EVALUATING ANTI-MÜLLERIAN HORMONE (AMH) AS A REPRODUCTIVE TOOL IN DAIRY CATTLE

by

KAYLA JEAN ALWARD

(Under the Direction of Jillian F. Bohlen)

ABSTRACT

One-hundred and five virgin Holstein heifers were sampled pre-breeding for circulating AMH concentration and transrectal ultrasonography was performed to determine AFC and cyclicity status. Post-calving, heifers were sampled at 5-20 days in milk for AMH. At 45-60 days in milk, two-hundred and fifty-two primiparous and multiparous were enrolled and all animals were sampled for AMH concentration and transrectal ultrasonography was performed. Animals were assigned to an estrous detection or TAI breeding protocol and grouped based on pre-breeding circulating AMH. AMH was positively correlated with AFC, lactation number, age and milk-weight on sampling and breeding day ($P<0.0001$). Conception risk to first service in cows and heifers was not impacted by breeding protocol or AMH category ($P>0.05$). Older, greater lactation animals were more likely to cycle and be bred on an estrous detection protocol ($P<0.01$). Heifers maintained their AMH categorization post-calving and pre-breeding but dropped in AMH post-calving ($P<0.0001$).

INDEX WORDS: Anti-Müllerian Hormone, Breeding Protocol, Fertility

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DEDICATION

To My Family and Friends

Thank you being my source of support and strength and encouraging my dreams throughout this process.

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First, I would like to thank Dr. Jillian Bohlen for the opportunity she provided me to learn under her. From our first year together at UGA as an undergraduate until now, you have been a wonderful mentor and friend, who has always been available and willing to help me through my problems and celebrate my achievements with me. I cannot thank you enough for all of your effort to shape me into a successful graduate student. Thank you Dr. Graves for being a guiding hand throughout this process – your opinions and experiences have been invaluable. Thank you Dr. Palomares for your insight and advice throughout this process. Thank you to the Godfrey Dairy Farm crew for all of your help, from locking to breeding cows and heifers. Thank you especially to Cuyler for putting up with our inconveniencies with a smile and always making us laugh.

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CHAPTER 1

INTRODUCTION AND LITERATURE REIVEW

An Evaluation of Cyclicity and Endocrinology in Cattle

Cattle are classified as polyestrous, with a singular estrous cycle lasting 21 days on average (Armstrong and Hansel, 1958). Unlike sheep and goats which are constrained by seasonal factors, cattle cycle year-round (Ortavant et al., 1985). The estrous cycle can be broken into 2 phases: the follicular phase and the luteal phase. The follicular phase is dominated by the ovulatory follicle as the primary ovarian structure, with estrogen as the main hormone produced. While the luteal phase is dominated by the corpus luteum (CL) as the primary ovarian structure, with progesterone being the main hormone produced (Wettemann et al., 1972). Each phase is also subdivided into two stages. The follicular phase is made up of the stages proestrus and estrus, and the luteal phase is made up of the stages metestrus and diestrus (Ireland et al., 1980).

Together, proestrus and estrus, both stages of the follicular phase, account for approximately 20% of the cows' estrous cycle (Veronesi MC et al., 2002). Luteolysis, or the functional and physical breakdown of the CL marks the beginning of the follicular phase, and specifically proestrus. Proestrus is the transition period beginning on about day 17, as circulating progesterone is decreasing due to loss of a functional CL, and concurrently, circulating estrogen is increasing with follicular growth (Ireland et al., 1979). As progesterone declines with the removal of the CL, the developing follicles on the ovary have the opportunity to not only achieve dominance, but now ovulate. (Peters,

1985). As estrogen continues to increase, the cow then transitions into estrus, the most recognizable stage of the estrous cycle due to marked visible behavioral signs and temperament change of the animal (Hurnik et al., 1975). The main sign of estrus is standing to be mounted by other cattle and chin resting, while there are other indicators including a rise in spontaneous activity, mounting other cattle and clear vulvar discharge (Rao et al., 2013). Due to these physical markers indicating estrus, the onset of estrus is usually described as the first day of the cycle (day 1). During this phase, the follicle reaches peak estradiol production, causing sexual receptivity and the visible behavioral signs. Estrus typically lasts 6 – 24 hours, with an average duration of 15 hours (Dransfield et al., 1998). The end of estrus and the follicular phase is ovulation, occurring 24 to 32 hours after the onset of estrus (Senger, 2012).

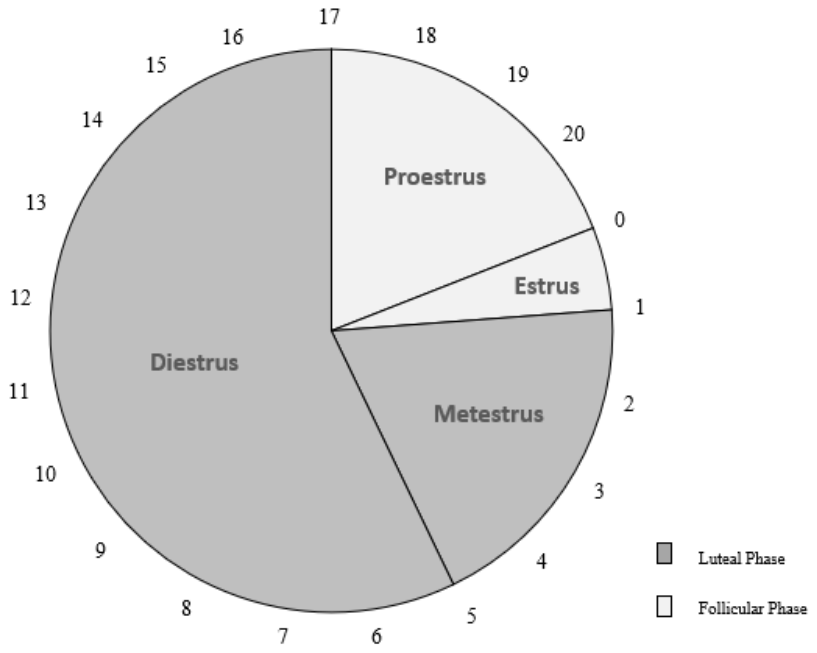

Figure 1.1: Graphic representation of the phases and stages of the bovine estrous cycle. Numbers surrounding the graph correspond to the day of the estrous cycle. The stages are labeled within their corresponding phase.

The luteal phase begins immediately after ovulation and comprises the remaining 80% of the cycle, starting with the stage metestrus (Ireland et al., 1980). During ovulation, blood vessels rupture as the oocyte is released from the dominant follicle. The ruptured blood vessels leave a bloody appearance, known as a corpus hemorrhagicum or CH. Following the expulsion of fluid and the oocyte from the follicle, the follicular walls collapse inward and the cells undergo luteinization (Senger, 2012). During luteinization, the CL forms from the remnant follicular tissue of the recently ovulated follicle. As follicular cells transform into luteal cells and begin producing progesterone, progesterone levels rise concurrently (Peters, 1985). In the cow, complete luteinization and development of a fully functional CL takes 2-5 days (Senger, 2012). The beginning of diestrus is marked by a fully functional CL. Diestrus is marked by a high and sustained level of progesterone secretion and is the longest stage of the estrous cycle in both pregnant and nonpregnant animals. This period lasts until luteolysis of the CL removes progesterone production, and the animal reenters proestrus. (Senger, 2012).

Hypothalamic-Pituitary-Gonadal Axis

In the cow, the estrous cycle is driven by both the production and feedback of hormones which cause the characteristic traits associated with each stage of the estrous cycle. The interaction and control of reproductive hormones is collectively known as the Hypothalamic Pituitary Gonadal (HPG) axis, shown in Figure 1.2. The HPG Axis structurally involves the tonic and surge centers of the hypothalamus, the anterior lobe of the pituitary gland and the ovaries. The chief hormones involved in the HPG axis are Gonadotropin-releasing Hormone (GnRH), Luteinizing Hormone (LH), Follicle

Stimulating Hormone (FSH), Estradiol (E_2), Inhibin and Progesterone (P_4) (Christensen et al., 2012).

GnRH is released by the tonic and surge center of the hypothalamus and acts directly on the anterior lobe of the pituitary via a portal blood system. This blood system prevents GnRH from entering the circulatory to reach its target tissue, allowing small changes in concentrations to have a significant impact on the release and production of substances from the anterior pituitary (Carmel et al., 1976). The tonic center releases gonadotropin at low levels with little fluctuation throughout most of the cycle. In contrast, the surge center releases high levels of gonadotropins for a short period of time. The differing nature of secretion from these centers is in due to their unique responses to hormone feedback. The tonic center is receptive to low levels of estrogen and negative feedback by P_4. It allows the release of low levels of pulsatile GnRH secretion (Goodman 1978). Low levels of estrogen are present when the CL is functioning, representing developing follicles, and P_4 levels are high, as described previously. This low, pulsatile level of GnRH is critical for follicular growth. In contrast, the surge center is receptive to high levels of estrogen and positive feedback. So, when estrogen levels are low, the surge center is suppressed.

In response to GnRH, the anterior lobe pituitary releases both FSH and LH which enter the blood stream to travel to their target tissue, the ovary. Together, they act positively on the ovary to promote the growth and development of follicles. As these follicles develop, they begin producing increasing amounts of E_2. At low levels, E_2 acts in a suppressive manner on the surge center as previously described. However, the low levels of E_2 also feed back positively to the tonic center of the hypothalamus to cause the

release of low, pulsatile levels of GnRH. GnRH then acts on the anterior lobe pituitary positively to release FSH and LH, thus continuing the feedback loop. GnRH and E_2 together create a positive feedback loop, as each causes an increase in the production of the other (Vadakkadath Meethal et al., 2005; Bearden et al., 2004).

During the luteal phase, when a CL is present on the ovary, P_4 production is high and E_2 is low. Together, this hormone profile plays a negative feedback role on the HPG axis by inhibiting GnRH neurons of the surge center, which in turn decreases the release of LH preferentially from the anterior lobe pituitary (Savoy-Moore and Swartz, 1987; Wildt et al., 1981). Decreased LH levels coupled with high levels of P_4 create an environment in which follicles can not achieve ovulation. Only once the high level of P_4 is removed is LH pulse frequency allowed to increase, leading to ovulation of a dominant follicle.

In the follicular phase, the growing follicles produce increasing amounts of E_2 until the surge center of the hypothalamus responds in a positive feedback manner by releasing large amounts of GnRH. This high frequency GnRH amplitude preferentially increases LH production, allowing for final growth of the follicles and ovulation (Roche, 1996). In addition, during the follicular phase, inhibin is being produced by the developing follicles on the ovary and acts in a negative feedback role to the anterior pituitary. Follicles that progress through recruitment and selection and proceed towards dominance produce high levels of inhibin, which feeds back to the anterior pituitary to selectively inhibit the release of FSH. In the presence of low FSH levels when follicles have already been selected and are growing, other primordial follicles are not stimulated to grow and be recruited to the follicle pool in order to conserve the follicular pool (Peper

et al., 2009). These pathways are examples of positive and negative feedback loops occurring simultaneously within the HPG Axis.

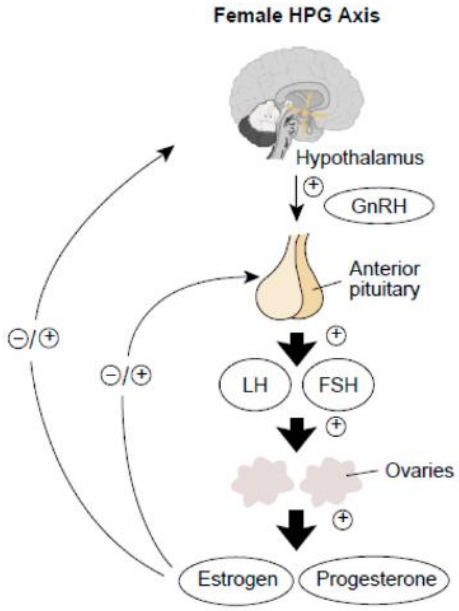

Figure 1.2: Diagram of the female HPG axis with related structures and hormones. (Kong et al., 2014)

CL Formation

As the luteal phase begins, the follicular tissue remaining from the recently ovulated follicle is transformed into the CL via luteinization. This process occurs under the influence of LH. While critical for ovulation, LH plays an additional important role for transforming the remaining tissue that surrounded the oocyte into functional luteal tissue, which will make up the CL (Alila et al., 1984). The tissue left behind after ovulation is made up of granulosa cells and thecal cells. These are transformed into large luteal cells and small luteal cells, respectively (Murphy, 2000). LH receptors are primarily located on small luteal cells and LH receptor number increases during early luteal development (less than 4.5 days post-LH surge) (Schams and Berisha, 2004). The

number of LH receptors present has a direct, positive relationship with the amount of progesterone secreted by the CL during early development (Spicer et al., 1981). A greater number of LH receptors and therefore more LH binding allows more effective transformation of the follicular tissue into functional, luteal tissue. Inhibition of the LH pulses or a reduced number of LH receptors present reduces both the size and functional capability of the developing CL (Peters et al., 1994). Both of the luteal cell types have the capability to produce progesterone, however large luteal cells are also capable of producing oxytocin, which is critical for cycle regulation (Harrison et al., 1987). As previously described, formation of a functional CL is critical for normal cyclicity in addition to obtaining a pregnancy.

Folliculogenesis and Follicular Waves

The growth and development of follicles occurs in 4 stages: recruitment, selection, dominance and atresia; collectively known as folliculogenesis. This process occurs in "waves" 2 to 3 times during a single estrous cycle depending on cycle length. During the first wave, a cohort of small antral follicles are recruited and begin to grow beyond 4mm and secrete E_2 in response to elevated FSH, caused by increased GnRH pulse amplitude. The emergence of this new cohort occurs during days 1-2 of the estrous cycle. Most of these recruited follicles will undergo atresia or death via apoptosis as a result of decreasing FSH over the following 2-3 days of the cycle (Austin et al., 2001). Follicles at this stage are predominantly FSH dependent, as FSH stimulates the recruitment of small antral follicles. Therefore, decreased FSH levels causes many small follicles to undergo atresia. The few remaining follicles continue growing until a single follicle achieves deviation (the dominant follicle). This follicle that does not undergo

atresia following selection proceeds towards dominance. The number of LH receptors present is a key factor between follicles that undergo atresia and the follicle that will achieve deviation, with those undergoing atresia having significantly fewer LH receptors (Beg et al., 2001 and Xu et al., 1995).

