fertility outcomes in synchronized reproductive programs.

CONCLUSION

Taken together, plasma anti-Müllerian hormone concentrations were highly repeatable within synchronized cows and associated with physiological indicators of ovarian function, including parity, body condition score, luteal volume, and GDPR. While AMH was associated with ovarian structural features and GDPR in a non-linear fashion, it did not reliably predict ovulatory response to the first GnRH or P/AI under a DO protocol. These results confirm that AMH is a stable endocrine marker of follicular activity but highlight its limited utility as a standalone predictor of fertility outcomes in synchronized lactating dairy cows. The integration of AMH with genomic traits for fertility purposes to improve selection for pregnancy outcomes was not supported by the current study's findings.

FIGURES

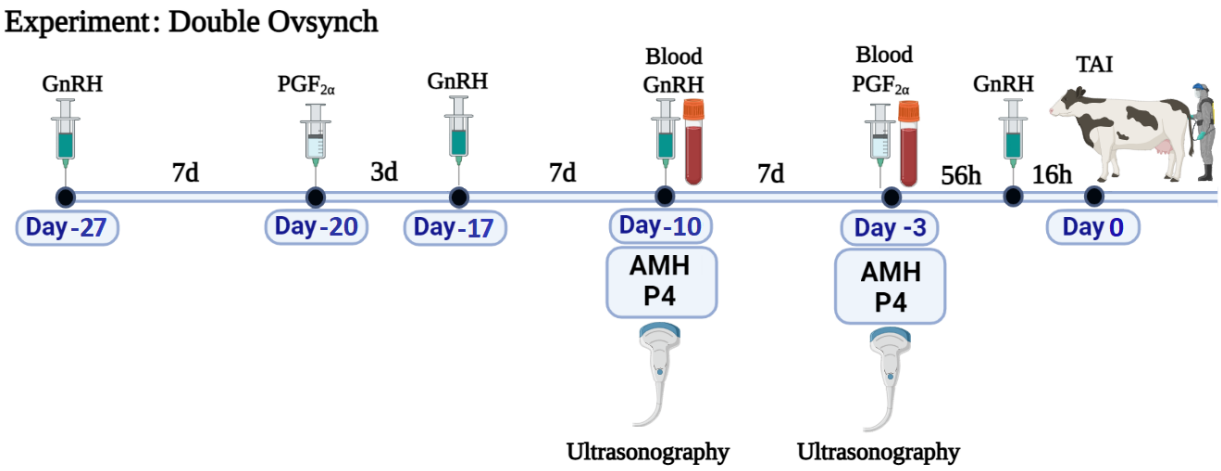


Figure 1

Figure 1: Timeline of experimental procedures relative to timed AI; Day 0. Lactating Holstein cows were enrolled in a Double-Ovsynch protocol for synchronization of ovulation. Blood samples were collected on days −10 and −3 relative to AI for quantification of plasma concentrations of anti-Müllerian hormone (AMH) and progesterone (P4) using bovine-validated ELISA kits. Transrectal ultrasonography was performed on days −10 and −3 to evaluate ovarian structures.

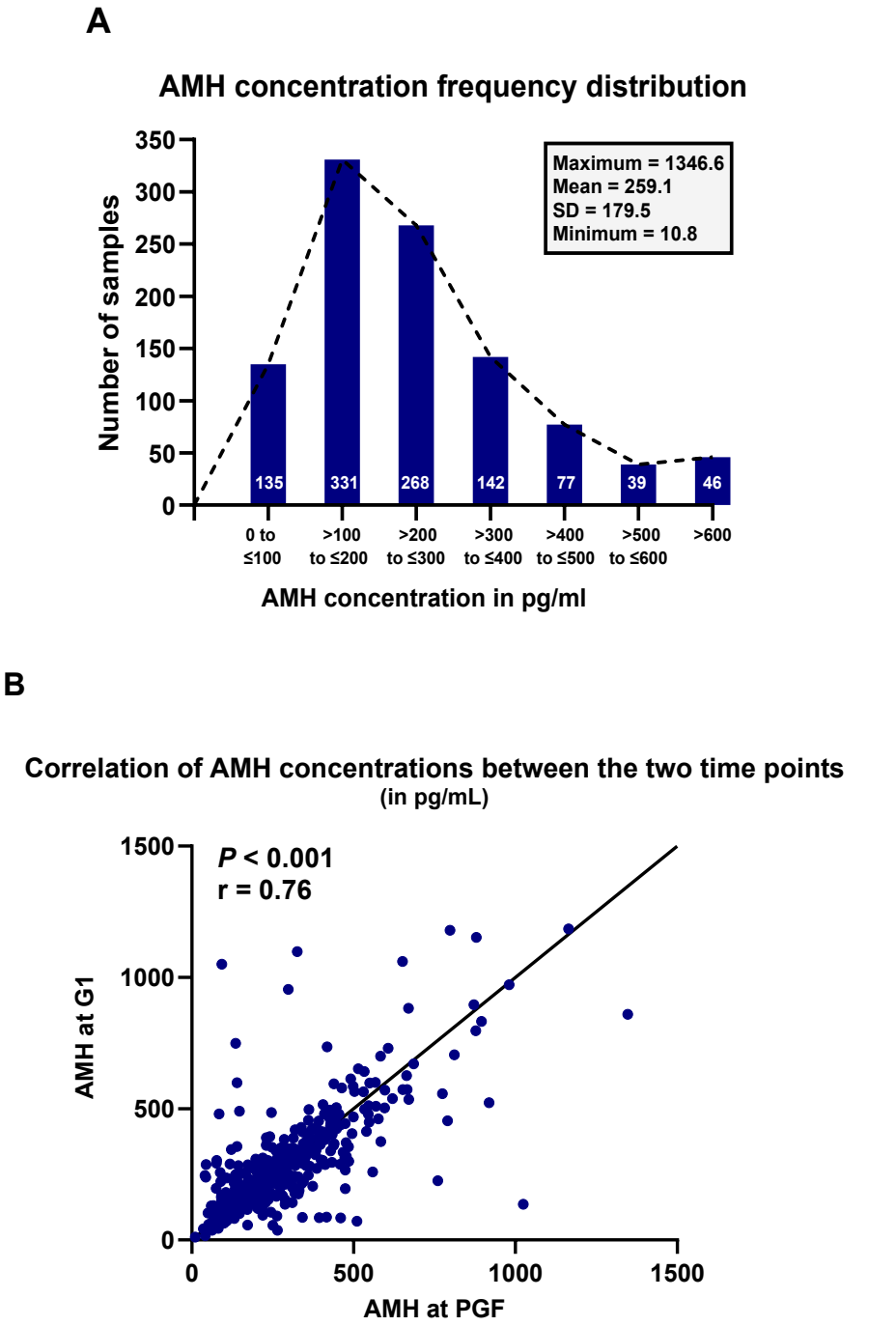
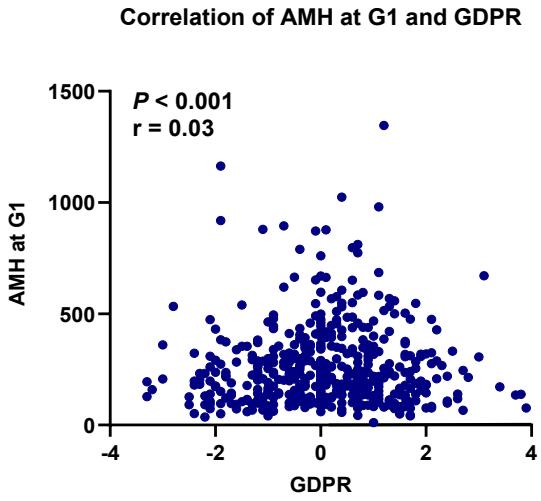


Figure 2

Figure 2. Distribution and correlation of anti-Müllerian hormone (AMH) concentrations in plasma of lactating Holstein cows sampled at two time points during the Double-Ovsynch protocol. **A)** Frequency distribution of AMH concentrations from a subset of 519 cows (n = 1,038 total samples). **B)** Pearson correlation between AMH concentrations measured at the 1st GnRH D −10 and $PGF_{2\alpha}$ D −3 time points in the breeding protocol r = 0.76; $P < 0.001$.

A


Correlation of AMH at G1 and GDPR
P < 0.001
r = 0.03
AMH at G1
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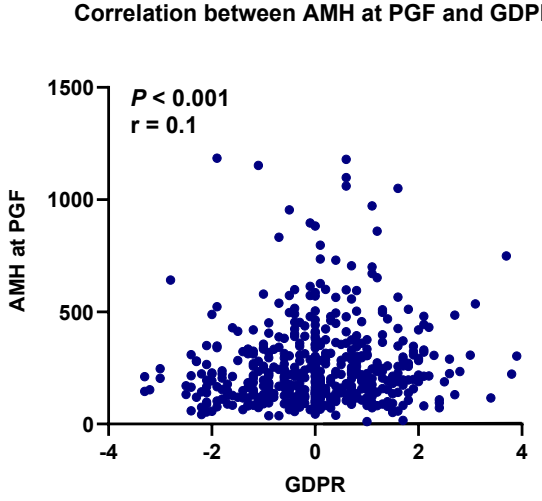
1000
500
0
-4
-2
0
2
4
GDPR

B


Correlation between AMH at PGF and GDPR
P < 0.001
r = 0.1
AMH at PGF
1500
1000
500
0
-4
-2
0
2
4
GDPR

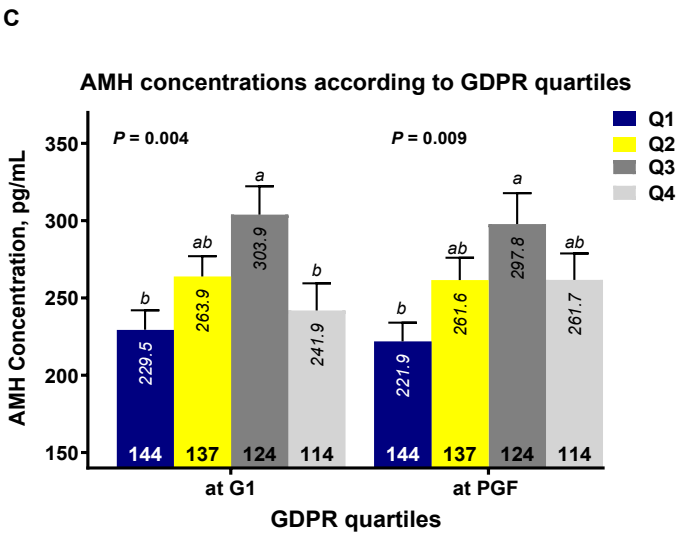


Figure 3

Figure 3. Correlation and association of anti-Müllerian hormone (AMH) concentrations with genomic daughter pregnancy rate (GDPR) in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **A)** Pearson correlation between GDPR and plasma AMH concentrations measured at the first GnRH d −10**, B)** Pearson correlation between GDPR and plasma AMH concentrations measured $PGF_{2\alpha}$ d −3 time points. **C)** Mean AMH concentrations by GDPR quartile at each time point. The error bars represent the standard mean error for AMH concentration in each GDPR quartile. Different superscripts indicate $P < 0.05$.