During this time, the dominant follicle secretes increasing amounts of both estradiol and inhibin. Estradiol feeds back positively as previously described to allow elevated LH levels necessary for final maturation. Inhibin negatively feeds back to the anterior pituitary to selectively inhibit FSH secretion to prevent any other cohort growth. The dominant follicle continues growing for 3-4 days but will eventually become atretic during this portion of the estrous cycle, due to the presence of a functional CL and high P_4 levels coupled with low E_2 levels which regulates the LH pulse pattern and prevents the LH surge required for ovulation. Large selected follicles and dominant follicles are predominantly LH dependent, as an important role of LH is to promote final growth and maturation of dominant follicles and to stimulate ovulation (Senger, 2012).

As the dominant follicle undergoes atresia, E_2 levels begin declining on day 6 of the estrous cycle and the follicle loses dominance between days 7 and 9, allowing for another rise in FSH, thus causing a new wave of follicles to be recruited and emerge. The selection of a new dominant follicle occurs simultaneously as the old dominant follicle regresses. If luteolysis occurs here, then an LH surge will be able to occur now in the absence of high P_4 and the follicle will ovulate. However, if the CL is still present, LH pulse frequency will be suppressed and this follicle will also undergo atresia, giving rise to a third wave.

During the final wave, following regression of the CL, a frequent LH pulse pattern will support a dominant follicle achieving final differentiation and ovulation. In the cow, generally and ideally 1 follicle will become dominant, but 2 follicles becoming dominant has been described and results in twinning. One study looking at double ovulators in Holsteins found that 66% of the double ovulations occurred from the same ovary, while 33% double ovulate a follicle from each ovary (Kusaka et al., 2017). The mechanisms and consequences of multiple follicular ovulation will be discussed later.

Cow vs Heifer Cyclicity Differences

Multiple studies have confirmed that cows generally have 2 follicular waves, while heifers have 3 (Sirois and Fortune, 1988; Savio et al., 1987; Taylor and Rajamahendran, 1990). Due to the differing number of follicular waves during an estrous cycle, cows and heifers have different cycle characteristics. Three wave animals generally have a longer estrous cycle, with research showing a difference of 2.4 to 3.3 days in cycle length between 2 wave and 3 wave animals (Ginther et al., 1989b; Ahmad et al., 1997; Townson et al., 2002). The start of follicular waves in 3 wave animals occurs on average on Day 2, 9, and 16 of the estrous cycle, while follicular waves in 2 wave animals occurs on average on Day 2.5 and 12 of the estrous cycle (Rajakoski, 1960; Sirois and Fortune, 1988). This results in 2 wave animals generally ovulating an older and larger follicle when compared with animals having 3 waves (Ahmad et al., 1997, Townson et al., 2002). Despite similar growth rates of ovulatory follicles in 2 and 3 wave animals, the difference in duration of dominance allows for a larger, older follicle to be ovulated by 2 wave animals. The older age of the ovulatory follicle in 2 wave animals is thought to impact fertility, as the extended growth period of the ovulatory follicle prior to

ovulation has been associated with lower fertility evidenced by depressed pregnancy rates. Researchers contribute this lower fertility of older follicles to elevated estradiol levels and increased duration of LH pulse frequency (Ahmad et al., 1995; Mihm et al., 1994; Townson et al., 2002).

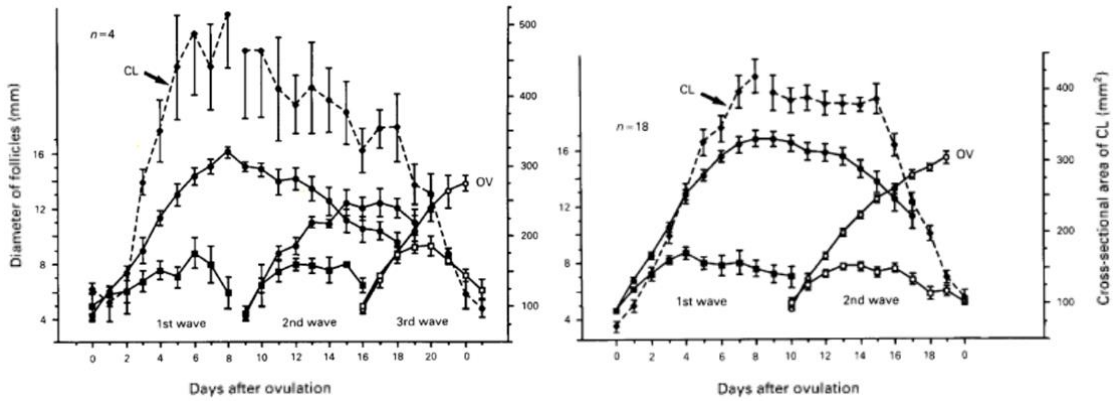

Figure 1.3: Graphs depicting 2 and 3 follicular waves within a cycle in heifers where the x- axis is day of the cycle after ovulation and the left y-axis depicts the diameter of the follicles in mm and the right y-axis depicts the cross-sectional area of the CL in mm^2 (Ginther et al., 1989b).

Luteal regression occurs later in animals with 3 waves than those with 2 waves, causing a lengthened estrous cycle. One study details that the length of the luteal phase was on average 2 days shorter in 2 wave cows with day of peak progesterone occurring ~2 days earlier in the cycle (day 13.8 vs. day16.1) (Ginther et al., 1989a; Taylor and Rajamahendran, 1991; Townson et al., 2002). However, peak progesterone concentrations and total area under the curve of progesterone concentrations did not differ between 2 and 3 wave animals. This indicates that duration of luteal progesterone

and the day of peak progesterone are key players in determining whether 2 or 3 waves of follicular growth will occur (Townson et al., 2002).

Table 1.1: Description of the day of peak progesterone, peak progesterone level, length of the luteal phase and progesterone area under the curve in two-wave and three-wave animals and pregnant vs. non-pregnant animals (Townson et al., 2002).

Group	No.	Peak progesterone (ng/mL)	Day of peak progesterone	Luteal length (days)	Progesterone area under curve to d 17
All two-wave	48	6.3 ± 0.4	13.8 ± 0.4[a]	17.6 ± 0.4[a]	51 ± 3
All three-wave	17	7.1 ± 0.6	16.1 ± 0.8[b]	19.3 ± 0.6[b]	62 ± 5
All nonpregnant	18	6.6 ± 0.6	15.2 ± 0.7	19.3 ± 0.8[c]	59 ± 5
All pregnant	47	6.9 ± 0.4	14.6 ± 0.5	17.5 ± 0.4[d]	54 ± 3

[a,b]Two-wave vs three-wave, $P < 0.05$. [c,d]Nonpregnant vs pregnant, $P < 0.05$.

In addition, the stressors of milk production impact cyclicity in cows, causing cyclic differences that prevent us from managing cows and heifers similarly from a reproductive standpoint. Milk production, particularly in high producing animals, negatively impacts cyclicity. To produce high quantities of milk, feed intake must be elevated and blood is required to circulate the body at a much higher rate in order to deliver the required nutrients to the mammary system, so that these substrates can be compiled to produce milk. A higher rate of blood circulation also means that the blood is passing through the liver more often, which results in the breakdown of steroids (Sangsritavong et al., 2002). This is the mechanism by which hormones such as E_2 and P_4 cleared more rapidly from the body, ultimately resulting in multiple negative effects including increased multiple ovulation rate, decreased behavioral estrus and less productive ovulatory follicles and CLs. (Lopez et al., 2004; Lopez et al., 2005; Wiltbank

et al., 2006). Wiltbank and collaborators (2006) elucidated the associations of milk production and fertility, finding that after the LH surge, increased clearance of E_2 led to a shortened duration of estrus. In addition, the levels of circulating E_2 preovulation rise at a slower rate due to increased steroid metabolism, meaning E_2 would continue to rise over a longer period of time in order to reach a sufficiently elevated level to induce a GnRH and LH surge. Thus, a larger, older, "persistent" follicle would be ovulated that would have been exposed to a longer period of LH pulses, potentially meaning that it has been overstimulated or prematurely activated and resulting in decreased fertility (Ahmad et al., 1995; Ahmad et al., 1996; Revah et al., 1996).

Cows post-calving also exist in a state of negative energy balance. For these animals, circulating blood glucose concentration is depressed which also decreases insulin response and IGF1 levels. With insulin and IGF1 responsible for controlling the activity of LH and FSH receptors on the ovary, these depressed levels are theorized to decrease the ovary's receptiveness to LH and FSH and therefore delay ovulation in the cow as seen in in vitro studies (Dumesic and Richards, 2013; Lucy et al., 2013; Lucy et al., 2014; Butler et al., 2003). Our post calving animals are also more prone to uterine health issues such as metritis due to calving, and those animals with poor postpartum uterine health are at greater risk for early embryonic loss later postpartum (Santos et al., 2004). In addition, these animals have a longer postpartum anestrous period, longer duration to first ovulation, and lower pregnancy rates compared to animals in a lesser state of negative energy balance. (Roche et al., 2009; Pryce et al., 2002; Buckley et al., 2003; Roche et al., 2007).

Studies have also confirmed that the number one contributing factor to twinning in lactating dairy cattle is high milk production (Kinsel et al., 1998). Again, increased steroid metabolism is the main contributor to double ovulations in cattle. Lower circulating P_4 level during development of the follicular wave housing the ovulatory follicle due to increased steroid metabolism results in multiple instead of single ovulations (Macmillan et al., 2018). The decreased P_4 level allows for an increase in GnRH and LH pulse frequency and thus allow more than one follicle to deviate and become dominant (Lopez et al., 2005). While more research is still needed to confirm other processes, it is widely known that these issues are unique to lactating cattle as opposed to heifers, who do not exhibit alterations of cyclicity associated with milk production and previous calvings.

Factors that Impact Cyclicity

A host of factors can impact cyclicity and cause an animal to vary from a "normal" estrous cycle. These factors can be divided into environmental and genetic factors.

Environmental effects that impact cyclicity chiefly include metabolic stressors. An inadequate plane of nutrition can reduce LH pulse frequency, follicular diameter, circulating estrogen and result in anovulation (Bossis et al., 1999; Diskin et al., 2003; Roche, 2006). Acute under-nutrition also drastically affects follicular growth and cyclicity. Cyclic heifers restricted for 3.5 days had reduced follicular growth and reduced maximum diameter of the first dominant follicle wave of the subsequent estrous cycle (Mackey et al., 1999). After 13-15 days of nutrient restriction, 60% of these heifers failed to ovulate the dominant follicle. However, when heifers are returned to an appropriate

plane of nutrition, they will return to normal cyclicity after a shortened, abnormal cycle (Bossis et al., 2000). In addition, specific ingredients in the diet may contribute to altered cyclicity. By altering the nonstructural-to-structural carbohydrate ratio in early lactation animals, the period of anestrus can be reduced (Garnsworthy et al., 2008). When providing nonstructural carbohydrates, the concentration of both IGF1 and insulin increase, allowing greater follicular sensitivity to gonadotropins and resumption of normal cyclicity (Gong et al., 2002). The level of fat in the diet may also impact cyclicity positively by allowing increased metabolizable energy intake as well as maximizing any positive physiological effects of fatty acids in reproductive tissues (Lucy et al., 1992; Mattos et al., 2000; Wathes et al., 2007). Enhanced growth and function of the dominant follicle has been described in cows supplemented with long-chain unsaturated fatty acids (Lucy et al., 1991).

Heat stress also alters cyclicity in both cows and heifers. Generally, cattle experience heat stress at 72 and above on the temperature humidity index (THI) scale (Ravagnolo and Misztal 2000). Animals experiencing heat stress for short periods can alter cyclicity short term, but long-term heat stress exposure has lasting negative effects on all aspects of fertility including a reduction in circulating estradiol and compromised follicular growth. An immediate effect of heat stress on cyclicity is that dominant follicles are generally smaller with less fluid than those from animals not experiencing heat stress. In addition, a lack of suppression on smaller follicles has been seen even though dominant follicles from heat stressed animals are larger and contain more fluid. This indicates disruption to follicular selection and dominance and the potential for multiple ovulations (Badinga et al., 1993). Heat stress also alters LH pulse amplitude and

frequency and plasma inhibin concentrations. Heat stressed animals have lower levels and frequency of LH which may impact the animals' ability to ovulate a follicle (Gilad et al., 1993 and Wise et al, 1988). The depressed inhibin levels explain the lack of suppression on smaller follicles (Wolfenson et al., 1995).

However, animals can also be genetically predisposed to abnormal cyclicity and therefore poor reproductive performance. Published data show that the chance of developing cystic ovaries, which can prevent an animal from ovulating and keep her in a constant follicular phase (follicular cyst) or luteal phase (luteal cyst) are lowly heritable (Lin et al., 1989; Ashmawy et al., 1990; Garverick, 1997). In addition, the pelvic angle and vulvar conformation, which have a genetic influence, can also affect an animal's chances of contracting uterine infections, which can alter cyclicity (Gautam and Nakao, 2009 and Gaviria and Zuluaga, 2013). Haplotypes within breeds have also been discovered that are directly linked with infertility. Specifically, the JH1 haplotype in the Jersey breed causes a mutation in the CWC15 gene which is important for cell signal processing. Embryos that receive a mutated CWC15 gene from both dam and sire are not able to complete development and die during gestation (Sonstegard et al., 2013). Similar haplotypes have also been discovered in Holstein and Brown Swiss cattle. (VanRaden et al., 2011). Other genetic parameters such as Antral Follicle Count (AFC) and Anti-Müllerian Hormone (AMH) will be discussed later.