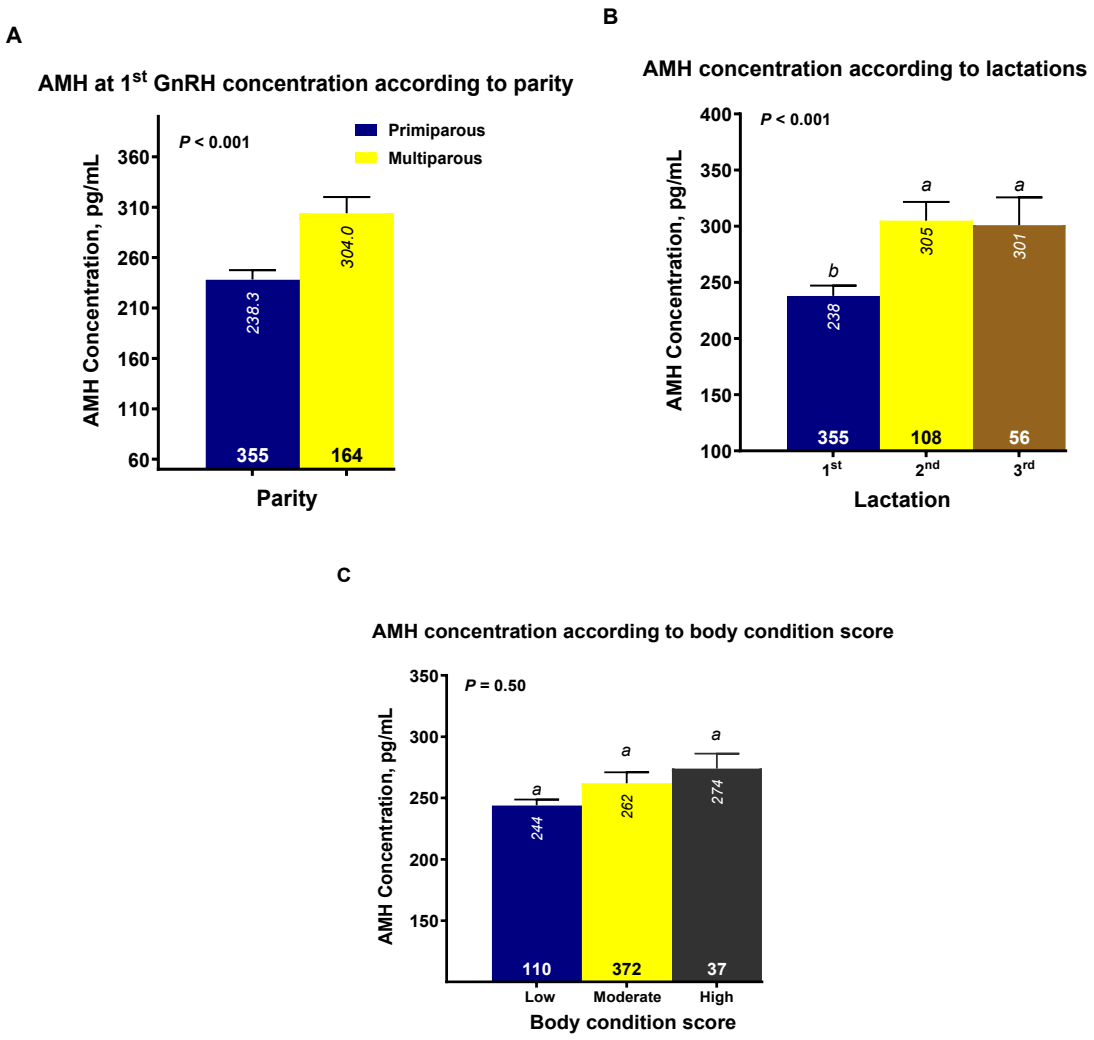


Figure 4

Figure 4. Association of anti-Müllerian hormone AMH concentrations with parity, lactation number, and body condition score BCS in lactating Holstein cows enrolled in a Double-Ovsynch protocol. **A)** Mean ±SE AMH concentration at the 1st GnRH D −10 according to parity group. **B)** AMH concentrations according to lactation number. The error bars represent the standard mean error for AMH concentration in each category. Different superscripts indicate $P < 0.05$. **C)** AMH concentrations according to BCS category.

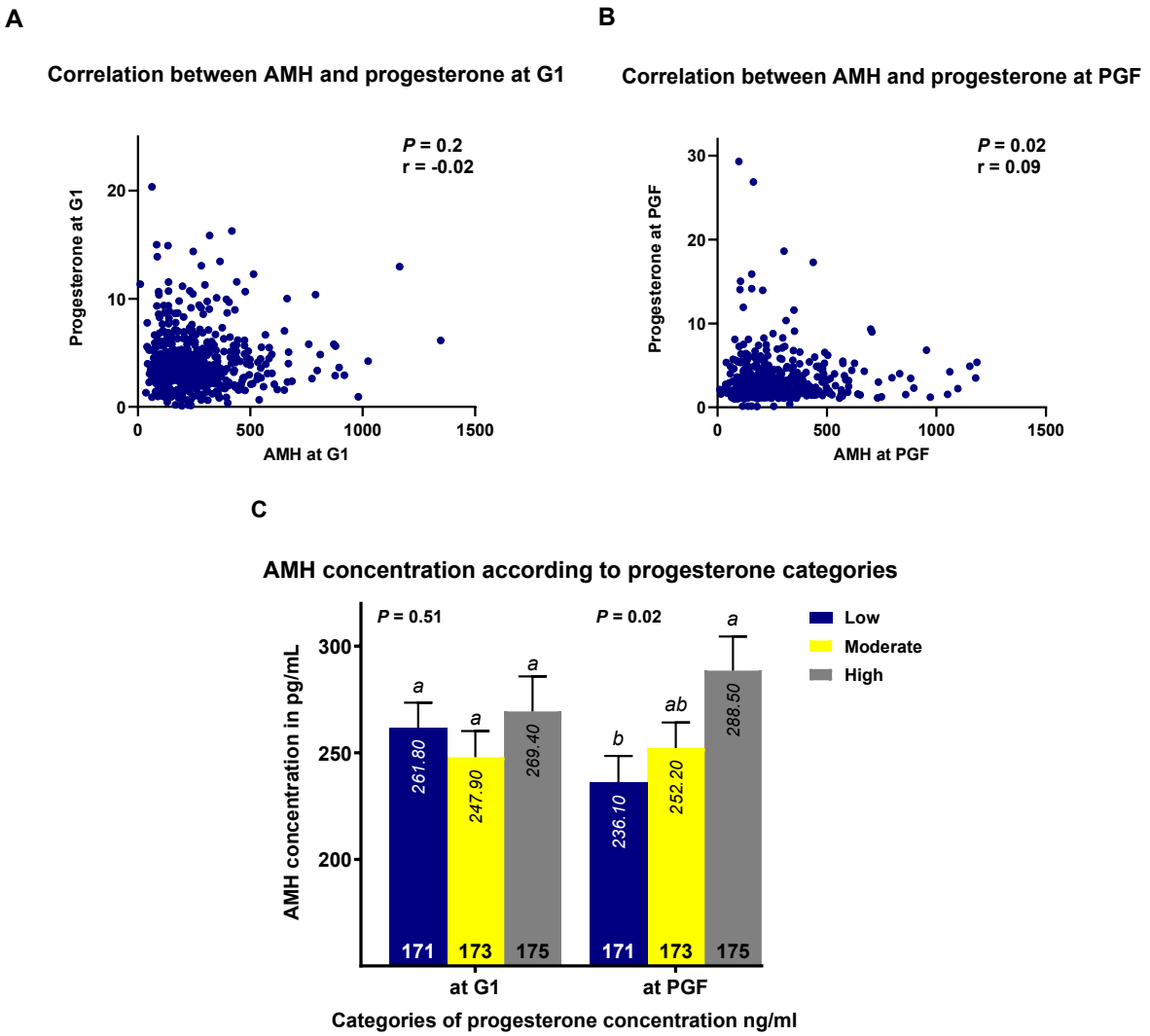


Figure 5

Figure 5. Association of anti-Müllerian hormone (AMH) concentrations mean ± SE with circulating progesterone (P4) in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **A)** Pearson correlations between AMH and P4 concentrations at the first GnRH (D −10). **B)** Pearson correlation between GDPR and plasma AMH concentrations measured and $PGF_{2\alpha}$ (D −3). **C)** Mean AMH concentrations are categorized by plasma P4 concentration at each time point. The error bars represent the standard mean error for AMH concentration in each category. Different superscripts indicate $P < 0.05$.

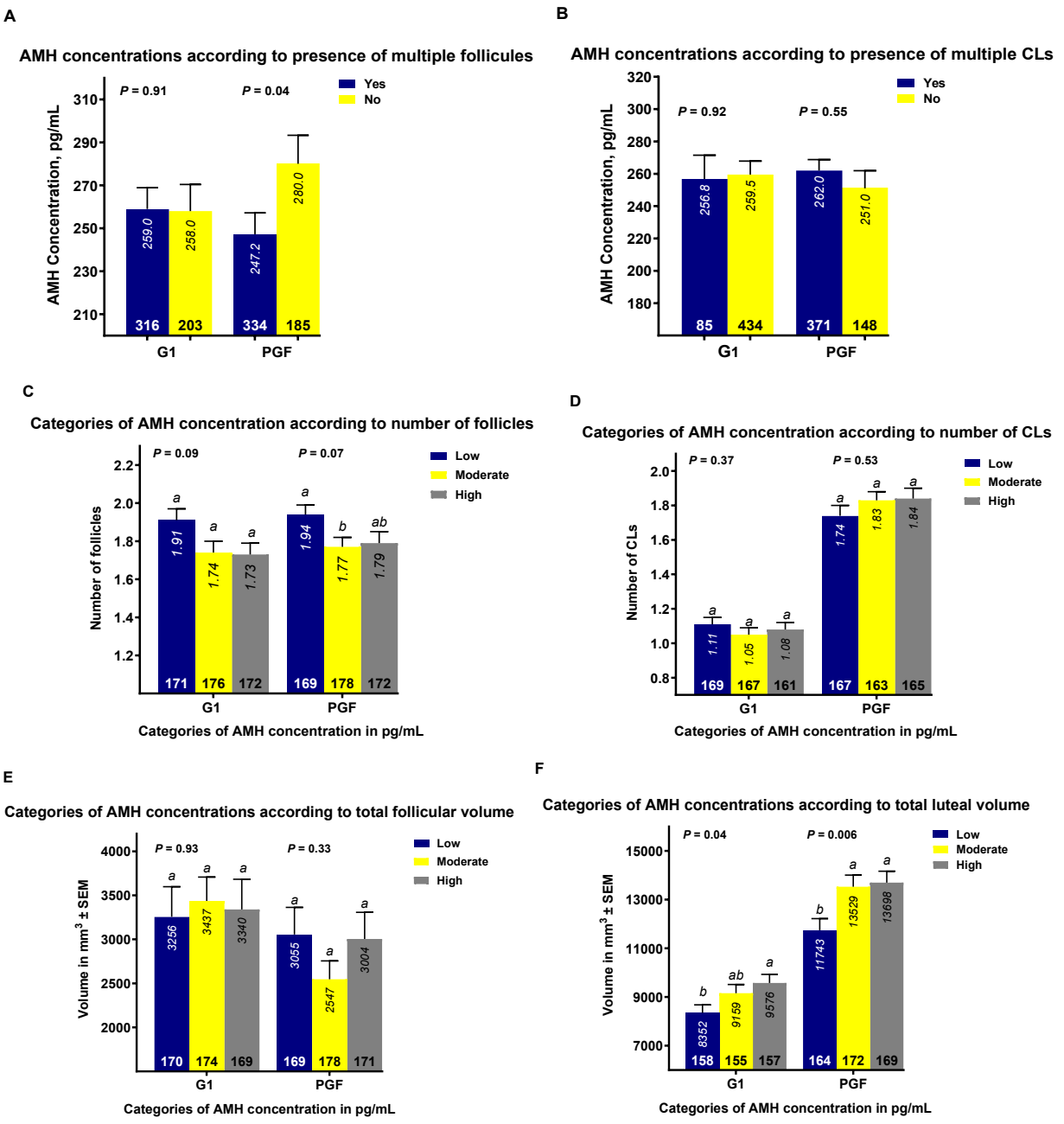


Figure 6

Figure 6. Associations between anti-Müllerian hormone (AMH) concentrations and follicular and luteal parameters in the Double-Ovsynch protocol. **A)** AMH concentrations at G1 and **B)** PGF according to the presence of multiple follicles and CLs, respectively. **C)** Categories of AMH at G1 and PGF according to the presence of multiple follicles and **D)** CLs. **E)** Categories of AMH concentrations at G1 and PGF according to total follicular and **F)** luteal volumes. The error bars represent the standard mean error for each category. Different superscripts indicate $P < 0.05$**.**