Manipulating Cyclicity

Variations in cyclicity of cattle occur due to a variety of factors previously discussed. This makes using a single tool to manipulate cyclicity to obtain pregnancy, embryo flushes and transfers, etc. difficult for producers. Thus, there are multiple

programs targeting improved reproductive efficiency with overall effectiveness varying from animal to animal and farm to farm. Current reproductive programs utilize hormones or a combination of hormones to manipulate cyclicity. The main hormones available include prostaglandin $F_{2\alpha}$, GnRH and progesterone.

Table 1.2: Lists the most commonly utilized hormones to manipulate cyclicity, their route of administration and effect on cyclicity.

Hormone	**Route of Administration**	**Desired Outcome**
Prostaglandin $F_{2\alpha}$	IM injection	Targets the corpus luteum to induce luteolysis and the start of a new follicular wave
Progesterone	CIDR	Targets the hypothalamus to inhibit GnRH release to either: 1. prevent the animal from ovulating a follicle and coming into estrus or 2. allow animal to build up follicles and ovulate at the same time upon progesterone removal
GnRH	IM injection	Targets the anterior pituitary to release FSH and LH to either: 1. cause ovulation or 2. cause the start of a new follicular wave

Used in conjunction with artificial insemination, these hormones allow producers to synchronize estrus or ovulation to increase economic returns and offer management benefits (Xu et al., 1996). Estrous synchronization is generally less involved in terms of a shot schedule than Timed Artificial Insemination (TAI) and functions to bring animals into heat within a window of time. This reduces a producer's window of heat checking, decreases days to first insemination and decreases labor costs (Holm et al., 2007). TAI programs utilize the hormones to synchronize ovulation and thus results in animals being

bred at a specified day and time according to the program. TAI programs can allow producers to eliminate heat checking entirely while reducing days to first service.

New tools beyond traditional reproductive programs have emerged to attack reproductive inefficiencies from another angle. Activity monitoring animals allows us to identify those exhibiting estrus via increased steps or activity but who otherwise do not display visible signs of estrus. Activity monitoring programs can detect small increases in activity and determine if they are significant, thus alerting the producer when an animal is in heat, which otherwise may have gone undetected. Genetic testing to identify animals that will have superior reproductive performance in the herd and eliminating those with genes associated with depressed fertility are another tool at a producer's disposal.

Anti-Müllerian Hormone as a Fertility Marker

Of the new tools being utilized, Anti-Müllerian Hormone or AMH shows potential as an indicator of fertility. AMH was originally discovered for its role in fetal sex differentiation, as it is produced by the Sertoli cells in the testis of males (Blanchard and Josso, 1974). When an oocyte is fertilized with sperm carrying male DNA, the Y protein of the XY chromosomal pair has a region known as the Sex Determining Region on the Y chromosome (SRY). This region coordinates synthesis of SRY protein by the sex cords within the primitive gonad, which in turn drives development of testes. The Sertoli cells of the testes secrete AMH, which causes degeneration of the paramesonephric duct, also known as the Müllerian duct (Jost, 1947). Regression of the Müllerian duct while allowing the mesonephric duct to develop allows for the male reproductive system to form. This process is described in Figure 1.4. This force of

regression of the Müllerian ducts is the manner by which Anti-Müllerian Hormone came to have this name.

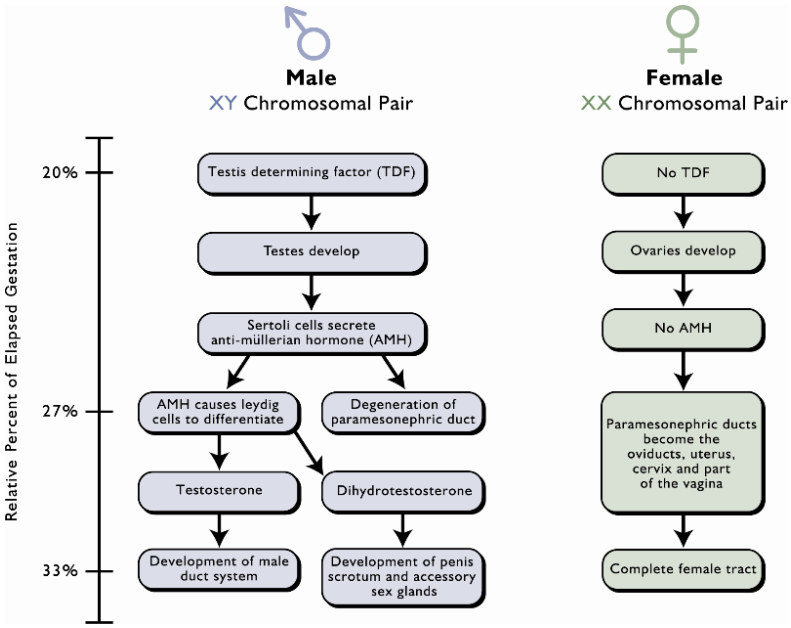

Figure 1.4: A flow chart depicting the process by which the SRY gene (also known as TDF) ultimately leads to AMH production and regression of the paramesonephric ducts. (Senger, 2012)

AMH production by the testes begins during the 7th to 8th week of gestation in male fetuses and in cattle, is the main culprit in rendering female calves born twin to males as sterile, a condition known as Freemartinism (Vigier et al., 1984). This mechanism was first described in 1916 by Lillie. The fusion of the chorion and anastomosis of the fetal circulation of multiple calves in utero exposes the female calf to the hormone profile of the male calf. Therefore, the AMH produced by the male's testes causes partial regression of the Müllerian duct in the female which would go on to develop into the female reproductive tract, resulting in abnormal reproductive tract

development (Lillie 1917; Jost, 1947). The degree of abnormality varies but generally includes ovarian stunting, uterine and vaginal hypoplasia, presence of male genital tract derivatives and masculinization (Rota et al., 2002). This phenomenon occurs in 90% of female calves from pregnancies carrying twins of the opposite sex based on the degree of placental anastomoses (Schlafer DH and Foster RA, 2016).

Beyond males, AMH is also produced by the granulosa cells of pre-antral or early follicles on the ovary in cycling females (Rico et al., 2011). These cells surround the oocyte and proliferate during follicle maturation before being luteinized after ovulation (Turan et al., 2015). Thus, AMH is a product of those follicles that have undergone recruitment from the follicular pool. A dimeric glycoprotein, AMH is a member of the transforming growth factor β superfamily (Monniaux et al., 2013). This family of proteins is critical in regulation and mediation of cellular processes including proliferation, differentiation and apoptosis. (Kingsley, 1994). Following synthesis by granulosa cells, AMH is secreted into the blood stream.

AMH Receptors & AMH Level at the Follicle

AMH action requires the use of two receptors. AMH receptor type I (AMHR1) and AMH receptor type II (AMHR2) dimerize to initiate AMH signaling following AMH binding to the receptor complex. These AMH receptors are found in granulosa cells during follicle development and are found in greater quantities on smaller follicles (5 to 8 mm follicles) than larger follicles (13 to 24 mm follicles). This indicates that younger follicles produce more AMH than older follicles (Poole et al., 2016). Published data comparing subordinate and dominant follicles describes follicles that achieve dominance as expressing specifically more AMHR2 than AMHR1 when compared to subordinate

follicles. This suggests that AMHR2 expression during growth is critical for a follicle to achieve dominance (Iiha et al., 2014). The preferential expression of AMHR2 over AMHR1 is contributed to the use of AMHR1 as a receptor for other TGF-B family proteins in addition to AMH, while AMHR2 is specific to AMH only (Baarends et al., 1994).

After initial production begins at the early follicle/pre-antral follicular stage, AMH production and AMH mRNA expression gradually decrease over the course of a follicle's development (Rico et al., 2009). This is common for most species and can be seen in Figure 1.5 below. However, when comparing atretic and healthy follicles, healthy follicles have significantly higher levels of AMH at each stage of growth than atretic follicles, indicating that a threshold AMH level is still critical for follicle maturation even though overall AMH production declines with follicular age as seen in Figure 1.6 (Monniaux et al., 2013).

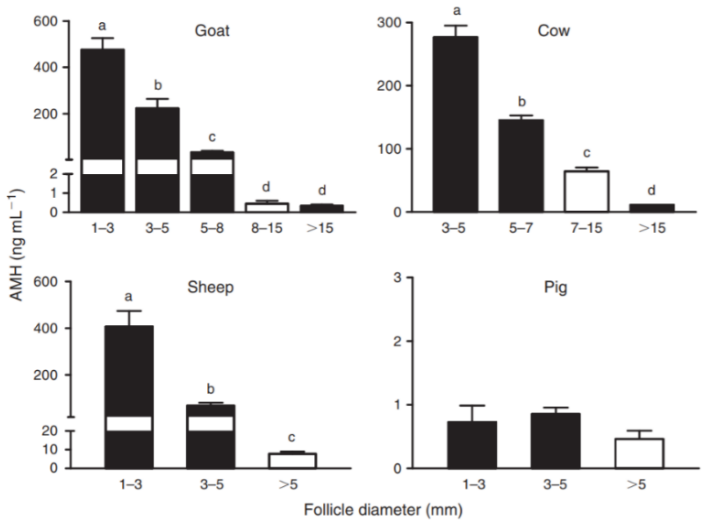

Figure 1.5: AMH production by follicle size for various species. The white bars indicate follicles of the preovulatory size class. The largest follicle in the goat and cow correspond to the size class of cyst (Rico et al., 2009).

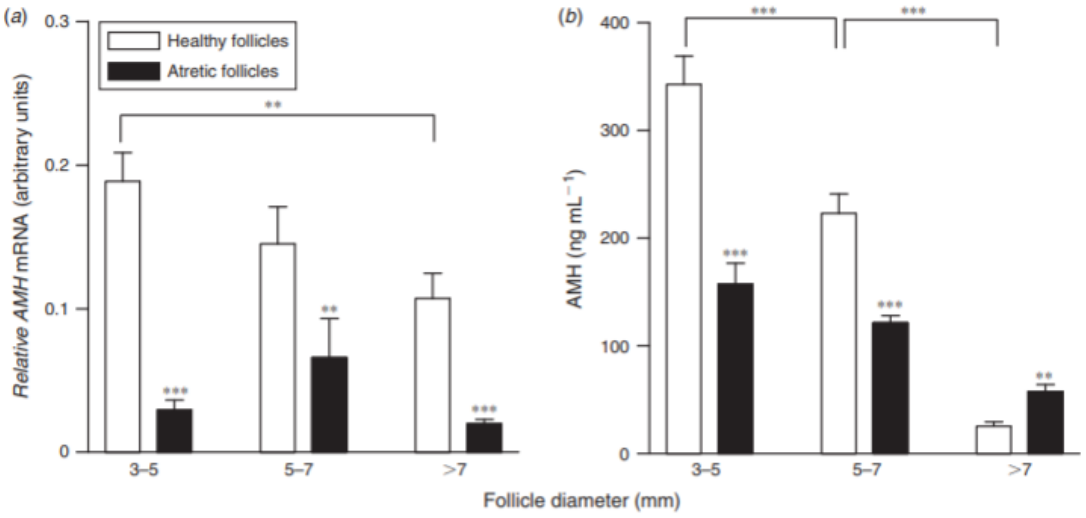

Figure 1.6: AMH production and AMH mRNA expression in various sized follicles from Holstein cows (Monniaux et al., 2013).

AMH Interaction with other Hormones & Impact at the Ovary

Research findings indicate that AMH has been shown to have an inhibitory effect on the recruitment of primordial follicles into the pool of growing follicles in species such as rats, mice and humans by decreasing the responsiveness of growing follicles to FSH (Durlinger et al., 1999; Durlinger et al, 2001; Durlinger et al., 2002; Carlsson et al., 2006). Durlinger's research also administered exogenous FSH to wild-type mice and mice that lack the gene for AMH production. Mice lacking the gene for AMH production had a higher rate of early follicular growth than in wild-type mice. This suggests that growing follicles are more responsive to FSH in the absence of AMH. Furthermore, that AMH could inhibit the sensitivity of preantral follicles to FSH and therefore negatively regulate follicular growth. Likewise, Gigli and collaborators (2005) found that primordial follicles did not initiate growth when bovine ovarian cortex tissue was exposed to AMH

in vitro. The suppression of primordial follicles to initiate growth by AMH is thought to be the body's way of preserving the follicular pool by only selecting a few follicles to grow at any time (Lebbe and Woodruff, 2013; Monniaux et al., 2013).

A negative effect by FSH on AMH has been described in in vitro bovine granulosa cells when the cells were treated with FSH. In follicles measuring 3 to 5 mm, AMH secretion was reduced by 50% following FSH administration. AMH gene expression was also inhibited in 5 to 10 mm follicles by ~30% after treatment with FSH. (Rico et al., 2011). These findings follow the observations seen both in vitro and in vivo in females of other species including humans, rats, mice and fish (Kuroda et al., 1990; Baarends et al., 1995; Pellatt et al., 2011).

Based on data from research in rats which have been shown to respond similarly to various hormones and extract similar responses from AMH, it is theorized that estradiol also plays a role in regulating AMH expression in granulosa cells (Baarends et al., 1995; Ikeda et al., 2002). However, the role is not fully understood and needs more investigation as initial studies have garnered conflicting data. An inverse relationship exists between AMH and aromatase expression and estradiol production, however, the mechanism for this relationship is not understood (Ireland et al., 2009; Monniaux et al., 2013)

AMH Profiles in Cattle

Immediately after birth, AMH levels in females calves are low. A 2013 study traced plasma AMH level in female crossbred beef calves after birth. Blood samples were pulled at age 7 weeks, 18 weeks, 35 weeks, 56 weeks and 86 weeks and tested for circulating AMH. AMH was highest at 7 weeks and decreased until 56 weeks of age.

From 56 weeks to 86 weeks however, AMH levels began increasing. These levels then drop off before climbing again as animals reach puberty.

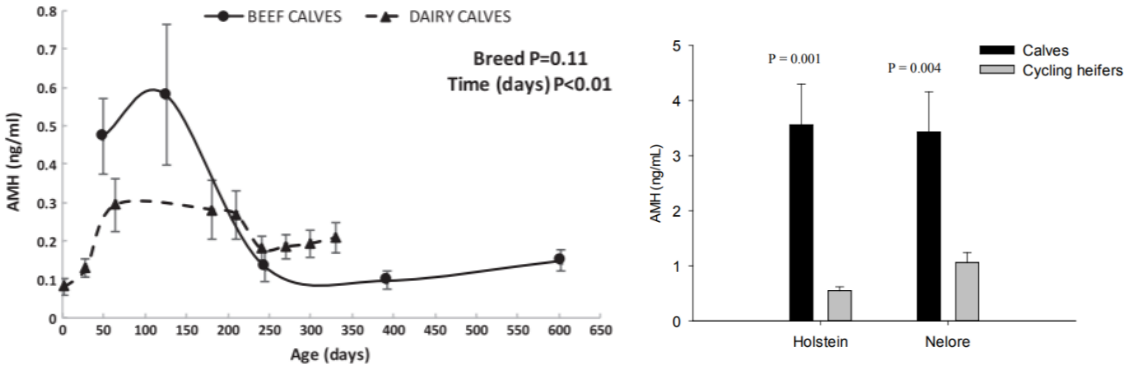

Figure 1.7 and 1.8: Figure 1.7 (left) shows the circulating AMH concentrations in dairy (n = 9_ and cross bred beef (n = 13) female calves (Mossa et al., 2013). Figure 1.8 (right) shows the plasma concentration in calves aged 2 – 4 months (n = 24 Holstein; n = 30 Nelore) and cycling heifers (n = 10 Holstein; n = 12 Nelore) (Baruselli et al., 2015).

Despite fluctuations in growing animals, after reaching puberty and beginning normal cyclicity patterns, published data indicates there is little variation within a cycle. A blood sample taken at any point of the estrous cycle in female cattle is indicative of accurate AMH level. This concept is evidenced in work by Souza et al, who found that circulating AMH was not affected by phase of the estrous cycle with 3 samples being pulled in estrous synchronized animals at a random stage (40 $\pm$ 3 DIM), proestrus (50 $\pm$ 3 DIM) and diestrus (57 $\pm$ 3 DIM). In addition, they found within-cow repeatability of circulating AMH to be 0.91 within cycles.

Few studies have described the impact of pregnancy and sex of offspring on an animal's AMH level. Monniaux and coworkers (2013) conducted a comprehensive study

following 21 Holstein heifers and cows through gestation and postpartum for two calving cycles. Their study saw that AMH levels increased during the first 3 months of gestation before declining until parturition. Post-partum, AMH levels rose slightly. This same trend was seen during the animal's second gestation and post-partum data collection period as described in Figure 1.9. A 2017 study found that the sex of the offspring played a role in the change in maternal AMH level during pregnancy. Maternal AMH level at day 35, day 135 and day 275 of gestation in 8 Zebu cows was analyzed. The 5 cows carrying male fetuses had a large plasma AMH level increase from day 35 to day 135 of gestation, while the 3 cows carrying female fetuses had a decreased plasma AMH level. For the male fetus carrying cows, the plasma AMH level increased on average 255.4 pg/ml from day 35 to day 135, while for the female fetus carrying cows, plasma AMH levels decreased on average 181.3 pg/ml. The study also looked at plasma AMH levels in 20 male and 19 female fetuses 54 to 220 days old and found that male fetuses had significantly higher plasma AMH when compared to female fetuses (193,561.6 $\pm$ 13,416.2 pg/ml vs. 147.1 $\pm$ 24.1 pg/mL). (Stojsin-Carter et al., 2017).

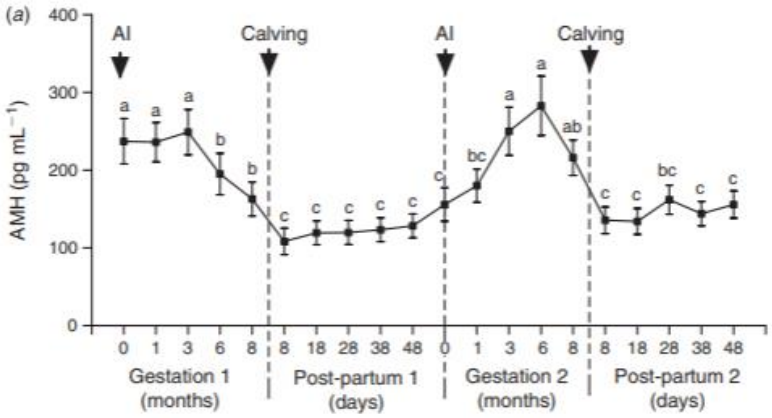

Figure 1.9: Plasma AMH level of Holstein cows and heifers during gestation and post-partum through two calving cycles. (Monniaux et al., 2013)

AMH and Antral Follicle Count (AFC)

Prior to AMH sampling, it was previously established that the Antral Follicle Count (AFC) of a mature animal is directly and positively correlated with the superstimulatory response in cows (Singh et al., 2004). Animals with a greater AFC have more follicles that can respond to a hyperstimulation protocol and will therefore ovulate more follicles. Published data also indicates that the follicles from high AFC animals are more fertile (Morotti et al., 2017). In addition, it has been determined that the number of follicles on the ovary 3 mm or greater in diameter is maintained during both the ovulatory and nonovulatory follicular waves of individual animals (Burns, et al., 2005). Thus, ultrasound determination of AFC has widely been used to determine optimal animals for hyperstimulation, with the potential for a large number of ovulated follicles, as it can be done at any point of the animal's estrous cycle with a great amount of accuracy and reliability. However, variations in ultrasound operators and operator-defined criteria for counting antral follicles has led the search for an equally reliable yet more user-friendly means of determining candidates for hyperstimulation (Souza et al., 2015).

Because AMH is produced by granulosa cells of antral follicles, it was theorized that circulating AMH levels could be indicative of AFC, and therefore, an animal's superstimulatory response. Multiple studies have since found that circulating blood AMH concentration is a reliable endocrine marker of the size of the antral follicle population, or essentially, the AFC (Ireland et al., 2011; Rico et al., 2011; Monniaux et al., 2013; Souza et al., 2015).

	Bos taurus (Holstein)	*Bos taurus* (Holstein)	*Bos indicus* (Nelore)	*Bos indicus* (Nelore)
AFP category	Low AFP	High AFP	Low AFP	High AFP
No. heifers	8	7	8	7
No. follicles	13.4 $\pm$ 1.4	34.3 $\pm$ 3.12	28.4 $\pm$ 2.15	48.1 $\pm$ 2.33
Plasmatic AMH (ng/ml)	0.06 $\pm$ 0.02	0.57 $\pm$ 0.26	0.78 $\pm$ 0.16	1.20 $\pm$ 0.16

Table 1.3: Comparison of Low and High Antral Follicle Population (AFP) groups and their respective plasma AMH levels. (Batista et al., 2014).

Beyond being a reliable predictor of AFC in cows, the collection process used to acquire a sample to determine AMH level has the capacity for less human error. Typically, blood is sampled by puncture of the coccygeal vein or artery into evacuated serum blood collection tubes and then immediately stored on ice and transported to a lab for processing. This method of sample collection is easy to train, fast and efficient, making it a viable option over ultrasonography (University of Queensland, 2016). There are a variety of bovine AMH ELISA tests available that can detect 0.5 ng/ml up to 2240 pg/ml, with a sensitivity of 0.04 pg/ml. These kits can be bought and processed in house, or samples can be shipped to a lab for processing.

AMH levels vary considerably from animal to animal and one study on 1,200 cows found that AMH levels ranged from 10 to 3,198 pg/mL, with the mean concentration being 320.3 $\pm$ 251.1 pg/mL. When these animals were grouped into the top 20% and bottom 20% based on AMH level, they found average AMH to be 85 pg/ml with a range of 10 to 140 pg/ml for the low group, and 631 pg/ml with a range of 451 to 3,198 pg/ml for the high group (Ribeiro et al., 2014). These were proposed to be the averages of truly “low” and “high” AMH animals. The intermediate group which

comprised 60% of the animals ranged from 141 to 450 pg/mL with an average of 263 pg/mL. However numerous other studies indicate normal ranges in AMH for any herd to be around 0.01 to 400 pg/ml, with few animals achieving levels over 400 pg/ml (Souza et al., 2015; Rico et al., 2009). Due to the wide variation in ranges from herd to herd, no hard benchmark AMH level has been established as of yet. Instead, animals are compared to their contemporary groups to give them a classification of high or low AMH.

Factors that Impact AMH Levels

Published data indicates that multiple factors including breed, lactation number, age, stage of life, and dam's milk production and environmental factors while gestating all impact an animals AMH level and contribute to the variability in AMH level described above. Published data indicates that AMH varies by breed with Bos indicus breeds having the highest AMH, followed by the Bos taurus breeds in the following order: Jerseys, Jersey/Holstein crossbreds, Holsteins (Figure 1.10) (Ribeiro et al., 2014; Batista et al., 2014). Bos indicus breeds also showed an elevated AFC, indicating that these breeds may be more fertile than the Bos taurus breeds. Beef breeds fall in between Bos indicus breeds and dairy breeds for AMH level (Jiminez et al., unpublished; Batista et al., 2014; Pfeiffer et al., 2014). Previous studies have shown that high milk producing cows have reduced fertility due to a variety of issues including poor expression of estrus and defects with the oocytes or embryo after fertilization. For this reason, some argue that the high demands of milk production play a role in depressing AMH in dairy cattle, but studies have not yet been done to isolate this effect (Dobson et al., 2009).

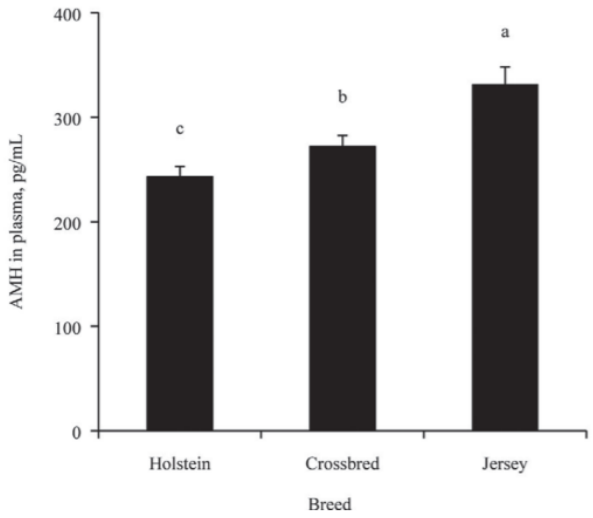

Figure 1.10: AMH levels by breed all statistically significant ($p \leq 0.05$) and bars with different letters are statistically significant from each other. (Ribeiro et al., 2014)

An effect of lactation number and age on AMH level has also been described. One study found that cows on their 2nd and 3rd lactation had greater AMH levels pre-breeding than those on their 1st or 4+ lactation (Ribeiro et al., 2014). Another study found similar results, when looking at 1st, 2nd, and 3rd lactation animals, with 3rd lactation animals having the highest circulating AMH. (Koizumi et al., 2017). However, a third study found conflicting results after tracking animals from heifers at 13-18 months throughout adulthood (up to 16 years), finding that overall, AMH decreased throughout the lifespan of the animals. (Hirayama et al., 2017).

Other factors such as maternal nutrition during pregnancy also impact AFC and AMH in offspring. Mossa and coworkers (2013) found that nutrient restriction shortly before conception to the end of the 1st trimester of pregnancy negatively impact offspring AFC and circulating AMH concentrations. When they compared the offspring from a

control group and a nutrient restricted group that was fed to only 60% of their maintenance energy requirements, researchers found that the birthweight of offspring was unaffected. However, calves born to the nutrient restricted mothers had a 60% lower AFC and therefore circulating AMH concentrations when compared with calves born to control mothers. Preliminary research on dam Somatic Cell Count, milk fat concentration and milk fat to protein ratio show an influence on daughter AFC that cannot yet be accurately described and has not yet been replicated, so it has not been included (Walsh et al., 2014; Evans et al., 2012).

AMH as a Marker of Fertility

As previously stated, published data elucidates a direct correlation between AMH and an animals Antral Follicle Count (AFC), and therefore the animal's superstimulatory response. While this information is useful for selecting animals to flush embryos from with a high rate of success, its practical application is otherwise not fully outlined or understood. Because of this, multiple researchers investigated whether a singular AMH sample can predict overall fertility of an animal. One study (Ribeiro et al., 2014) looked at a variety of parameters after enrolling cows to a pre-synch and ovsynch, followed by a period of heat detection. Any that came into heat in the next 19 to 35 days after the TAI were bred off heats before being placed with a Holstein and Jersey bull on day 35. Animals were then broken into a high (top 20%), middle (60%) and low (bottom 20%) group by AMH. They found that a higher percentage of animals from the low group exhibited estrus at the time of breeding to the TAI protocol (49.3% vs. 34.5%) (P <0.01), which authors theorized the negative association between AMH level and estrus at TAI was a result of the TAI protocol optimizing ovarian performance through forced follicular

development, luteal regression and ovulation. Furthermore, more of the low AMH animals became pregnant to this breeding than high AMH animals (37.4% vs. 34.8%) (P =0.58). During the period of estrous detection following the TAI protocol, more animals from the high AMH group exhibited estrus and were rebred than animals from the low group (20.8% vs. 7.3%) (P =0.17). In the scenarios where breeding was based on the animal's ability to naturally exhibit estrus, the high group performed better, whereas when ovulation was timed, the low group performed better. Finally, at the end of the breeding season, 88.7% of the high AMH group was pregnant compared 83.3% of the low AMH group ($P < 0.10$). In addition, pregnancy loss between d 30 and 65 of gestation was found to be 16.7% for the low group and 8.0% for the high group ($P \leq 0.05$). High AMH animals had lower pregnancy loss and low AMH animals needed the synchronization aids to achieve the same level of fertility as the high group. This information could change the way that producers manage their reproductive program on the farm.

Jimenez-Krassel and coworkers (2015) found that the concentration of AMH in dairy heifers was positively associated with their productive life as a member of the herd. After synchronizing estrus in Holstein heifers 11-15 months old (n=281), an AMH sample was pulled and heifers were later bred. These same heifers were tracked through pregnancy, calving, and into their subsequent lactations. They were broken into quartiles based on their AMH level and researchers found that Q1 animals (lowest AMH) completed fewer lactations when compared with Q3 cows. Q1 animals also had a significantly shorter productive herd life when compared to Q2 and Q3 (196 days less).