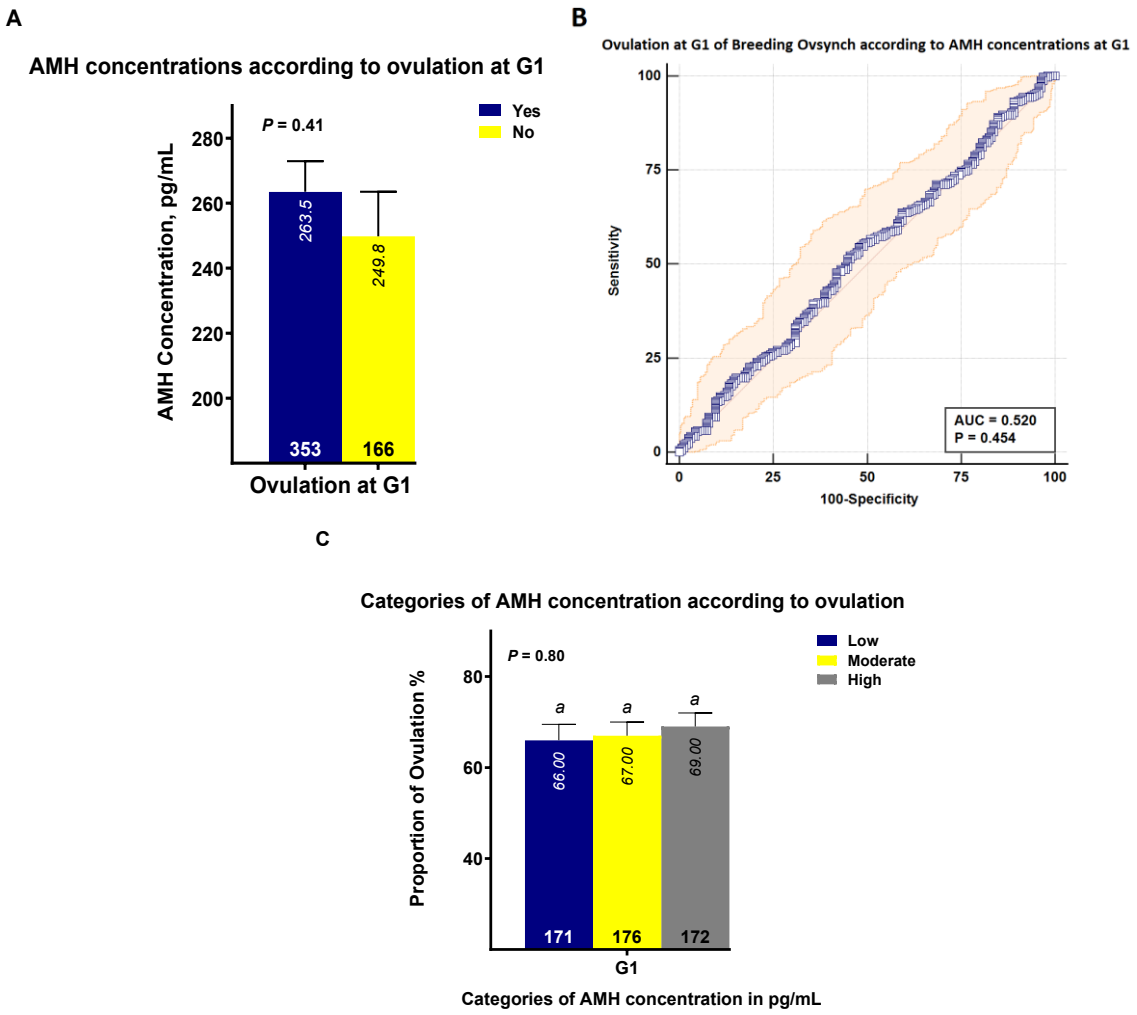


Figure 7

Figure 7: A) Anti-Müllerian hormone (AMH) field concentrations association with ovulation at 1st GnRH of the breeding Ovsynch in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **B)** Receiver Operating Characteristic (ROC) analysis showed that AMH concentrations did not predict ovulation in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **C)** Ovulation was not associated with the categories of AMH concentration.

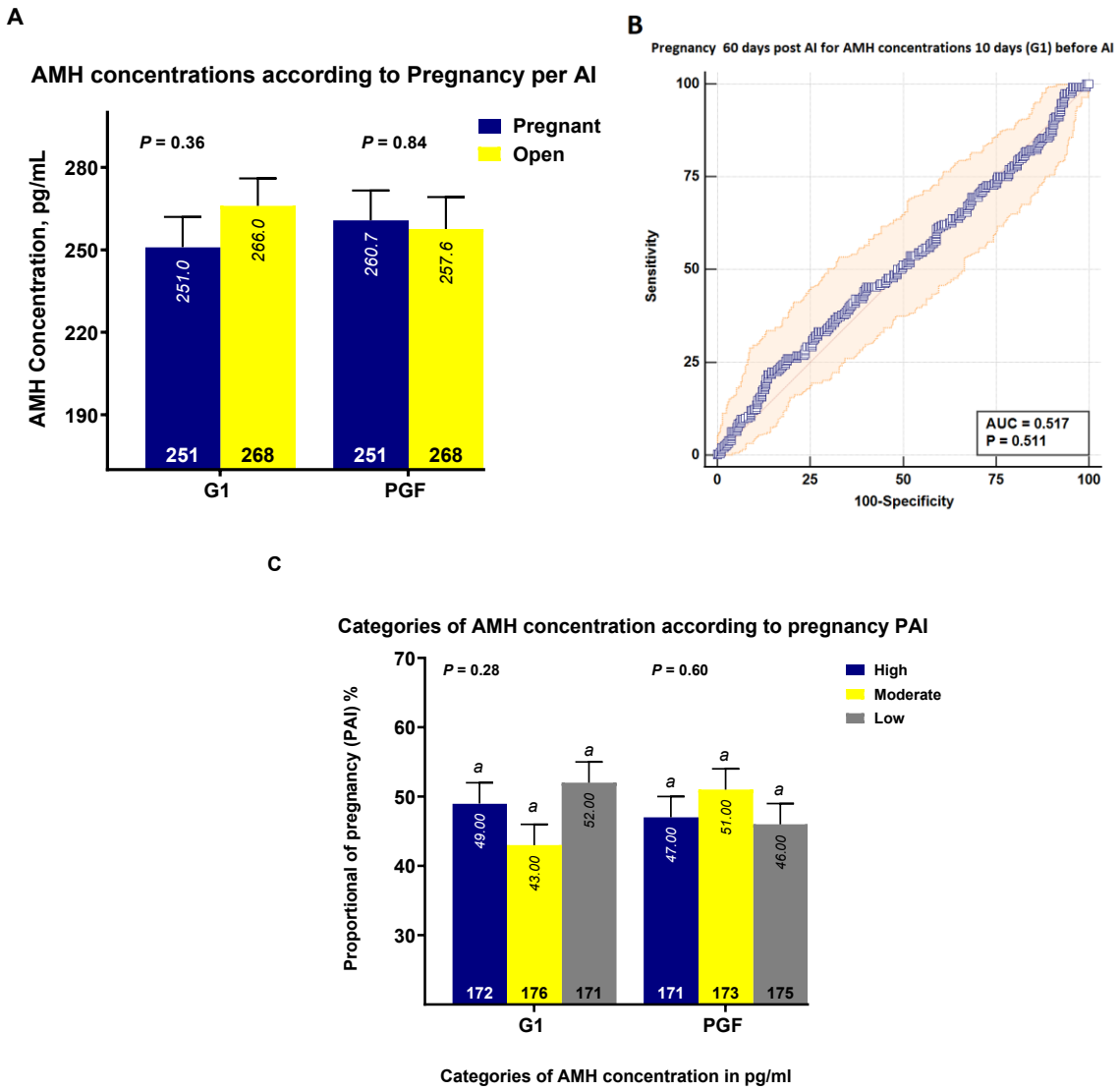


Figure 8

Figure 8. A) Association between anti-Müllerian hormone (AMH) concentrations and pregnancy per artificial insemination (AI) in lactating Holstein cows enrolled in a Double-Ovsynch protocol. **B)** Receiver operating characteristic ROC curve evaluating the predictive capacity of AMH concentrations for pregnancy outcome at 60 d post-AI. **C)** Association of pregnancy per AI with the categories of AMH concentration.

CHAPTER III

Interpretative Summary

Association of inhibin A and B hormones with genomic prediction of daughter pregnancy rate, ovarian responses, and pregnancy per AI in lactating dairy cows *By Salman et al.,* Inhibin (Inh) A and B are fertility-related hormones that are suggested as predictors of the antral follicle count, while genomic prediction of daughter pregnancy rate (GDPR) and ovulation at the beginning of OvSynch (G1) are makers of fertility potential in dairy cows. Herein, we performed a study to test the hypothesis that Inh A and B are positively associated with GDPR, ovulation at G1, and pregnancy per AI. The results of the current study indicated that Inh A is positively associated with GDPR and ovulation in G1; however, it was unable to predict pregnancy using AI.

Association of Inhibin A and B hormones with genomic prediction of daughter pregnancy rate, ovarian responses, and pregnancy per AI in lactating dairy cows

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INTRODUCTION

Inhibin A and B are dimeric glycoproteins composed of a subunit linked to either a βA or βB subunit, forming inhibin A and inhibin B, respectively (Juengel et al., 1993, Walentowicz et al., 2014). These hormones are members of the transforming growth factor β (TGF-β) superfamily and are synthesized predominantly by granulosa cells of growing preantral and antral follicles (Wrathall and Knight, 1993). Inhibin A and B play a central role in regulating follicular development by exerting negative feedback on pituitary follicle-stimulating hormone (FSH) secretion (Robertson et al., 1986, Lu et al., 2009), thereby influencing follicle selection and recruitment dynamics. At least six forms of Inhibin were initially characterized in bovine follicular fluid (Miyamoto et al., 1986), with up to nine variants subsequently identified (Good et al., 1995a). Inhibin A is primarily associated with dominant follicles and functional corpora lutea, increasing during the periovulatory and luteal phases (Bleach et al., 2001). In contrast, inhibin B is secreted from small- and medium-sized follicles, peaking during the mid-follicular phase (Beg et al., 2002). Collectively, these hormones reflect different stages of follicular growth, and changes in their circulating concentrations mirror ovarian status and endocrine function (Bleach et al., 2001, Geng et al., 2008a).

Inhibin A and B have been studied for their role in regulating fertility and enhancing reproductive outcomes in cattle. Studies have shown that inhibin A is negatively associated with FSH but positively correlated with estradiol during the estrous cycle (Bleach et al., 2001). Functional studies have demonstrated that immunization against the inhibin α-subunit alters follicular dynamics, disrupts ovulation, and increases interferon-tau concentration at maternal recognition of pregnancy (Morris et al., 1997, Chen et al., 2022b), further suggesting a role for Inhibin A and B in early gestation success. Additionally, inhibin A has been positively associated

with the number and diameter of small and medium-sized follicles and has been explored as a potential additive to in vitro maturation media (Stock et al., 1997).

Despite their critical role in ovarian physiology, the endocrine profiles of inhibin A and B have not been fully investigated in the context of modern reproductive technologies in dairy cattle. While AMH has emerged as a reliable endocrine marker of ovarian reserve and fertility potential (Garcia-Guerra et al., 2017, Alward and Bohlen, 2020), similar comprehensive evaluations of Inhibin A and B are lacking. Recent work in humans has demonstrated a strong association between inhibin B and antral follicle count (Penarrubia et al., 2010, Walentowicz et al., 2014), although equivalent studies in cattle are scarce. Several endocrine and physiological factors modulate ovarian responses to synchronization programs in dairy cows. Ovulation in response to the first GnRH of a Double-Ovsynch protocol has been positively associated with follicle size and circulating progesterone concentrations and is considered a key determinant of fertility (Souza et al., 2008). Moreover, follicular and luteal parameters such as follicle size, number of follicles, presence of multiple corpora lutea, and total follicular and luteal volumes have been widely used to assess ovarian function and reproductive potential (Fortune, 1986). Notably, inhibin gene expression and protein secretion have been shown to respond to gonadotropin stimulation, including FSH and GnRH (Juengel et al., 1993, Good et al., 1995a). However, the direction and magnitude of these changes may vary by reproductive stage (Bleach et al., 2001). Inhibin exert a strong suppressive effect on FSH secretion by ovine pituitary cell, but not luteinizing hormone (LH), in a dose-dependent manner (Robertson et al., 1986), suggesting that fluctuations in endogenous inhibin levels could influence both follicular recruitment and dominant follicle selection.