This information may point to AMH as a useful tool for predicting the success of heifers as cows in the herd and could be used to make early culling decisions for producers.

Heritability

In addition to its ability to predict reproductive success, superovulation ability and livability in the herd, AMH's heritability has also been studied indirectly. Studies have looked at the heritability of AFC and therefore AMH since these two parameters are directly correlated. Overall, heritability was found to be 0.31 $\pm$ 0.14 and 0.25 $\pm$ 0.13 for a group of Holstein dairy cows and heifers, respectively (Walsh et al., 2014). This is much higher than the heritability of other reproductive parameters in dairy cattle such as days open, calving interval, survival to 1st freshening, number of services, days in milk at 1st breeding and age at 1st cavling which have a heritability of .01 to 0.13 (Campos et al., 1994; Hermas et al., 1987; Jamrozik et al., 2005). This indicates that AFC and essentially AMH is a trait that can be selected for within cattle more reliably than other reproductive traits, but that it is also impacted by a variety of environmental factors as evidenced by the previous section. This information along with previous data showing a correlation between AMH and fertility indicates that producers could select for more fertile animals by selecting for AMH.

All of this data culminates the idea that AMH samples can be used for more than just determining a donor animal to flush for ET. Its use as a predictor of fertility, which includes a variety of specific reproductive parameters could change the management practices of producers when it comes to reproduction to achieve greater reproductive success, allowing a more profitable and efficient herd.

Objectives of the Study

The objectives of the upcoming study were to examine the relationship between circulating AMH level and reproductive success within the herd, as well as the impact of life events on AMH level. The first part of the study focuses on the timing of sampling to determine if stage of life or life events at sampling impact AMH level as well as describing changes in AMH level from pre-breeding heifers to lactating cows. Changes in AMH level will indicate the ideal sampling time and whether sampling a pre-breeding heifer for AMH is an accurate marker for lifetime AMH level. The second piece of the study was used to investigate whether high and low AMH animals can achieve greater pregnancy rates when assigned to different breeding strategies (TAI vs. estrous detection) by using AMH as an indicator of fertility.

CHAPTER 2

EVALUATING THE IMPACT OF LIFE EVENTS AND STAGE OF LIFE ON ANTI-MÜLLERIAN HORMONE IN DAIRY CATTLE

Alward, Kayla. To be submitted to *Translational Animal Science.*

Abstract

The objective of this study was to examine the impact of life events and stage of life at sampling on circulating Anti-Müllerian Hormone concentration in Holstein heifers. Virgin, Holstein heifers (n=105) of breeding age (13 $\pm$ 0.8 months) were enrolled prior to first service in the trial. Animals were heat detected using tail-chalk and bred via artificial insemination and pregnancy checked at 32+ days. Serum samples for AMH were collected at three time points: upon enrollment (heifer), at 5-20 days in milk (fresh) and at 45-60 days in milk (pre-breeding). Transrectal ultrasonography was performed upon enrollment (heifer) and at 45-60 days in milk (pre-breeding) to determine antral follicle count (AFC), cyclicity status, and uterine health. Heifers were blocked into a top, middle and bottom third by AMH concentration. LOW (<183 pg/mL; n=36), MID (183-354 pg/mL; n=35) and HIGH (>354 pg/mL; n=34) groupings. Data were analyzed with the PROC GLIMMIX procedure of SAS. As heifers, age at 1st service and conception risk to first service were not impacted by AMH concentration (P>0.05). Reason for leaving the herd, health incidences, sex of offspring and calving difficulty were also not impacted by AMH concentration (P>0.05). AFC and cyclicity had a positive impact on heifer AMH concentration (P<0.01). Total AFC for heifers differed by AMH group with the HIGH group having the most follicles (8.76), followed by the MID (5.87) and then the LOW (3.53) group (P<0.0001). This confirms previous studies that AFC is directly correlated with circulating AMH concentration. However, AFC was not different by AMH group pre-breeding (P>0.05). From the heifer sample to the fresh sample, average AMH concentration dropped from 313.15 pg/mL to 160.01 pg/mL (P<0.0001). Average AMH concentration at the pre-breeding sample was 183.23 pg/mL, which was lower than the

heifer sample ($P<0.0001$), but not different from the fresh sample ($P>0.05$). AFC and AMH at the heifer sample had a positive impact on AMH at the fresh sample ($P<0.01$). Pre-breeding AMH was positively impacted by both the fresh and heifer AMH concentration ($P<0.001$). Most animals kept their AMH categorization as HIGH, MID or LOW through all two time points with more of the LOW AMH animals maintaining their categorization than the other groups. Although no differences were seen in circulating AMH concentration based on health events, differences in AMH concentration across three time points indicate a drop in circulating AMH concentration post-calving.

Introduction

Identifying heifers that will survive in the herd after calving and perform well as a lactating cow is a challenge that many producers face. On average, producers will spend just over $2,200 to raise or buy a replacement heifer (Tranel, 2017). However, producers will not see a return on their investment until heifers enter the lactating herd (Gardner et al., 1988). Therefore, it is in a producer's best interest to aim to invest only in those animals that will become pregnant, survive calving, and have a longer productive life in order to increase the producer's return on investment of each animal (Tozer and Heinrichs, 2001). With net income increasing by approximately $500 - $1,000 after the first lactation a dairy cow completes, maintaining an animal in the herd longer is more profitable (DeVries, 2013).

Heritability estimates of productive life are very low (VanRaden et al., 2006) and therefore not a reliable indicator. Reliable biomarkers predictive of productive herd life and survivability in the herd have not yet been well researched in dairy cows. To identify other indicators of productive life, examining the reasons dairy animals are culled from

the herd is useful. According to the national 2014 United States Department of Agriculture National Animal Health Monitoring System (NAHMS) study, the number one reason for removing animals from the herd was infertility (21.2%) followed by poor production (21.1%), mastitis (16.5%), animals sold as dairy replacements (9.5%) and lameness (7.2%) making up for 75% of the culling reasons. With fertility accounting for more than 20% of dairy culls, selecting fertile animals greatly impacts a producer's profitability and the herd's overall productive herd life.

Thus far there has been no reliable and consistent test for an animal's fertility. However, recent research indicates that inherent fertility of animals may be measured with a simple Anti-Müllerian Hormone (AMH) assessment and serve as a biomarker of fertility and therefore productive herd life and survivability as well. AMH is produced by follicles on the ovary and is secreted into the blood stream (Rico et al., 2011). A simple blood sample can be pulled at any point during a cycling animal's estrous cycle as a reliable predictor of that animal's AMH level (Ireland et al., 2008; Rico et al., 2009, 2011). Recent work by Jimenez-Krassel and coworkers (2015) indicates a positive correlation between AMH and productive herd life in heifers. In this study, heifers were sampled for AMH pre-breeding and broken into quartiles and followed through life. Q1 animals had the lowest AMH concentrations with Q4 being the highest. Q1 animals completed fewer lactations, had a shorter productive life, and a higher probability of being culled after the birth of their first calf compared to Q3 cows, and a tendency to perform better when compared to Q4 cows.

This data suggests that AMH could be a reliable tool for identifying heifers that will have a longer productive life. With this knowledge, producers would be able to

increase their profits by investing in animals that will survive in the herd, while potentially culling those that would have a shorter productive life.

However, to date, there is little published data describing AMH concentration over the entire course of a single animal's life, and any effects of various life events on AMH concentration. One study (Monniaux et al, 2013) following animals through gestation, calving and 48 days post-partum through two calving cycles saw a drop in circulating AMH during the 2nd trimester and through calving, before rising again post-calving. However, this study did not record any health events of animals in relation to circulating AMH level and no other published data exists elucidating the impact of various life and health events on circulating AMH. One study which profiled circulating AMH in animals aged 2 months to 200 months, but without tracking AMH on any single animal saw a negative correlation between age and AMH level ($r = -0.082$, $P > 0.05$) (Hirayama et al., 2017). However, other studies found a positive correlation between age and lactation number with AMH (Ribeiro et al., 2014; Koizumi et al., 2017). Without knowing how AMH fluctuates in the long-term, or how life events impact AMH level, a recommendation on ideal sampling time or ensure that a sample taken at any point in time is representative of that animal's true AMH level. These data and remaining questions about AMH led to the development of the following study.

The objectives of this study were to 1) determine the impact of stage of life on circulating AMH levels, 2) establish if circulating AMH levels are impacted by life events such as dystocia or metabolic disorders as well as milk production level, and 3) to investigate the value of AMH from a single animal at different time points to evaluate changes over time and repeatability. It was hypothesized that heifers would have the

highest circulating AMH level, which would be depressed post-calving, specifically in animals that had severe negative health events, but that this AMH level would rise and remain steady after remediation. It was also theorized that heifers with high circulating AMH levels would remain higher post-calving and pre-breeding when compared to heifers with low circulating AMH levels.

Materials and Methods

Animal Management

Virgin, Holstein heifers (n=105) 12 to 15 months of age (13 $\pm$ 0.8 months) were selected for enrollment in the trial. The heifers were born and housed on a commercial dairy operation in Madison, Georgia milking ~1,100 100% Holstein cows with an RHA of 31,364 lbs. The heifers were housed on pasture and fed a heifer TMR designed to meet their maintenance and growth requirements. After meeting age and weight requirements, heifers were moved into the breeding pen and enrolled in the trial. As per farm protocol, heifers were then tail-chalked and checked for heats once per day. Heifers with most (>50%) of the tail chalked were determined to be in heat and immediately bred. All animals were bred via artificial insemination by two experienced inseminators employed by the farm. All animals' 1st and 2nd service were bred to sexed semen, with subsequent services to conventional semen. All heifers were checked for pregnancy via palpation at 32+ days after breeding. Number of breedings and pregnancy outcome was recorded for each animal.

Ultrasonography Data Collection

Transrectal ultrasonography was performed at two different time points: when heifers were moved into the breeding pen (heifer) and at 45-60 days in milk after calving

(pre-breeding). Transrectal ultrasonography was performed to ensure cyclicity and assess reproductive health with the following observations recorded: antral follicle count (AFC), presence and number of corpora lutea, and any uterine or ovarian abnormalities. Any animals with a reproductive anomalies were excluded from the study.

Blood Collection for AMH

Blood was collected for AMH analysis at three different time points: when heifers were moved into the breeding pen (heifer), at 5-20 days in milk after calving (fresh) and at 45-60 days in milk (pre-breeding). Blood was collected via coccygeal venipuncture into vacutainer tubes containing no additives for AMH analysis.

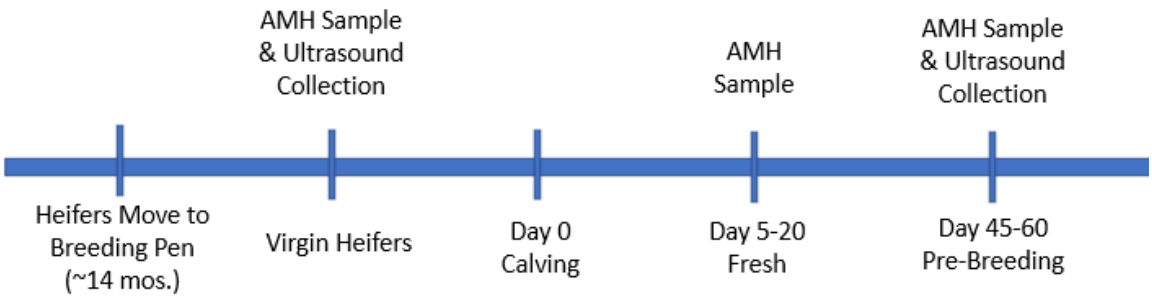

Figure 2.1: Experimental timeline of events

Blood Processing, Storage and Hormone Analysis

After collection, samples were immediately placed and maintained on ice and transported to the lab for processing within 2 hours of collection. Samples were centrifuged at 3400 rpm for 15 minutes to separate serum. Serum was then extracted and transferred to storage tubes in duplicate labeled with animal number and sampling date. Samples were frozen at -20 C for future analysis. Frozen serum was shipped to and analyzed for AMH by Ansh Labs (Webster, TX) utilizing their developed enzyme-linked

immunosorbent assay for AMH analysis specific to bovines which has been validated for Holsteins and detects a range of 11 to 2240 pg/mL using 50 µL of blood serum.

Statistical Analysis

Data were analyzed with the PROC GLIMMIX procedure of SAS 9.4 (Cary, NC). Variables incorporated into the model included age, days in milk, number of follicles and CLs present at sampling and seven-day average milk-weight on the sampling day. AMH concentration, AMH categorization, number of times bred, conception rate, reason for leaving the herd, sex of offspring and health events were also evaluated. Pregnancy status to 1st service conception was run as a binomial and services per conception was run with a poisson distribution. The gamma distribution was used for the heifer, fresh and pre-breeding AMH concentration as well as to analyze AMH concentration over time. Correlations were run for all associated variables. Heifers were blocked by top, middle and bottom third into a LOW (<183 pg/mL), MID (183-354 pg/mL) and HIGH (>354 pg/mL) AMH group based on the distribution of AMH concentrations at the heifer sample for analysis. For analysis of AMH level at the fresh and pre-breeding sample, categories were reassigned based on the distribution of AMH concentration at these time points in the same manner described above. Animal number and AMH level by time point can be seen in Table 2.1.

Table 2.1: AMH categorization by sampling time point with animal number by level.

	n	Low	n	Mid	n	High
Heifer	36	≤ 183 pg/mL	35	196-354 pg/mL	34	≥ 358 pg/mL
Fresh	27	≤ 98 pg/mL	29	112-169 pg/mL	26	≥ 173 pg/mL
Pre-Breeding	18	≤ 123 pg/mL	19	124-194 pg/mL	19	≥ 197 pg/mL

Results

Circulating AMH Over Time

The distribution of AMH concentrations for the heifer (Figure 2.2), fresh (Figure 2.3) and pre-breeding (Figure 2.4) samples are displayed below.