Another factor that might contribute to variable pregnancy outcomes is genetics. In early 2010, with the exponential increase in genotyping of dairy cows (Weller et al., 2017), dairy farms began to utilize tools such as genomic predictor of daughter pregnancy rate (GDPR) to improve selection for improved fertility in dairy cows (Norman et al., 2009). A series of studies confirmed that GDPR is positively associated with estrus characteristics, estradiol, and pregnancy outcomes in heifers and parous cows (Veronese et al., 2019b, Lima et al., 2020, Madureira et al., 2022, Melo et al., 2025), independent of the breeding program, estrus detection, or timed AI. Still, potential for GDPR as a useful predictor of fertility are yet to be fully explored. Although metrics such as daughter pregnancy rate are not highly heritable, incorporating them into genomic selection frameworks has proven beneficial (Cole et al., 2011). Previous studies have shown positive associations between GDPR and estrus expression, ovulation, conception rate, and pregnancy per AI in heifers and lactating cows (Veronese et al., 2019b, Lima et al., 2020, Melo et al., 2025). However, the physiological and endocrine basis underlying genomic fertility traits, such as GDPR, remains to be fully elucidated. Furthermore, the relationship between the plasma concentration of inhibin A and B and GDPR warrants further investigation.

To date, few studies have characterized inhibin A and B concentrations during synchronization programs or explored their associations with ovarian responses and fertility metrics such as GDPR or P/AI. We hypothesized that circulating concentrations of inhibin A and B at 1st GnRH of the breeding Ovsynch **(G1)** of the Double-Ovsynch (DO) protocol are associated positively with ovulation, pregnancy per AI, and GDPR in Holstein dairy cows. Our objectives are to evaluate the association of plasma concentrations of inhibin A and B at the G1 and PGF of the breeding Ovsynch in a DO protocol with GDPR, ovulation at G1, and P/AI. We also aimed to

evaluate the association of Inhibin A and Inhibin B with ovarian structures at G1, at PGF, and with P4 at G1 and PGF time points, parity, BCS, and lactation.

MATERIALS AND METHODS

The procedures in this experiment are similar to what was described in the AMH chapter (Chapter II). Animals, housing, diet, blood sampling and DO synchronization protocol were previously described in the AMH chapter. Key differences in the hormone assay and statistics are listed here. Briefly, plasma concentrations of inhibin A and B were performed using the standard bovine protocol for RIA as previously validated for use in cattle by Ansh Labs LLC, Webster, Texas. Briefly, the concentrations of Inhibin A and B were analyzed using a commercial chemiluminescence immunoassay developed for specific binding and accurate quantitation of bovine inhibin A and B. The monoclonal antibody pair used in this assay detects pro-mature covalent and nonconvex-lent bovine inhibin complexes and does not have cross-reactivity to other proteins of the transforming growth factor family, such as AMH and activins A, B, and AB.

Statistical analysis

All data were analyzed using JMP version 17.2 (SAS/STAT; SAS Institute Inc., Cary, NC). A 3-step approach was used to assess the association of inhibin A and B concentrations in plasma and responses of interest to the variables. First, descriptive statistics and distributions were performed for all continuous variables, and normality of residuals was evaluated using histograms, Q-Q plots, and the Shapiro-Wilk test. Analyses have revealed that the data is right-skewed with several statistical and biological outliers. Outliers were further confirmed using standard deviation thresholds and boxplots but given the biological relevance of maintaining values in pg/mL. To

address skewness and stabilize variance, data were log-transformed to minimize the influence of extreme values. The data was back-transformed to values in pg/mL to preserve the interpretability of hormone concentrations. Inhibin A and B plasma concentrations were also categorized into terciles for further investigation into their associations with ovarian response and fertility outcomes. Inhibin A concentrations at the G1 were classified as: low (2.9–5.94; $\bar{x}$ = 4.22, n = 172), moderate (5.94–8.02; $\bar{x}$ = 7.04, n = 171), and high (8.03–16.32; $\bar{x}$ = 10.64, n = 176) pg/mL. Inhibin A at PGF was grouped as: low (2.9–5.69; $\bar{x}$ = 4.11, n = 175), moderate (5.69–7.79; $\bar{x}$ = 6.81, n = 173), and high (7.79–17.02; $\bar{x}$ = 10.42, n = 171) pg/mL. Inhibin B at the G1 was categorized as: low (2.6–7.36; $\bar{x}$ = 5.50, n = 172), moderate (7.36–11.66; $\bar{x}$ = 9.16, n = 172), and high (11.7–55.53; $\bar{x}$ = 24.79, n = 175) pg/mL. Inhibin B at PGF was classified as: low (2.34–7.2; $\bar{x}$ = 5.74, n = 175), moderate (7.2–12.34; $\bar{x}$ = 9.22, n = 169), and high (12.34–61.51; $\bar{x}$ = 26.46, n = 175) pg/mL. We also categorized the GDPR into quartiles as follows: Q1: (-3.3 to -0.6; $\bar{x}$ = -1.4; n = 142); Q2: (-0.5 to 0.0; $\bar{x}$ = -0.14; n = 137), Q3: (0.1 to 0.9; $\bar{x}$ = 0.48; n = 122), Q4: (1.0 to 3.9; $\bar{x}$ = 1.68; n = 118). The P4 concentrations at G1 were also categorized as: low: (0.09 to 2.85; $\bar{x}$ = 1.85, n = 171); moderate: (2.87 to 4.82; $\bar{x}$ = 3.83, n = 189); high: (4.83 to 20.3; $\bar{x}$ = 7.41; n = 160) ng/mL for further investigation.

Second, we performed univariable analyses for each independent variable to assess the association and correlations of this variable with the concentration of inhibin A and B. The relationship of inhibin A and B with parity, number of lactations, BCS, GDPR, presence of CL, size of CL, multiple CLs, total luteal volume, presence of follicles, size of the largest follicle, multiple follicles, follicular volume, ovulation, and plasma P4 concentration was analyzed. Third, multivariable logistic regression was used to assess the relationship between inhibin A and B and ovulation at G1 and P/AI, as well as other covariates associated with these responses. Additionally,

receiver operating characteristic (ROC) curves were performed to assess whether inhibin A and B could predict ovulation at G1 and P/AI. Multicollinearity was assessed using the variance inflation factor (VIF), and model selection was guided by the Akaike information criterion (AIC) and Bayesian information criterion (BIC). Significance was declared at $P < 0.05$, while a tendency was declared when $0.05 \leq P < 0.10$.

RESULTS

Characterization of plasma inhibin A and inhibin B concentrations

The distribution of inhibin A and B concentrations in a subset of 519 cows showed right-skewed patterns at both time points of the Double-Ovsynch protocol (Figure 10). For Inhibin A, the mean ± SD concentrations were 6.35 ± 4.1 pg/mL at G1 (Figures 10A) and 6.61 ± 6.4 pg/mL (Figures 10B). For Inhibin B, the mean ± SD concentrations were 12.1 ± 13.1 pg/mL at G1 (Figures 10C) and 13.18 ± 21.34 pg/mL at PGF (Figures 10D). Approximately 90% of inhibin A samples were <15 pg/mL, while ~95% of inhibin B samples were <40 pg/mL, highlighting a narrow but variable circulating range across the herd.

Correlations of inhibin A and B concentrations across the two sampling points of the DO protocol are shown in Figure 11. Inhibin A concentrations were positively correlated between the first G1 and PGF (r = 0.64; $P < 0.001$; Figure 11A), and a stronger correlation was detected for inhibin B between the same time points (r = 0.73; $P < 0.001$; Figure 11B). Positive correlations were observed between inhibin A and B at G1 (r = 0.14; $P = 0.02$, Figure 11C) and PGF (r = 0.19; $P < 0.001$, Figure 11D).

Characterization of inhibin A and B association with GDPR

Correlations between GDPR and inhibin A or B were below or equal to 0.08 (Figure 12). There was a tendency ($P = 0.06$) for a correlation between inhibin A and GDPR at the PGF time point (Figure 12B). No other correlations were observed between inhibin A and B and GDPR. For categorized data, the High inhibin B group at PGF had greater ($P = 0.01$) GDPR than the Low Inhibin A group (Figure 12E). However, the categorized data at G1 for inhibin A and B and inhibin B at PGF had no differences (Figure 12E).

Characterization of inhibin A and B association with luteal parameters, follicular parameters, and hormonal concentrations

Correlations between inhibin A and B concentrations and ovarian structures at G1 and PGF are presented in Table 1. For luteal parameters, except for inhibin B at PGF, which was positively associated with the volume of the third CL (r = 0.44; $P = 0.001$) and total luteal volume (r = 0.32; $P = 0.047$), no other correlation was found between luteal parameters and inhibin A and inhibin B (Table 1). For follicular parameters, no correlations were observed (Table 1). For hormonal parameters, a trend was also observed between inhibin A and circulating P4 (r = –0.08; $P = 0.05$) at the G1 time point (Table 1). However, no meaningful correlations were detected between inhibin A and B and AMH plasma concentrations at either time point. When inhibin A and B concentrations were put in tercile groups, cows in the High inhibin A group at G1 had a greater number of follicles compared with those in the Low group ($P = 0.02$), while no differences were detected for inhibin A at PGF or for inhibin B at G1 and PGF (Figure 13A). Moreover, cows in the High Inhibin A group had a smaller size for the largest CL than cows in the low Inhibin A group at G1 ($P = 0.02$), but no differences were observed at PGF or for inhibin B (Figure 13B).

Characterization of inhibin A and B association parity, number of lactations, and BCS

Inhibin A at PGF was greater in multiparous cows (8.32 ± 0.20 pg/mL) than in primiparous cows (7.98 ± 0.17 pg/mL; $P = 0.006$), with no differences observed for inhibin B (Figure 14A). Inhibin A concentration at PGF was also affected by the number of lactations ($P = 0.02$; Figure 14B). Neither inhibin A nor B concentrations were significantly affected by BCS category (Figure 14C).

Characterization of inhibin A and B concentrations and their association with ovulation

Plasma concentrations of inhibin A and B at G1 were not associated with ovulation (Figure 15A). Predictive models were developed to assess whether there was a threshold of inhibin A or inhibin B that could predict ovulation at G1. Results of the ROC analysis demonstrated that neither inhibin A (AUC = 0.53; $P = 0.28$, Figure 15B) nor inhibin B (AUC = 0.53; $P = 0.28$, Figure 15C) could predict ovulation at G1 in lactating Holstein cows.

Characterization of inhibin A and B association with pregnancy after AI

Our univariate analysis indicated that plasma concentrations of inhibin B at G1 were greater for cows that became non-pregnant after AI when compared to cows that became pregnant (Figure 16A). However, there are no differences for Inhibin B at PGF or for Inhibin A at G1 and PGF for cows that become pregnant or not. However, the ROC analyses demonstrated that inhibin A at G1 and PGF, and inhibin B at G1 and PGF could not predict P/AI in lactating Holstein cows enrolled in the DO protocol (Figures 16B to 16E).

DISCUSSION

This study was designed to gain a better understanding of the relationship between the plasma concentration of inhibin A and B at G1 and PGF of breeding Ovsynch in a DO protocol with GDPR, ovulation at G1, and P/AI. Unlike AMH, inhibin A and B have not been associated with the antral follicle population and ovarian reserve in cattle. However, considering its essential role in the estrous cycle and regulation of FSH, there has still been an interest in their potential utility for predicting reproductive performance in cattle.

The plasma concentrations of inhibin A and B were observed to be not normally distributed and have a right-skewed distribution, which reflects physiological variation in follicular activity and luteal regression during the synchronization protocol. Inhibin B exhibited a broader dynamic range and higher variability than inhibin A, consistent with its predominant secretion from small- and medium-sized antral follicles (Glister et al., 2001). The low circulating concentrations in most cows agree with previous studies that reported low peripheral inhibin levels outside the peri-ovulatory window (Shukovski et al., 1993, Wrathall and Knight, 1993). The increase in mean inhibin concentrations at PGF may relate to enhanced follicular turnover or differential luteal clearance patterns, as previously reported (Miyamoto et al., 1986, Hutchinson et al., 1987). These findings support the utility of inhibin B as a temporally responsive biomarker for follicular status. However, inhibin have high individual variation, suggesting that caution is necessary when interpreting their validity as a potential marker for follicular status (Taya et al., 1996, Knight and Glister, 2001).

The moderate-to-strong correlation of inhibin A and B concentrations between time points within the DO protocol indicates the potential biological stability of these hormones within individual cows. It also likely reflects the fact that these cows had their estrous cycle synchronized

with full Ovsynch (Pursley et al., 1995) for presynchronization, which ended seven days before G1. Every cow received GnRH at G1, seven days before PGF, which likely resulted in a high percentage of these cows being on days 7 or 8 of the estrous cycle, helping reduce variability on the stage of the cycle and follicular status across cows (Souza et al., 2008). As inhibin B is more reflective of small antral follicle activity, while inhibin A is associated with larger follicle and luteal dynamics, their modest relationship likely captures complementary—rather than redundant—physiological roles.