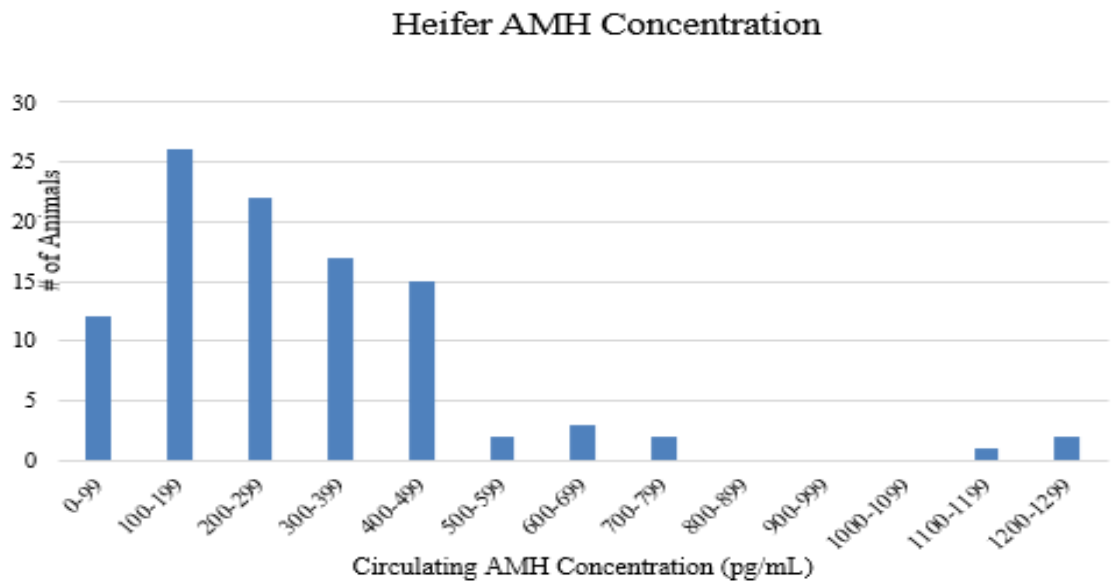

Figure 2.2: Distribution of animals by heifer AMH concentrations.

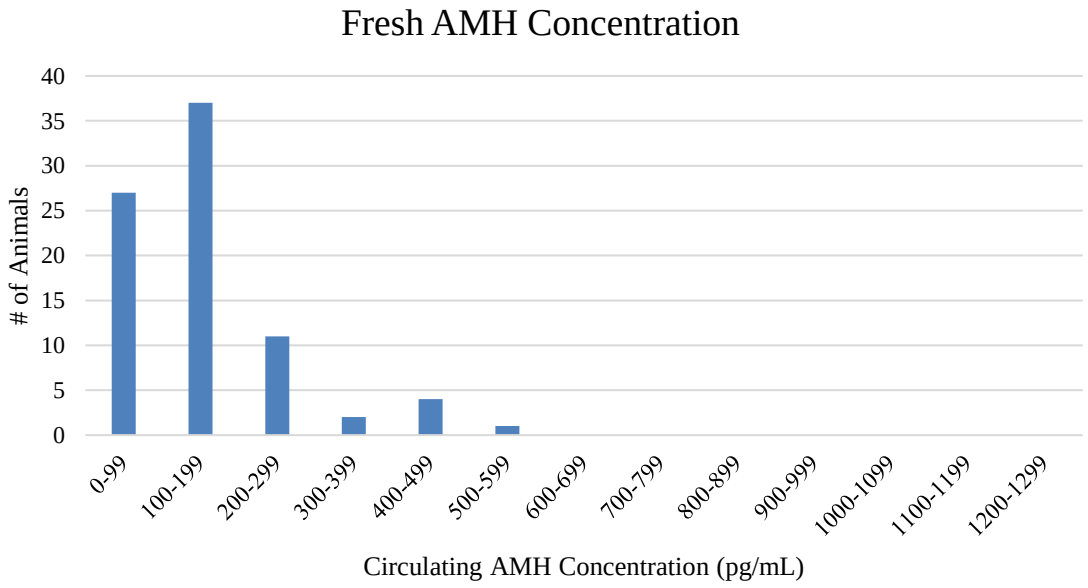

Figure 2.3: Distribution of animals by fresh AMH concentrations.

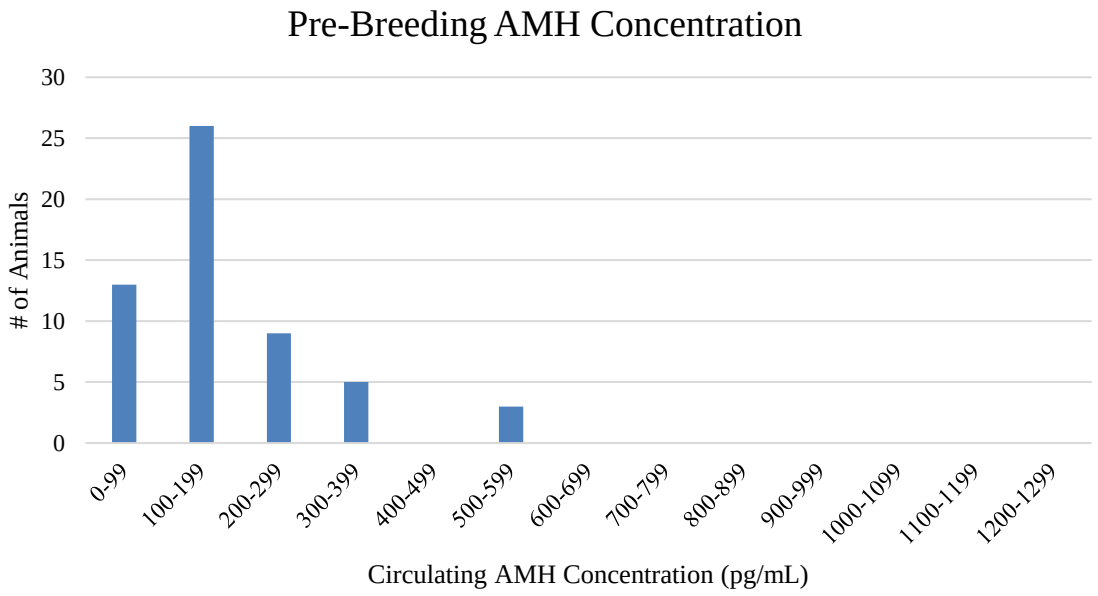

Figure 2.4: Distribution of animals by pre-breeding AMH concentrations.

AMH concentration was highly correlated across all sampling times (P<0.0001), with a significant drop seen in AMH concentration from heifer to fresh (P<0.0001). AMH remained depressed to the pre-breeding sample (P<0.0001), as seen in Figure 2.5. The average AMH concentration for the animals at the three different time points were 313 pg/mL (heifer), 160 pg/mL (fresh) and 183 pg/mL (pre-breeding). Correlation coefficients and p-values for these parameters are displayed in Table 2.2, while graphs show the correlations between heifer and fresh AMH (Figure 2.6), fresh and pre-breeding AMH (Figure 2.7) and heifer and pre-breeding AMH (Figure 2.8). Numerically, animals generally maintained their AMH level categorization over time despite seeing a drop in AMH concentration post-calving. More animals from the LOW group maintained their AMH level categorization across all three time points when compared with the MID or HIGH group numerically (Table 2.3).

Table 2.2: Correlation coefficients and p-values are displayed for the correlations between AMH concentrations.

	Heifer AMH	Fresh AMH	Pre-Breeding AMH
Heifer AMH	1.0	0.71774 <0.0001	0.64095 <0.0001
Fresh AMH	0.71774 <0.0001	1.0	0.83188 <0.0001
Pre-Breeding AMH	0.64095 <0.0001	0.83188 <0.0001	1.0

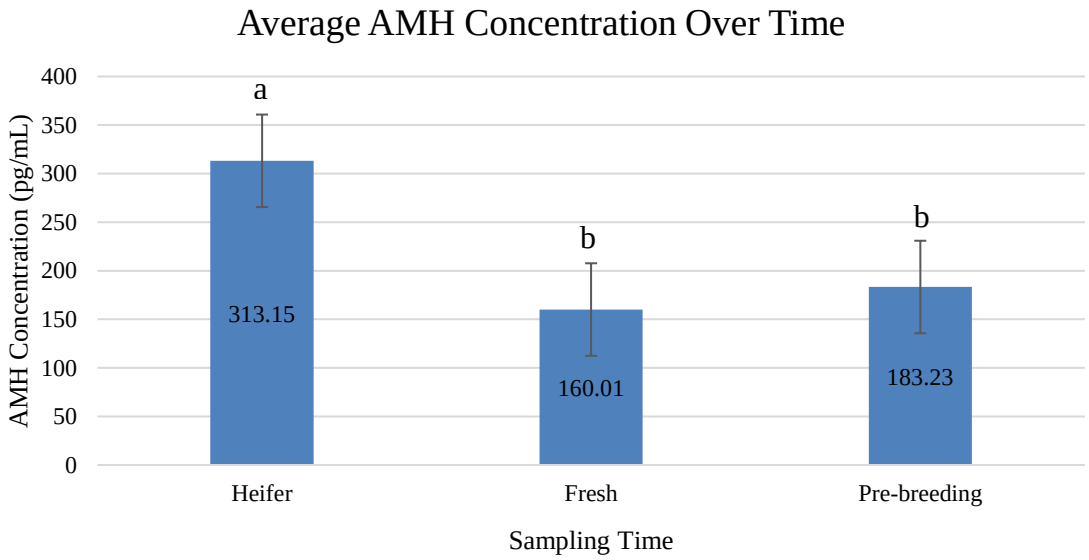

Figure 2.5: Average AMH concentration based on sampling time. Bars with different letters are different (P<0.0001).

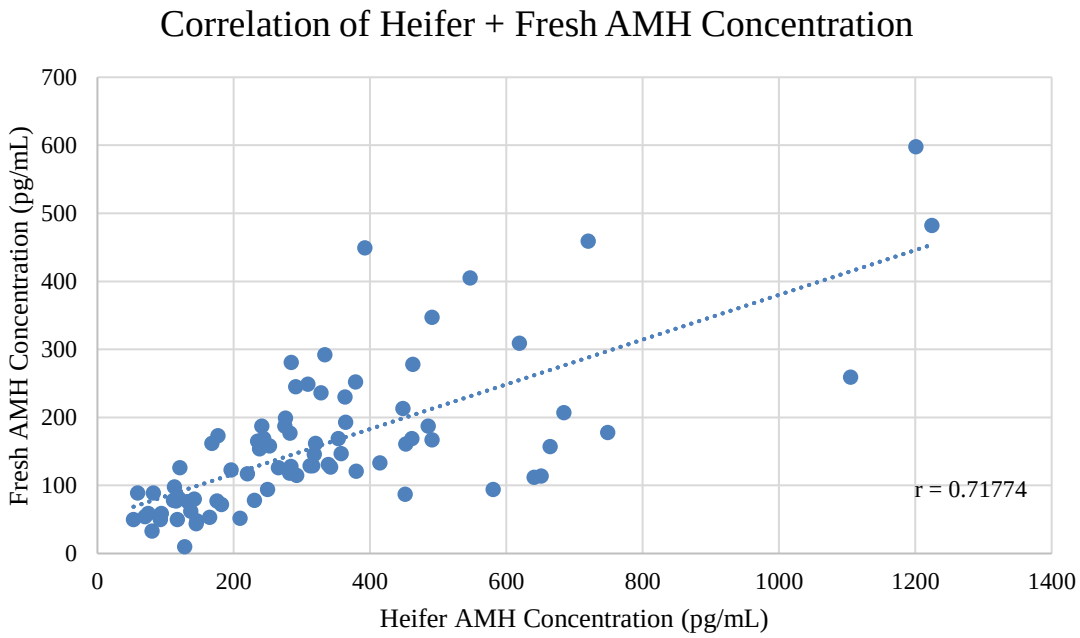

Figure 2.6: Correlation of AMH concentration from the heifer and fresh sample.

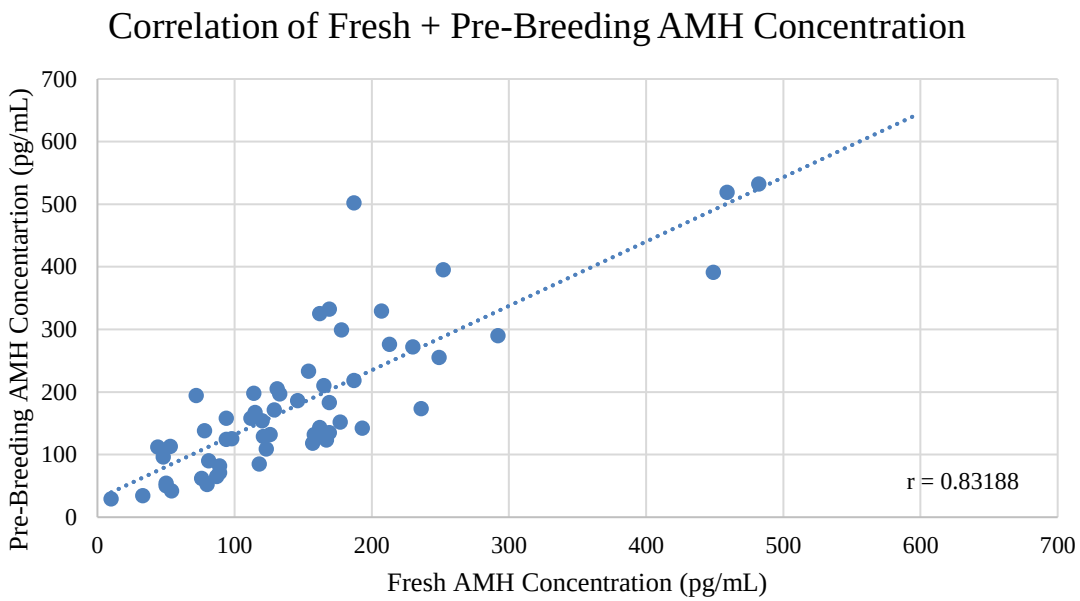

Figure 2.7: Correlation of AMH concentration from the fresh and pre-breeding sample.

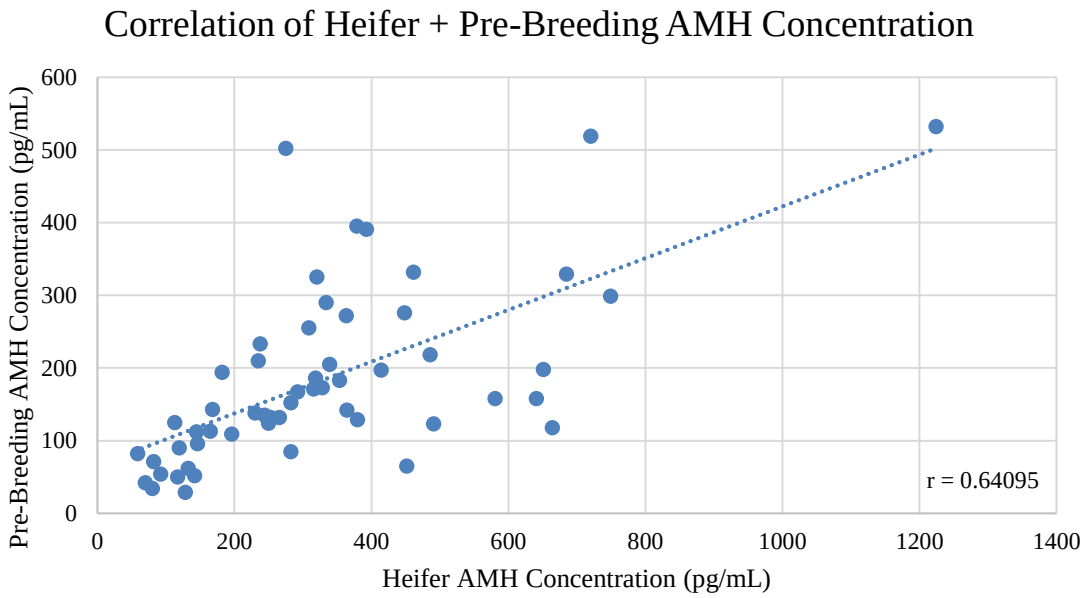

Figure 2.8: Correlation of AMH concentration from the heifer and pre-breeding sample.

Table 2.3: Retention of AMH level categorization from the heifer to fresh sample, the heifer to pre-breeding sample, and when looking at all 3 time points over time for the different AMH groups

	Heifer to Fresh	Heifer to Pre-Breeding	All 3 Time Points
LOW	88%	81.25%	81.25%
MID	58.62%	55%	35%
HIGH	59.26%	63.16%	47.37%

Health Events and Culling

An incidence rate of 12.2% was seen for health events of the 86 animals that calved. There was no impact of various health events, calving difficulty, or sex of offspring on circulating AMH level (P>0.05). At the conclusion of the study, 64.71% of the HIGH AMH heifers remained in the herd, 63.14% of the MID heifers remained and 54.3% of the LOW AMH group remained in the herd. However, culling rates nor reason

for leaving the herd were different between the AMH groups for animals that left the herd pre or post-calving (P>0.05).

AFC, AMH and Cyclicity as Heifers

AFC at the heifer sample was positively correlated with heifer AMH concentration (P<0.0001) as seen in Figure 2.9. No other variables impacted AFC and AMH and no associations between any parameters and cyclicity were found (P>0.05).

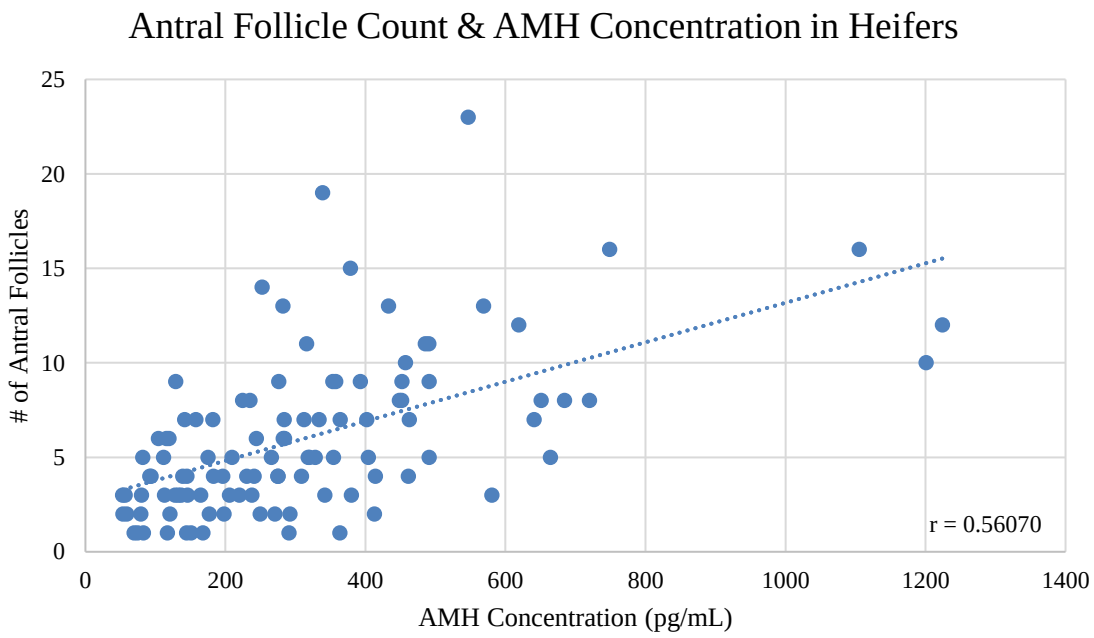

Figure 2.9: Graphic representation of AMH concentration and AFC for heifers

Breeding Parameters

As heifers, conception risk to 1st service and services per conception was not impacted by AMH concentration or categorization (P>0.05). Age at 1st service, number of CLs present, cyclicity status and AFC also did not impact conception risk or services

per conception (P>0.05). Age at 1st service, 1st service conception risk and services per conception by AMH group are displayed in Table 2.4.

Table 2.4: Breeding parameters by AMH level in heifers. Age at 1st service is shown in months.

		LOW		MID		HIGH
Parameter	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
Age 1st Service	36	14.2 ± 0.9	35	13.86 ± 0.69	34	13.79 ± 1.0
1st Service Conception Risk	36	25.00% ± 4.4	35	40.00% ± 5.0	34	41.17% ± 5.0
Services per Conception	33	2.3 ± 1.16	34	2.06 ± 1.1	34	1.91 ± 1.0

AFC, AMH and Cyclicity Post-Calving

Post-calving, days in milk nor age at either sampling-time impacted circulating AMH concentration (P>0.05). Neither the fresh nor pre-breeding AMH concentration was impacted by number of CLs, cyclicity status or seven-day average milk-weight on the day of sampling (P>0.05). A positive impact was found with AMH concentration as a heifer on AMH concentration at the fresh sample (P<0.0001). AMH concentration at both the heifer and fresh sample had a positive impact on AMH concentration at the pre-breeding sample (P=0.0006; P<0.0001). Together, AMH concentration at the heifer and fresh sample interact to have a negative impact on AMH concentration at the pre-breeding sample (P=0.0028). Means for post-calving parameters are depicted in Table 2.5, while regression coefficients and p-values for the variables impacting fresh AMH and pre-breeding AMH concentration are depicted in Table 2.6.

Table 2.5: Ovarian and cyclicity parameters post-calving by AMH group.

		LOW		MID		HIGH
Parameter	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
AFC	18	7.89 ± 2.8	19	8.53 ± 2.6	19	8.42 ± 4.6
# of CLs Present	18	0.44 ± 0.5	19	0.42 ± 0.61	19	0.42 ± 0.50
% of Animals Cycling	18	44.44% ± 0.51	19	36.84% ± 0.50	19	42.11% ± 0.60

Table 2.6: Regression coefficients and P-values for post-calving parameters.

Parameter	Regression Coefficient	P-value
Heifer AMH + Fresh AMH	0.002871	<0.0001
Heifer AMH + Pre-Breeding AMH	0.001716	0.0006
Fresh AMH + Pre-Breeding AMH	0.006563	<0.0001
Heifer AMH*Fresh AMH + Pre-Breeding AMH	-0.00000481	0.0028

Discussion

This data suggests that AMH is depressed significantly at 5-20 days post-calving and remains depressed compared to pre-calving concentrations at 45-60 days in milk. Numerically, the AMH concentration of the pre-breeding sample was slightly higher than the fresh sample. This may indicate that AMH is only temporarily depressed post-calving and begins making a recovery around 45-60 days in milk. Previous work by Monniaux et al (2013) represented a similar temporary depression in circulating AMH concentration during the last trimester of gestation and into the early post-calving period before a slight increase in AMH concentration towards 48 days post-calving. However, the rise in AMH after the fresh period was not elucidated in this study. While AMH concentration did drop significantly post-calving, animals seem to maintain their categorization of HIGH, MID, or LOW from pre-breeding as a heifer, through calving and into the 45-60 days

post-calving period. Specifically, this study saw that the LOW AMH animals remained in the LOW categorization throughout the different sampling times. The MID and HIGH group changed AMH categorization more often and were less predictable.

This study also shows that the drop in AMH post-calving was not related to negative health incidences, as the drop in AMH post-calving was seen in 95.3% of the heifers, despite only 12.2% experiencing negative health events. This drop could be a result of depressed immune function and increased metabolic stress which is a normal post-calving response (Wathers, 2010; Goff; 2008; Goff, 2006). This theory would explain Monniaux's work where the AMH depression post-calving was temporary. As animals recover from the stress of calving and move beyond the extreme negative energy balance period associated with the early periparturient period, AMH concentration would recover. Further studies following animals later into lactation and tracking AMH concentration would elucidate the duration of this depression and whether calving permanently or temporarily lowers AMH concentration. Evaluating cyclicity of these animals at longer days in milk would also elucidate whether animals begin cycling later as a result of the stressors of calving for the first time In addition, a larger sample size to allow for more health incidences are needed to rule out the theory that negative health events cause a greater depression in AMH than those animals that do not experience any negative health events.

It is important to note that during this trial, the commercial dairy operation saw higher than usual culling rates in an attempt to stay under quota with their milk processor. This elevated culling led to decreased animal numbers but garnered no significant association between culling and AMH level or reason for leaving and other factors as

most animals were sold as voluntary dairy culls. Therefore, the lack of relationship between culling reason and productive life between AMH groups is likely explained, but still inconsistent with previous findings. Recent work by Jimenez-Krassel and coworkers (2015) described a positive relationship between AMH concentration and productive life in heifers, with the bottom quartile of animals based on AMH concentration completing the fewest lactations and having the shortest productive life ($P<0.02$). Animals that did survive in the herd longer had delayed days to second and third calving when compared with animals from the other quartiles. Interestingly, a second study by Jiminez-Krassel et al (2017) saw that animals with a high AFC (>25 follicles) had a 180-day shorter productive herd life ($P<0.02$). One major difference between this study and the present described is the AFC. Only one animal from the 105 heifers in this study had an AFC above 20. This may indicate that animals on the extreme high end have a shorter productive life as evidenced by Jimenez-Krassel's 2017 work, but those in a more normal-upper range have a longer productive life as evidenced by previous work (2015).

This study saw no relationship between AMH concentration and reproductive success in the heifers. This conflicts with Jimenez-Krassel's 2017 work which showed animals with higher AMH concentration having a higher pregnancy rate. However, this study's findings could be explained by the farm's exceptional reproductive management program with the heifers, which may have allowed lower AMH concentration animals to achieve greater pregnancy rates that were not different from the mid or high AMH concentration animals.

The correlation between AMH level and AFC in heifers is consistent with previous research (Ireland et al., 2011; Rico et al., 2011; Monniaux et al., 2013; Souza et

al., 2015). However, the lack of correlation between AMH level and AFC in cows is inconsistent. There was a numerical difference although not statistically significant, which could be attributed to reduced sample size of the animal population. This lack of relationship could also be attributed to stress post-calving, as a greater percentage of these animals were not cycling (evidenced by no CL present on the ovary) compared to multiparous animals at the 45-60 days in milk ultrasound sample. In working with this herd, there appears to be an anomaly that seems when 1st lactation animals reach a 90-pound threshold of milk production, they are less likely to be cycling. This could further indicate the presence of metabolic stress and explain depressed AMH concentration post-calving.

The correlation between AMH concentration at the heifer sample, fresh sample and pre-breeding sample demonstrate the repeatability and consistency of AMH concentration at different points during an animal's life. While we saw no differences in reproductive performance or productive life as seen by Jimenez Krassel and collaborators (2015), the repeatability findings are consistent with multiple other studies (Monniaux et al., 2013; Souza et al., 2015) that have looked at AMH concentration during different time points within animals. Similarly, AMH concentration at the heifer and fresh sample positively impact AMH concentration at the pre-breeding sample. Coupled with the LOW AMH animals remaining low across sampling times, this indicates a heifer AMH sample as a reliable predictor of future AMH concentration and an identifier specifically of animals that will remain in the LOW AMH category post-calving and most likely have a shorter productive life.