The weak correlations observed between circulating inhibin A and B concentrations and luteal or follicular metrics indicate that neither hormone was a strong linear predictor of ovarian structural parameters during the Double-Ovsynch protocol. However, the moderate correlation between inhibin B at PGF and volume of the third CL and total luteal volume although direct luteotropic effects of inhibin on luteal steroidogenesis have not been demonstrated (Shukovski et al., 1993, Wrathall and Knight, 1993). The negative association between inhibin A at G1 and P4 aligns with studies showing inverse dynamics between dominant follicle-derived inhibin A and B and luteal progesterone secretion. Overall, the data suggest that only minor associations exist between ovarian structures and inhibin A and B in lactating dairy cows in the current study.

Although plasma inhibin A and B concentrations were weakly correlated with GDPR, the categorized data of inhibin A and B showed that the High inhibin B group at PGF had greater GDPR than the Low Inhibin A group. This finding suggests a subtle physiological alignment between the endocrine output from the dominant follicles and underlying genomic fertility merit. Notably, inhibin A is predominantly secreted by the large, estrogen-active follicles and corpora lutea and serves to suppress FSH and reinforce follicular selection (Bleach et al., 2001). Thus, high inhibin A may indicate enhanced follicular selection and luteal functionality, both of which are

foundational to reproductive success. The GDPR itself has been positively associated with higher reproductive performance. Including parameters such as earlier resumption of cyclicity, higher estrus expression, and improved pregnancy outcomes in both heifers and lactating cows (Veronese et al., 2019b, Gobikrushanth et al., 2020, Lima et al., 2020). These physiological features align with the patterns observed in the current study, where greater inhibin concentrations were found in association with larger CL sizes. Therefore, higher plasma concentrations of inhibin A may indicate more competent follicular development in cows with higher genetic merit for fertility. However, the correlation between inhibin A and B at either time point was weak; thus, inhibin A alone is unlikely to serve as a predictive marker for GDPR in genomic selection programs. Further studies are needed to investigate this relationship.

Plasma concentrations of inhibin A and B at G1 were not associated with ovulation, but the multivariable analysis of the categorized terciles of inhibin has revealed that cows in the High inhibin A group at G1 had greater ovulation rates (52.0%) compared with the low group (13.6%). Although the ROC analysis confirmed that neither inhibin A nor B could reliably predict ovulatory response in lactating Holstein cows. These findings suggest that late-cycle inhibin secretion may be weakly associated with improved fertility potential in an Ov-Synch protocol, possibly reflecting greater follicular health and regulatory endocrine competence at the time of luteolysis (Hutchinson et al., 1987, Wrathall and Knight, 1993). It is well established that inhibin A is secreted from large antral follicles during the periovulatory window and acts to suppress FSH once a dominant follicle (Bleach et al., 2001) is selected.

The presence of elevated inhibin A at G1 likely mirrors the development of estrogen-active follicles that are more likely to respond to GnRH administration at G1 of the DO protocol. That time point is critical for the emergence of a synchronized follicular wave, the establishment of a

fertile ovulatory follicle, and ultimately, successful ovulation (Souza et al., 2008, Luchterhand et al., 2019). These results align with prior studies, which have shown high inter-animal variability and a lack of consistent association between inhibin A and reproductive success (Morris et al., 1997, Knight and Glister, 2001). Thus, although our data could not support a strong relationship between inhibin A and ovulation, the plasma concentration of inhibin A is a key player in regulating ovarian dynamics (Bleach et al., 2001) and, thus, may serve as a proxy for ovarian efficiency. Further research is warranted to assess this interesting relationship.

Plasma concentrations of inhibin B at G1 were greater for cows that later became non-pregnant 60 days after AI when compared to cows that became pregnant, but neither inhibin A nor B can be used as a predictor for P/AI. Multiple studies have reported that inhibin A's suppressive action on FSH may restrict secondary follicular recruitment, complicating its interpretation in the context of fertility outcomes (Shukovski et al., 1993, Lu et al., 2009). Furthermore, immunization against the inhibin-alpha subunit in dairy heifers resulted in increased ovulation rates and improved conception, supporting the suppressive role of inhibin on follicular development and fertility outcomes (Chen et al., 2022a). Our data confirms this negative association between inhibin B and reproductive outcomes and demonstrates the potential of inhibin as a negative biomarker for pregnancy. However, successful pregnancy involves a plethora of factors, such as oocyte competence, uterine environment, and early embryonic signaling, which are not directly captured by inhibin concentration in these *in vitro* studies (Shukovski et al., 1993, Wrathall and Knight, 1993, Guo et al., 2020). Therefore, further studies are necessary to investigate this relationship.

CONCLUSION

Taken together, plasma inhibin A and B concentrations were highly repeatable within cows and associated with physiological indicators of ovarian function, including parity, ovarian structural features; number of follicles and the size of the largest CL, and GDPR. While plasma concentrations of inhibin A and B were positively associated with ovarian parameters, GDPR, and PAI, neither reliably predicted ovulation at G1 or P/AI under a DO protocol. These results confirm the potential use of inhibin A and B as a biomarker endocrine for reproductive performance yet highlight their limited utility as a standalone predictor of fertility outcomes in synchronized lactating dairy cows. The integration of inhibin A and B with genomic traits for fertility purposes to improve selection for pregnancy outcomes was not supported by the current study's findings.

FIGURES

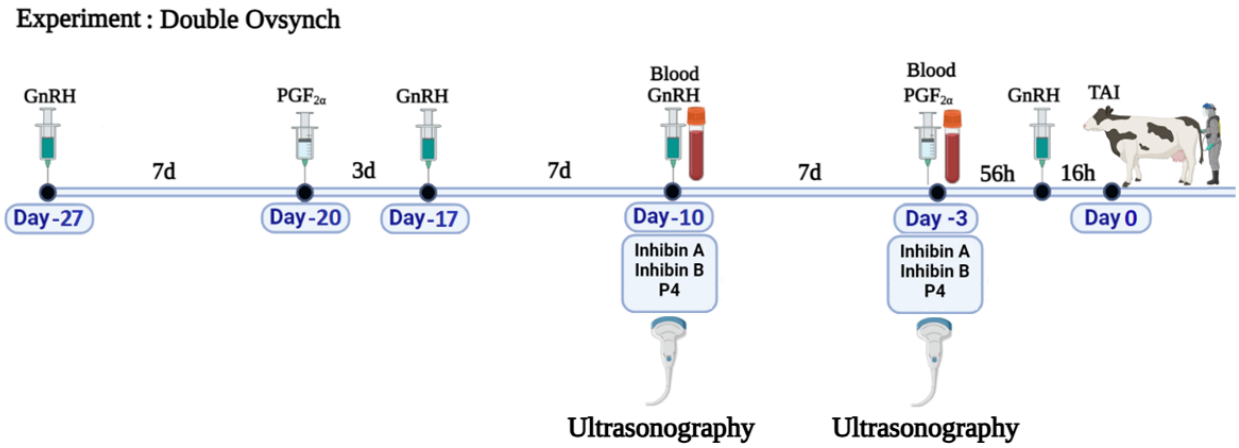


Figure 9

Figure 9: Timeline of experimental procedures relative to timed AI (TAI) at Day 0. Lactating Holstein cows were enrolled in an Ovsynch protocol for synchronization of ovulation. Blood samples were collected on days −10 and −3 relative to AI for quantification of plasma concentrations of inhibin A, inhibin B, and progesterone (P4) using bovine-validated ELISA kits. Transrectal ultrasonography was performed on days −10 and −3 to evaluate ovarian structures.

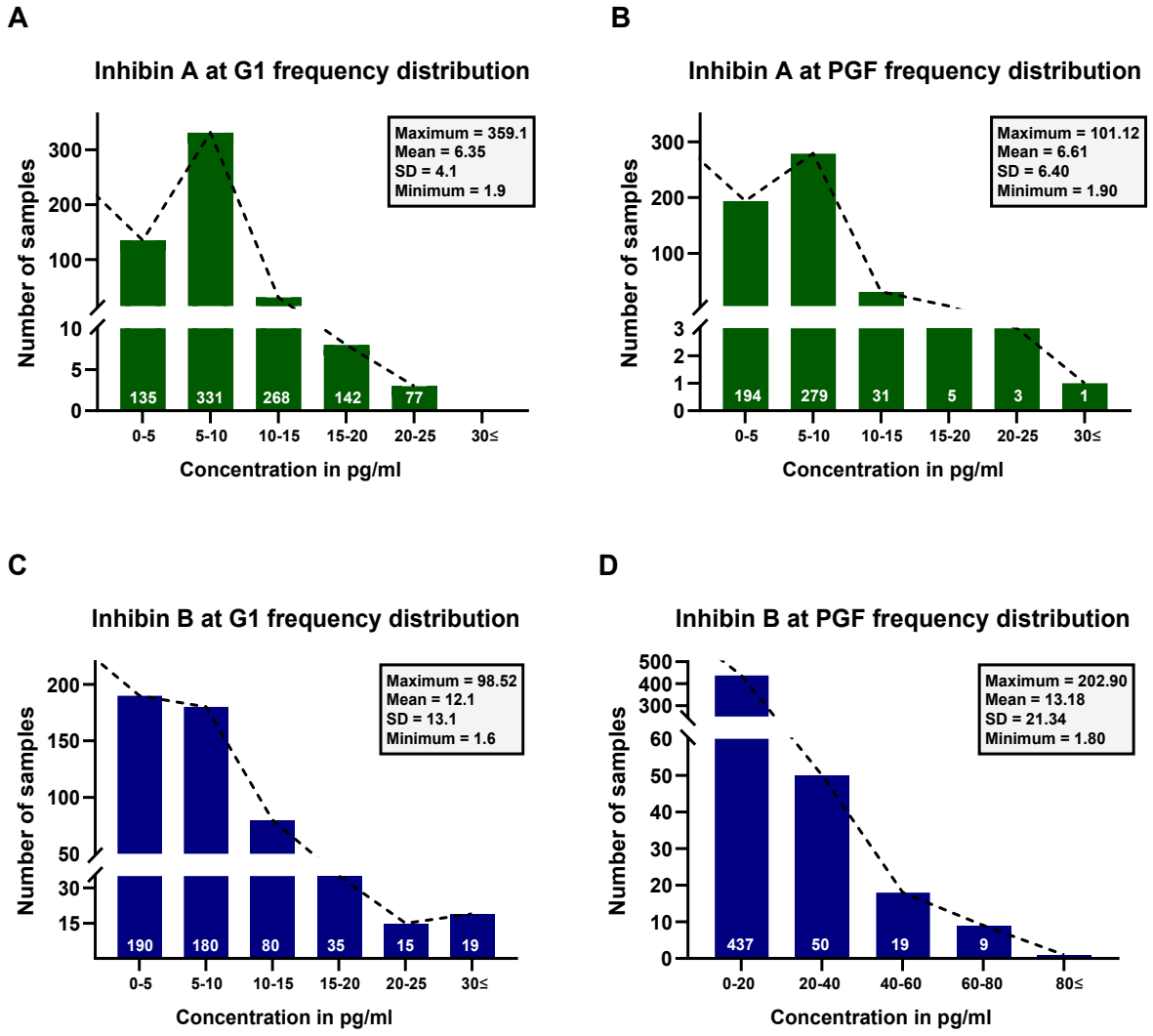


Figure 10

Figure 10. Distribution of inhibin A and B concentrations in plasma of lactating Holstein cows sampled at two time points during the Double-Ovsynch protocol. **A)** Frequency distribution of inhibin A at G1, **B)** inhibin A at PGF, **C)** inhibin B at G1, and **D)** inhibin B at PGF concentrations from a subset of 519 cows (n = 1,038 total samples).

Table 1. Pearson correlation (r) for Inhibin A and B in pg/mL and ovarian parameters for the first GnRH (G1) and $PGF_{2\alpha}$ (PGF) of breeding Ovsynch of the Double-Ovsynch protocol.

Parameters	**Inhibin A**				**Inhibin B**			
	At G1		At PGF		At G1		At PGF	
	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
Follicular parameters								
Volume of largest follicle	-0.04	0.30	-0.07	0.12	-0.01	0.79	-0.03	0.48
Volume of second follicle	0.004	0.93	-0.03	0.59	0.01	0.75	-0.02	0.61
Volume of third follicle	0.15	0.15	0.01	0.94	-0.07	0.50	0.03	0.79
Total follicular volume	-0.02	0.55	-0.06	0.16	-0.005	0.99	-0.03	0.53
Luteal parameters								
Volume of largest CL	0.004	0.92	-0.11	0.01	-0.04	0.71	0.06	0.15
Volume of second CL	-0.11	0.31	-0.06	0.23	-0.07	0.52	0.01	0.78
Volume of third CL	-0.10	0.79	0.43	0.27	0.44	0.27	0.001	0.99
Total luteal Volume	-0.02	0.57	-0.11	0.01	0.04	0.37	0.03	0.47
Hormonal concentration								
Progesterone, ng/mL	-0.08	0.05	0.01	0.73	-0.04	0.33	-0.03	0.40
Anti-Müllerian hormone, pg/ml	-0.001	0.98	-0.01	0.76	0.01	0.79	-0.02	0.54

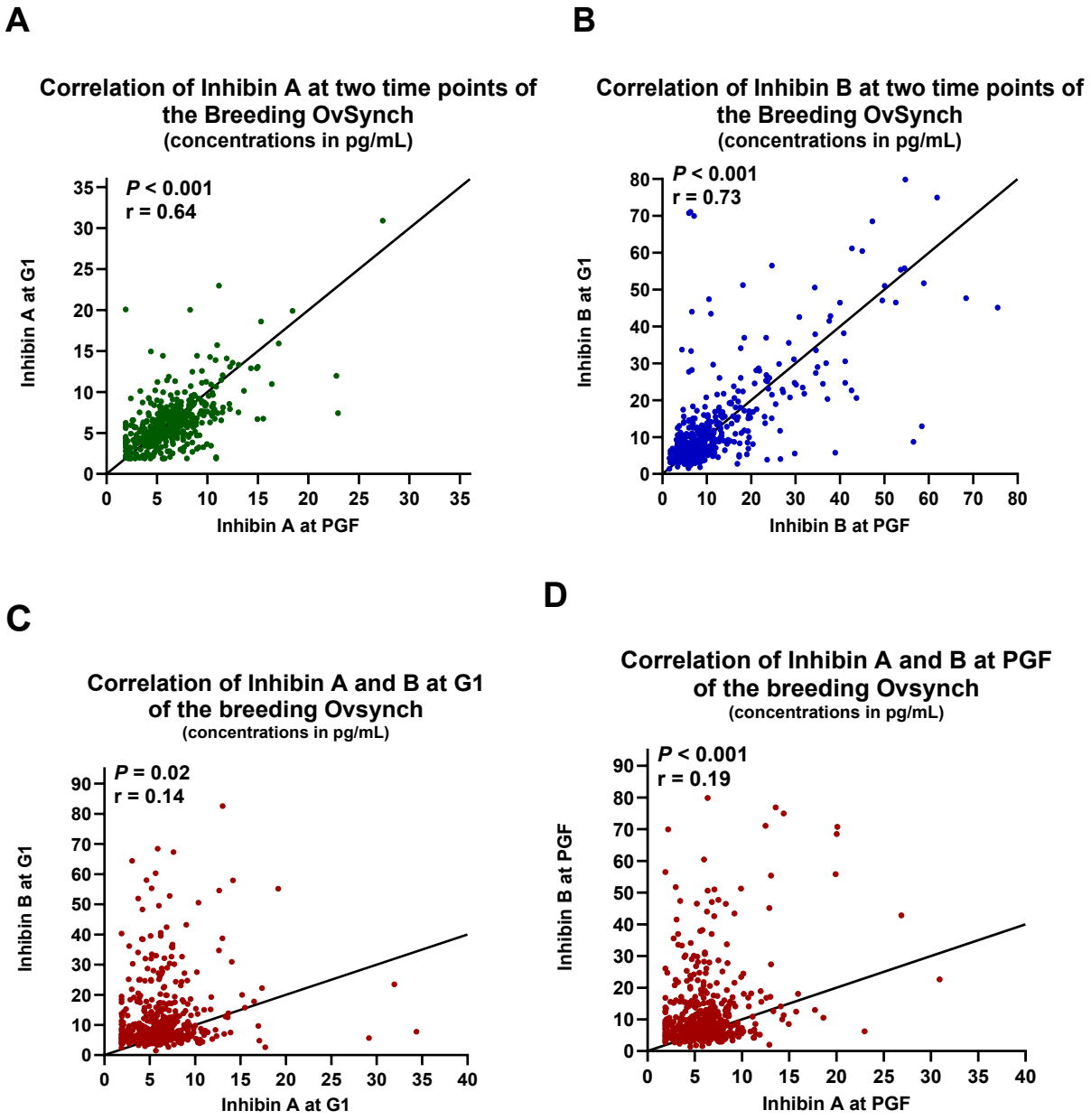
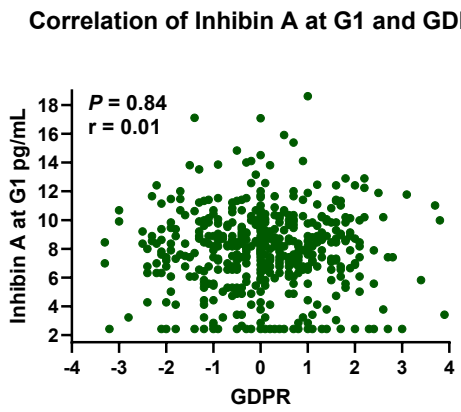


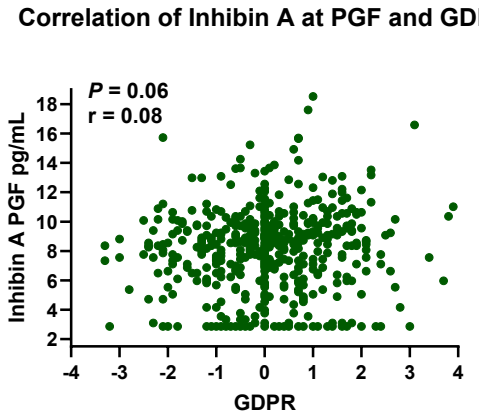
Figure 11

Figure 11: Pearson correlation between **A)** inhibin A, **B)** inhibin B concentrations measured at the G1 (D −10) and $PGF_{2\alpha}$ (D −3) time points in the breeding protocol r= 0.64; $P < 0.001$, and r= 0.73; $P < 0.001$, respectively. **C)** Inhibin A and inhibin B at G1 were correlated r= 0.14; $P = 0.02$, and **D)** Inhibin A and inhibin B at G1 were lowly correlated r= 0.19; $P < 0.001$.

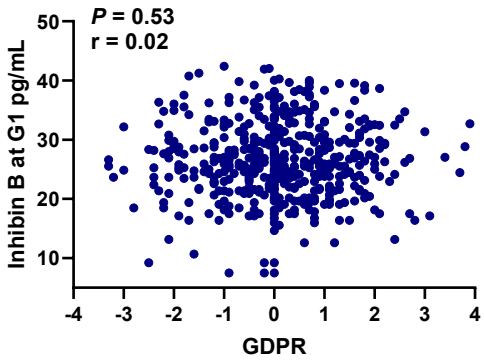
A


Correlation of Inhibin A at G1 and GDPR
P = 0.84
r = 0.01
Inhibin A at G1 pg/mL
GDPR

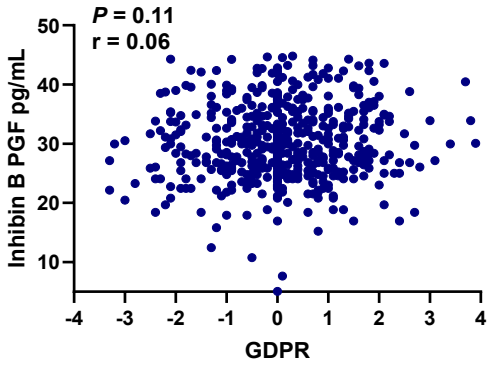
B


Correlation of Inhibin A at PGF and GDPR
P = 0.06
r = 0.08
Inhibin A PGF pg/mL
GDPR

C


Correlation of Inhibin B at G1 and GDPR
P = 0.53
r = 0.02
Inhibin B at G1 pg/mL
GDPR

D


Correlation of Inhibin B at PGF and GDPR
P = 0.11
r = 0.06
Inhibin B PGF pg/mL
GDPR

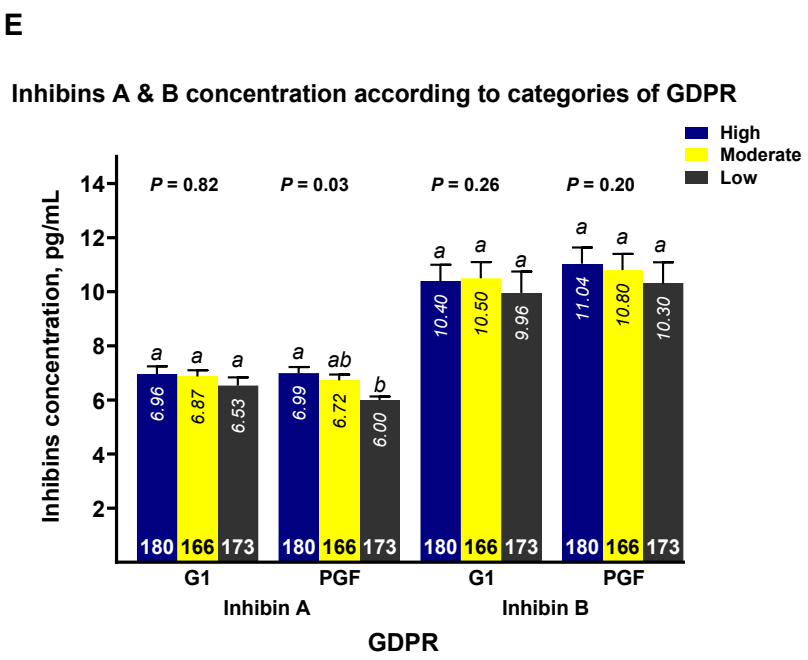


Figure 12

Figure 12. Correlation and association of inhibin A and B concentrations with genomic daughter pregnancy rate (GDPR) in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **A)** Pearson correlation between GDPR and plasma inhibin A concentrations measured at the first GnRH (D−10) d −10**, B)** at $PGF_{2\alpha}$ (D−3). **C)** Pearson correlation between GDPR and plasma inhibin A concentrations measured at the first GnRH (D−10), and **D)** at $PGF_{2\alpha}$ (D−3). **E)** Mean inhibin A and B concentrations by GDPR quartile at each time point. The error bars represent the standard mean error for inhibin A and B concentration in each GDPR quartile. Different superscripts indicate $P < 0.05$.

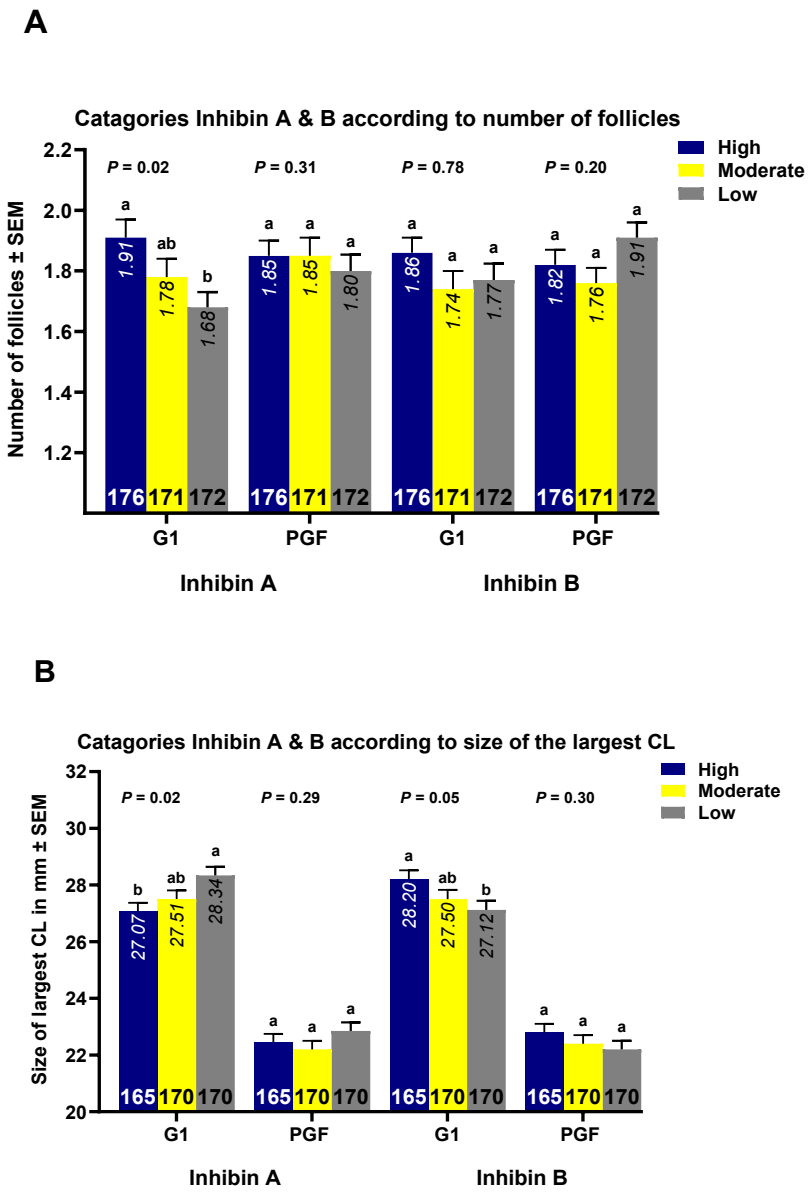


Figure 13

Figure 13. Association of the categories of inhibin A and B with ovarian parameters in lactating Holstein cows enrolled in a Double-Ovsynch protocol. **A)** Mean ± SEM of number of according to categories of inhibin A and B. **B)** Mean ±SEM of the size of the largest CL according to categories of inhibin A and B. The error bars represent the standard mean error for inhibin A & B concentration in each category. Different superscripts indicate $P < 0.05$.

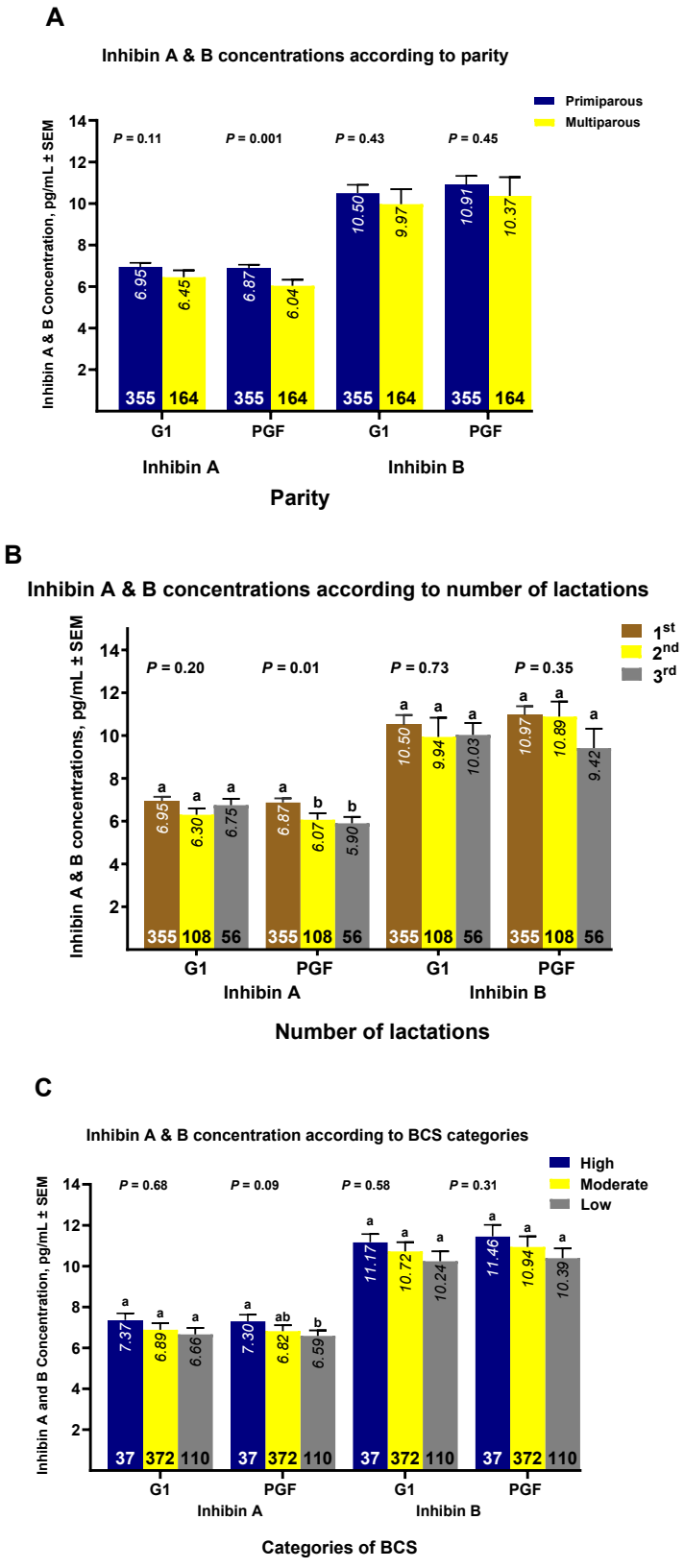

A
Inhibin A & B concentrations according to parity
Primiparous
Multiparous
Inhibin A & B Concentration, pg/mL ± SEM
P = 0.11
P = 0.001
P = 0.43
P = 0.45
6.95
6.45
6.87
6.04
10.50
9.97
10.91
10.37
355
164
G1
PGF
Inhibin A
Inhibin B
Parity
B
Inhibin A & B concentrations according to number of lactations
1st
2nd
3rd
Inhibin A & B concentrations, pg/mL ± SEM
P = 0.20
P = 0.01
P = 0.73
P = 0.35
6.95
6.30
6.75
6.87
6.07
5.90
10.50
9.94
10.03
10.97
10.89
9.42
355
108
56
Number of lactations
C
Inhibin A & B concentration according to BCS categories
High
Moderate
Low
Inhibin A and B Concentration, pg/mL ± SEM
P = 0.68
P = 0.09
P = 0.58
P = 0.31
7.37
6.89
6.66
7.30
6.82
6.59
11.17
10.72
10.24
11.46
10.94
10.39
37
372
110
Categories of BCS

Figure 14

Figure 14. Association of inhibin A and B concentrations with parity and number of lactations in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **A)** Parity according to inhibin A & B concentrations measured at the first GnRH (D−10) and at $PGF_{2\alpha}$ (D−3). **B)** Number of lactations according to inhibin A & B concentrations measured at the first GnRH (D−10) and at $PGF_{2\alpha}$ (D−3). **C)** Association of inhibin A and B concentrations with categories of body condition score and GnRH dose treatment in lactating Holstein cows enrolled in a Double-Ovsynch protocol. The error bars represent the standard mean error for inhibin A and B concentration. Different superscripts indicate $P < 0.05$.

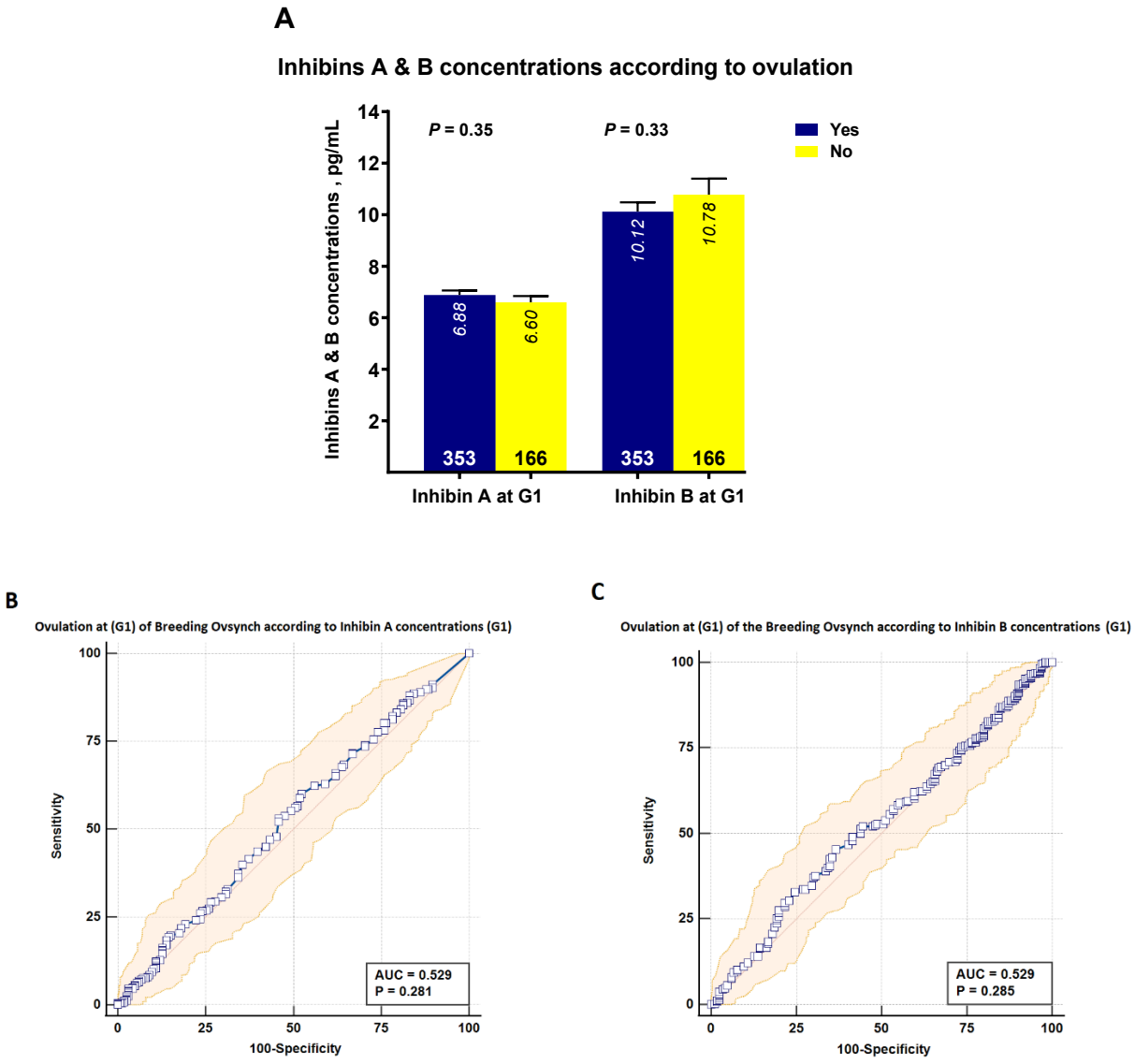
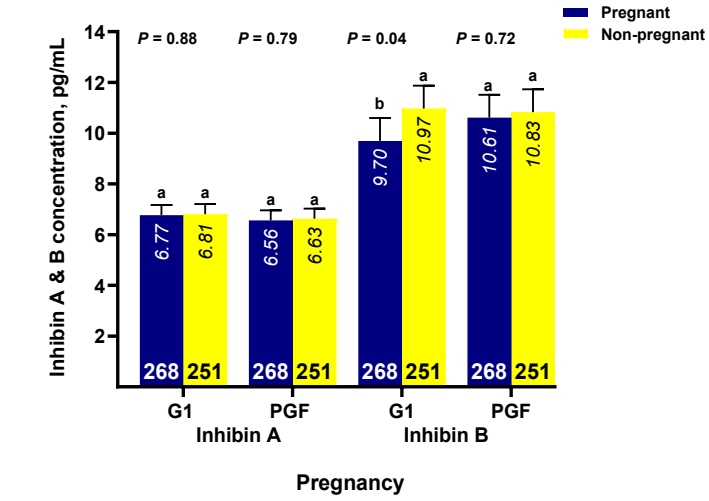


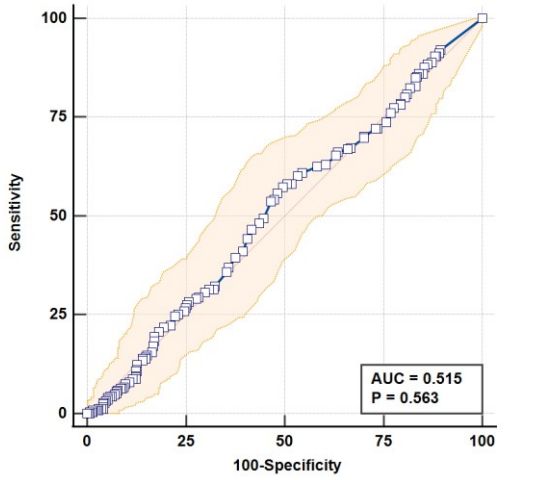
Figure 15

Figure 15: A) Inhibin A and B concentrations association with ovulation at G1 of the breeding Ovsynch in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **B)** Receiver Operating Characteristic (ROC) analysis showed that Inhibin A could not predict ovulation in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **C)** ROC for inhibin B could not predict ovulation. The error bars represent the standard mean error for inhibin A and B concentration. Different superscripts indicate $P < 0.05$.

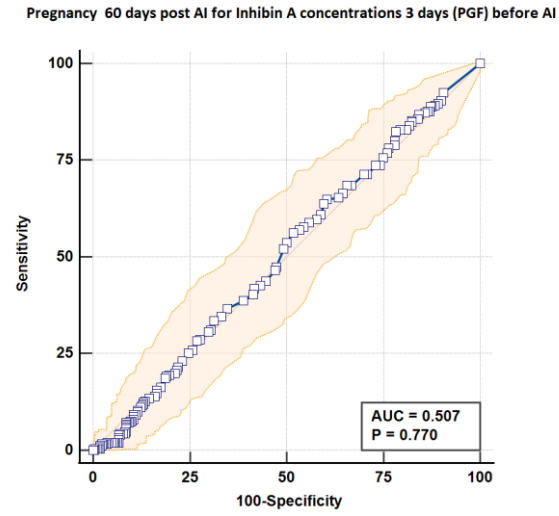
A


Inhibins A & B concentrations according to pregnancy per AI
Pregnant
Non-pregnant
P = 0.88
P = 0.79
P = 0.04
P = 0.72
Inhibin A & B concentration, pg/mL
14
12
10
8
6
4
2
a
a
a
a
b
a
a
a
6.77
6.81
6.56
6.63
9.70
10.97
10.61
10.83
268
251
268
251
268
251
268
251
G1
PGF
G1
PGF
Inhibin A
Inhibin B
Pregnancy

B


Pregnancy 60 days post AI for Inhibin A concentrations 10 days (G1) before AI
Sensitivity
100
75
50
25
0
0
25
50
75
100
100-Specificity
AUC = 0.515
P = 0.563

C


Pregnancy 60 days post AI for Inhibin A concentrations 3 days (PGF) before AI
Sensitivity
100
75
50
25
0
0
25
50
75
100
100-Specificity
AUC = 0.507
P = 0.770

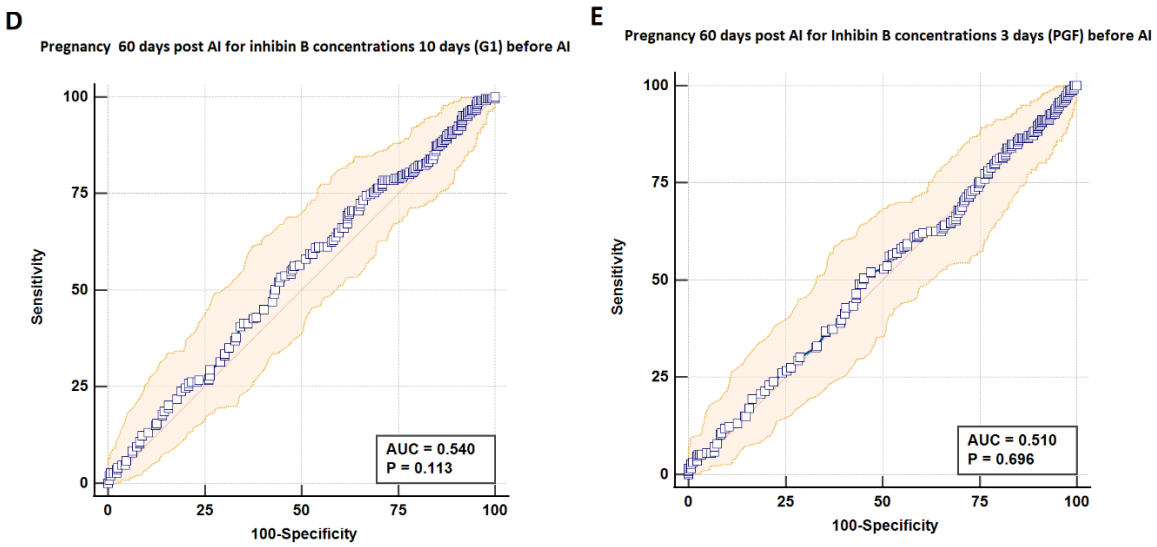


Figure 16

Figure 16. A) Association between inhibin A and B concentrations and pregnancy per artificial insemination (AI) in lactating Holstein cows enrolled in a Double-Ovsynch protocol. **B)** Receiver Operating Characteristic (ROC) analysis showed that Inhibin A at G1 could not predict P/AI in lactating Holstein cows enrolled in the Double-Ovsynch protocol. **C)** ROC analysis showed that Inhibin A at PGF could not predict P/AI. **D)** ROC analysis showed that Inhibin B at G1 could not predict P/AI. **E)** ROC analysis showed that Inhibin B at PGF could not predict P/AI. The error bars represent the standard mean error for inhibin A and B concentration. Different superscripts indicate $P < 0.05$.

CHAPTER IV

Clinical and production applications of AMH and Inhibin A&B in reproduction

The purpose of the dissertation was to investigate if AMH and inhibin A and B had a robust association with reproductive response in dairy cows, enough to be considered as biomarkers for improving decision-making in dairy herds. One of the key traits of these hormones as an endocrine indicators of antral follicle count, AMH and inhibin A and B plasma concentrations can be measured non-invasively early in the reproductive life of cows, enabling preselection of high-fertility individuals and the culling of sub-fertile animals. Numerous studies have reported that circulating AMH concentrations are moderate to strongly associated with the number of antral follicles, ovarian response to super-ovulation, and embryo yield, particularly in embryo donor cows. However, AMH plasma concentrations associations with fertility outcomes have been inconsistent and the current study did not advance its usefulness for prediction of reproductive outcomes. Inhibin A and B concentrations are hypothesized to reflect granulosa cell activity and ovarian health in bovine species and may complement AMH in assessing follicular function and reproductive potential. For instance, cows with high Inhibin A at G1 could be prioritized for fixed-time AI, while those with low AMH may be flagged for alternate reproductive strategies. Ultimately, refining our understanding of AMH and inhibin biology can help refine the next generation of precision reproductive management in dairy herds. Collectively, these biomarkers may allow for more precise and cost-effective reproductive strategies, especially in synchronized cows and embryo transfer programs.

Although both AMH and inhibin A and B are hypothesized to be secreted by granulosa cells in bovine species, their regulation of ovarian function, physiological timing, and functional roles is yet to be fully understood. The findings of the current study indicated that AMH is not predictive of ovulation and P/AI and has a very low relationship with GDPR. While Inhibin A—

particularly at G1— was associated with ovulation, while neither inhibin form showed consistent associations with GDPR or P/AI.

Comparison of predictive values and associations with GDPR, ovulation, and P/AI

In terms of predictive value, AMH showed low associations with GDPR at PGF, where cows in the highest AMH tercile tended to have higher GDPR values. AMH's association with P/AI was weak, likely due to the multifactorial relationship of pregnancy that includes periovulatory events and uterine environment that might not be represented by AMH concentrations. On the other hand, Inhibin A demonstrated an association with ovulation at G1, suggesting more potential for further exploration. There is also a further need to explore the value of these hormones as biomarkers for embryo transfer and IVF programs, where donor selection and follicular response prediction can be critical to the success of these programs.

Novelty and contribution to the field

To our knowledge, this is the first and largest study to simultaneously assess circulating concentrations of AMH and Inhibin A and B in relation to GDPR, ovulation, and P/AI in synchronized dairy cows. By evaluating hormone profiles at key stages of the DO protocol and integrating them with the genomic trait, this work provides a novel framework for linking endocrine and genomic data in reproductive performance assessment. Furthermore, this study introduces a new perspective on the utility of Inhibin A as a short-term predictor of ovulatory response. While AMH has been widely studied in the context of antral follicle count, its concurrent evaluation with inhibin forms enables a more nuanced understanding of follicular status and

synchronization outcomes. This dual-hormone approach represents a meaningful advancement in the design of reproductive diagnostics and genomic selection tools.

Study limitations and practical implications

One limitation of the current study is the observational design and the single-cycle hormone sampling strategy. Hormone concentrations were assessed at only two time points—G1 and PGF—which, while physiologically relevant, may not fully capture the dynamic endocrine shifts occurring throughout the estrous cycle. Another limitation was that only follicles >10mm were considered during ultrasonography, which limits the potential association with AMH and inhibin A and B plasma concentration with smaller early antral follicles. Additionally, the study population (n=519), although genetically and physiologically diverse, consisted of lactating Holstein cows within a commercial setting, which may limit generalizability to heifers or nonlactating cows. Despite these constraints, the practical implications of these findings are significant. It is reasonable to surmise that AMH can be considered a lowly variable within estrous cycle biomarker in dairy cattle that may be useful for heifer screening or embryo donor selection programs, while Inhibin A—especially when measured at the time of G1—may indicate ovulatory response, aiding synchronization protocol refinement. These results support the investigation of endocrine profiling into reproductive management strategies and genetic evaluations, particularly when tailored to specific reproductive outcomes warrant further investigation.

Future perspectives and applications

Future studies should aim to expand hormone profiling beyond two time points to include the periovulatory and luteal phases, which may yield additional insight into the hormonal

association with reproductive responses. Longitudinal hormone tracking across multiple time points during the estrous cycle along multiple cycles could also help refine predictive models and clarify the temporal dynamics of AMH and inhibin A and B. Moreover, mechanistic studies investigating AMH and inhibin signaling pathways in granulosa cells—particularly under different synchronization treatments—could further enhance biological interpretation. Furthermore, characterizing AMH and inhibin A and B in follicular fluid during the final stages of *in vivo* oocyte maturation may offer a more localized and physiologically relevant view of their functions than plasma measurements alone. Unlike peripheral blood, follicular fluid directly reflects the intrafollicular microenvironment where oocyte growth, maturation, and cumulus expansion occur. By assessing these hormones within the follicle, researchers can gain clearer insight into their paracrine roles in granulosa–oocyte signaling, steroidogenesis, and follicular competence. For example, AMH within the follicle may act locally to fine-tune the effects of FSH or prevent premature luteinization, while inhibin A and B concentrations may better indicate granulosa cell activity and dominant follicle readiness. This approach not only enhances the mechanistic understanding of endocrine interactions but could also refine predictive biomarkers for oocyte quality and IVF success, bridging the gap between physiology and reproductive biotechnology applications in cattle.

Broader industry utility of AMH and inhibin

Beyond the scope of this study, AMH plasma concentration may prove valuable in fertility restoration programs, where cows returning from metabolic or reproductive disorders could be screened for functional ovarian reserves before reintegration into breeding programs. AMH-based selection criteria may also align with genetic selection tools, offering a multi-layered evaluation

strategy that incorporates endocrine biomarkers into genomic-enhanced breeding indices. Its ability to predict embryo yield further enhances its application in IVF and *in vitro* embryo production systems, particularly for high-value animals.

Moreover, similar to equine and feline species, inhibin may be explored as potential markers for ovarian disorders. For instance, altered Inhibin A or B profiles could signal disrupted folliculogenesis or luteal insufficiency, providing an early warning system for subclinical reproductive problems. With further validation, inhibin profiles could be integrated into automated herd management tools, where milk or blood samples are routinely analyzed to detect emerging reproductive inefficiencies. Ultimately, both AMH and inhibin may evolve into accessible, cost-effective tools for improving fertility outcomes and profitability in dairy systems driven by data-informed decision-making.

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