CHAPTER 3

EVALUATING ANTI-MÜLLERIAN HORMONE AS A REPRODUCTIVE TOOL IN LACTATING DAIRY COWS

Alward, Kayla. To be submitted to *Translational Animal Science.*

Abstract

To examine the reproductive performance of animals based on variations in breeding programs and Anti-Müllerian Hormone (AMH) concentrations, primiparous and multiparous (n=308) purebred, lactating Holstein cows were enrolled after calving. At 45-60 days in milk (DIM) blood was pulled and analyzed for AMH concentration and transrectal ultrasonography was performed to record antral follicle count (AFC), presence of corpora lutea (CL) and cyclicity status, and any uterine or ovarian anomalies. Animals were then randomly assigned to either an estrous detection (n=155) or a timed artificial insemination (TAI) (n=98) breeding protocol. First service conception rate, days in milk at breeding, as well as 7-day average milk-weight on the day of sampling and breeding were recorded. Animals were blocked by AMH concentration into HIGH (>272 pg/mL; n=103) MID (158-272; n=102) and LOW (<158 pg/mL; n=103) groupings. Data were analyzed with the PROC GLIMMIX procedure of SAS. AMH concentration was positively correlated with AFC, lactation number, age and milk-weights ($P<0.001$). Conception risk to first service was not impacted by breeding protocol, AMH category or DIM ($P>0.05$); however, a numerical difference in conception risk by AMH level was seen with HIGH animal's having a 39.7% conception risk, MID animals being 40.2% and LOW animals achieving a 28.8% risk. AMH concentration for animals conceiving to 1st service averaged 276.82 $\pm$ 195.20 pg/mL while AMH concentration for open animals following 1st service averaged 245.35 + 152.75 pg/mL. As lactation number increased, so did the likelihood that animals were bred on an estrous detection protocol vs. the TAI protocol ($P=0.0018$) Cyclicity was positively correlated with lactation number ($P<0.0001$). Though conception risk to first service was not impacted by AMH

concentration, this study does potentially elucidate more information regarding variables correlated with AMH that were previously undescribed.

Introduction

As the demands for milk production increase, the industry has observed a negative impact on fertility in dairy cattle that is forcing producers and researchers to redefine what is considered "normal" for a variety of reproductive parameters (Walsh et al., 2011). High milk production inevitably has deleterious effects on fertility and reproductive management. Higher rates of blood circulation due to increased feed intake of high producing animals causes elevated depletion of steroid hormones, which results in decreased estrus expression, delayed ovulation and therefore ovulation of an older, less fertile follicle. This mechanism also results in multiple ovulations and twinning in cattle due to increased clearance of P_4 from circulation and removal of P_4's suppressive action on the follicular pool (Lopez et al., 2004; Wiltbank et al., 2006). In addition, negative energy balance associated with high milk production in fresh animals results in a prolonged anovulatory period and increased days to first service (Butler et al., 1981). In these negative energy balance animals, circulating blood glucose concentration is depressed which also decreases insulin response and IGF1 levels. With insulin and IGF1 responsible for controlling the activity of LH and FSH receptors on the ovary, these depressed levels are theorized to decrease the ovary's receptiveness to LH and FSH and therefore delay ovulation (Lucy et al., 2013; Lucy et al., 2014; Butler et al., 2003). Overall, these factors lead to altered cyclicity parameters. However, there are a number of tools utilized by high producing herds that allow them to also be reproductively efficient. These tools include the utilization of synchronization protocols, activity monitors,

embryo transfer (ET) as well as several other Assisted Reproductive Technologies (ART) (Saint-Dizier and Chastant-Maillard, 2012 and Lucy et al., 2004). Together these technologies can synchronize estrus and ovulation, detect animals in heat from slight activity changes, and increase overall reproductive efficiency. However, even with these technologies, there is still room for improvement and a need for new reproductive technologies that can combat fertility issues present in today's high producing herds.

Much like activity monitors identify animals in estrus that would be missed via visual heat detection, Anti-Müllerian Hormone (AMH) can be used to tailor a reproductive program to achieve greater reproductive success for an animal that we otherwise would not know how to achieve. AMH is produced by follicles on the ovary and is secreted into the blood stream (Rico et al., 2011). Previous works have established that AMH is directly and positively correlated with Antral Follicle Count (AFC) and the superstimulatory response in cows, with high AMH animals ovulating more follicles than low AMH animals in response to a superovulation protocol (Singh et al., 2004; Ireland et al., 2011; Rico et al., 2011). Further, published data has shown that a blood sample taken at any point of the estrous cycle in female cattle is a reliable measurement of overall AMH level and AFC, making it a more reliable and accessible form of measuring AFC than ultrasonography which requires a skillset and has a high level of variability between operators (Souza et al., 2015). AMH has thus far been utilized as a tool to identify animals that will respond well to superovulation protocols for embryo transfer (ET). Braganca and coworkers (2014) reported higher pregnancy rates to ET for high AMH vs. low AMH animals. Recent research indicates that AMH may prove useful beyond ET and also serve as a general marker for fertility in an animal.

Work by Ribeiro and collaborators (2014) suggest differences in the reproductive performance of cows by AMH level. In this study, blood was pulled and analyzed for AMH during the synchronization protocol. Later cows were bred to timed artificial insemination (TAI; pre-synch + ovsynch) with animals returning to estrus 19-35 days later bred again, before all animals were then placed with bulls on day 35. Pregnancy rates to each breeding were compared at the end of the breeding season. When comparing the high AMH (top 20%) and low AMH (bottom 20%) animals, low AMH animals had a greater incidence of pregnancy loss between d 30 and 65 of gestation ($P < 0.01$). Moreover, cows that failed to become pregnant by the end of the breeding season had lower concentrations of AMH in plasma than cows that became pregnant. Interestingly, a higher number of low AMH animals exhibited estrus at TAI than high AMH animals ($P < 0.01$) even though pregnancy risk to the TAI were not different between the groups. The authors theorized that the negative association between AMH level and estrus at TAI was likely a result of the TAI protocol optimizing ovarian performance through forced follicular development, luteal regression and ovulation. These data indicate that low AMH animals needed the synchronization aids to achieve the same level of fertility as the high AMH animals. This may indicate that reproductive factors and general fertility differs between high and low AMH animals and they can potentially be managed differently.

Other studies have confirmed this finding, determining no association between AFC (and essentially AMH) with conception rates after a timed artificial insemination protocol (TAI) (Baruselli et al., 2015). For this reason, research into whether producers

can minimize time and cost by properly assigning animals to breeding protocols is the purpose of this study.

The objectives of this study were to 1) determine the impact of circulating AMH level on ability to obtain a pregnancy, 2) to investigate whether high and low AMH animals respond differently to TAI vs. heat detection protocols and 3) to characterize the ability to predict success of an animal on a breeding protocol by AMH level while accounting for factors such as milk production, age and lactation number to determine their influence on AMH level. It was theorized that low AMH animals achieve greater pregnancy rates when bred to an ovulation synchronization protocol and exhibit lower pregnancy rates when bred off of a natural or induced heat. High AMH animals achieve high pregnancy rates when bred off of a natural or induced heat. High AMH animals do not achieve as high of pregnancy rates when bred off of an ovulation synchronization program, but still achieve greater pregnancy rates than the low AMH group.

Materials and Methods

Animal Management

Primiparous and multiparous Holstein cows (n= 308) 45 to 60 days in milk were selected on a rolling basis for enrollment in the trial between the Winter of 2017 and Spring of 2019. Sampling was suspended during the Summer of 2018 when THI was elevated to cause heat stress. All cows were born and housed on a commercial dairy operation in Madison, GA and were fed a TMR designed to meet their maintenance, growth and production needs. Cows were milked three times daily with an RHA of 31,364 pounds. Animals were grouped in freestall pens according to stage of lactation and lactation number with stocking density not exceeding 110%. During the trial,

insemination risk averaged 72%, conception rate averaged 32% and 21-day pregnancy rate averaged 15%. The farm employed Timed Artificial Insemination, activity monitoring via DeLaval's DelPro (Tumba, Sweden), and tail chalking for breeding.

Pre-Breeding Sample

At 45 to 60 days in milk, transrectal ultrasonography was performed to ensure cyclicity and assess uterine health with the following observations recorded: antral follicle count (AFC), presence and number of corpora lutea to determine cyclicity status, and observable fluid or uterine anomalies indicative of infection. Any animals with reproductive abnormalities were removed from the study. At this time, blood was also collected via coccygeal venipuncture into vacutainer tubes containing no additives for AMH analysis.

Breeding Protocol

All animals enrolled in the trial were bred via 1 of the 4 following methods: upon natural detected estrus (natural), detected estrus following prostaglandin injection (induced) or via one of two timed artificial insemination (TAI) protocols (Ovsynch, Double Ovsynch) between 53 and 115 days in milk. Estrus was determined through visual heat detection using tail-chalk as well as through monitoring of pedometer activity. All animals were bred via artificial insemination by 2 experienced inseminators employed by the farm. Animals were checked for pregnancy by palpation performed by a veterinarian 32+ days after breeding.

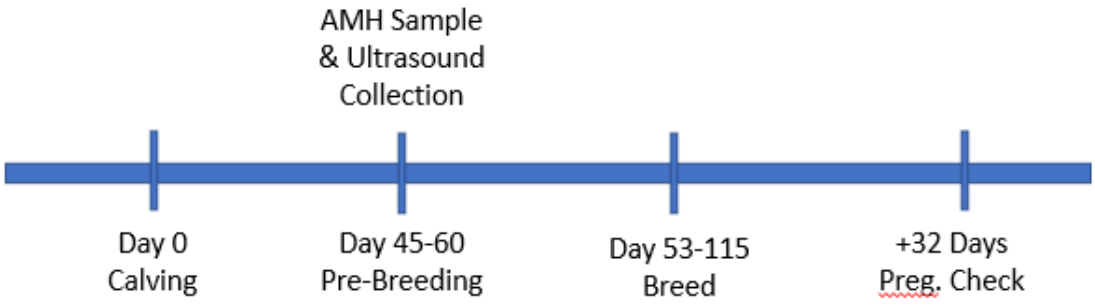

Figure 3.1: Experimental timeline of events.

Production Data Collected

Basic animal information including age at calving and lactation number for each animal were recorded. Seven-day average for milk production was recorded on the day that the animals were sampled as well as the day that the animals were bred. Breeding protocol, days in milk at breeding and pregnancy status (pregnant vs. open) to the protocol were recorded.

Blood Processing, Storage and Hormone Analysis

After collection, samples were immediately placed and maintained on ice and transported to the lab for processing within 2 hours of collection. Samples were centrifuged at 3400 rpm for 15 minutes to separate serum. Serum was then extracted and transferred to storage tubes in duplicate labeled with animal number and sampling date. Samples were frozen at -20 C for future analysis. Frozen serum was shipped to and analyzed for AMH by Ansh Labs (Webster, TX) utilizing their developed enzyme-linked immunosorbent assay for AMH analysis specific to bovines which has been validated for Holsteins and detects a range of 11 to 2240 pg/mL using 50 µL of blood serum.

Statistical Analysis

Data were analyzed with the PROC GLIMMIX procedure of SAS 9.4 (Cary, NC). Variables incorporated into the model include sampling date, sampling age, days in milk at sampling and breeding, number of follicles, number of CL's, protocol that the animal was bred to, status to the breeding and sampling day and breeding day seven-day average milk weights. Outcomes for cyclicity and pregnant or open to the 1st service breeding were run as a binomial distribution, while breeding protocol was run as a poisson distribution and AMH concentration was run as a gamma distribution. Correlations between all variables were also evaluated. Animals were blocked by top, middle and bottom third into a LOW (<158 pg/mL), MID (158-275 pg/mL) and HIGH (>276 pg/mL) AMH group based on the distribution of AMH concentrations for analysis. The distribution of AMH concentrations is seen in Figure 3.2. Breeding protocols were assigned numerically for statistical analysis and can be seen in Table 3.1.

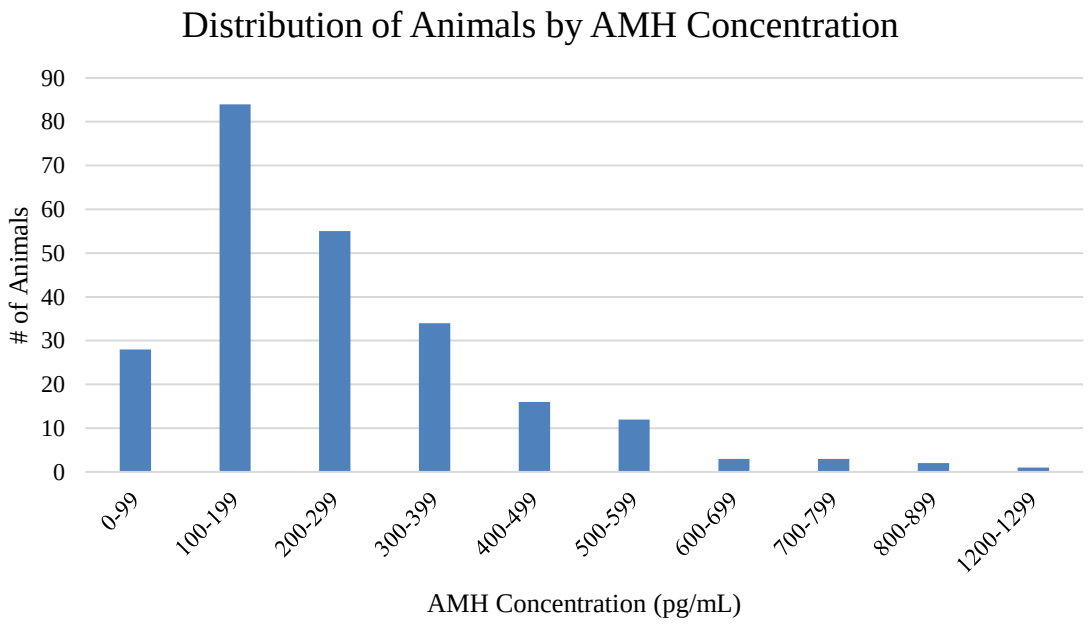

Figure 3.2: The distribution of animals by AMH concentration.

Table 3.1: Numerical assignment for breeding programs

1.	Natural Heat (Estrous detection)
2.	Induced Heat (Estrous detection)
3.	Double Ovsynch (TAI)
4.	Ovsynch (TAI)

Results

Lactation and Milk Production

Lactation number and age had a strong correlation ($P<0.0001$) as expected. Similarly, seven-day average milk-weight on sampling day and breeding day both had a strong correlation with lactation number and age ($P<0.0001$), indicating older and higher lactation number animals had greater milk production. Milk-weights also had a strong correlation with each other ($P<0.0001$). Correlation coefficients for these parameters are displayed in Table 3.2.

Table 3.2: Correlation coefficients and p-values displayed for parameters correlated with lactation and milk production.

Parameter	Correlation Coefficient	P-value
Lactation Number + Age	0.91381	<0.0001
Lactation Number + Sampling Milk-weight	0.77409	<0.0001
Lactation Number + Breeding Milk-weight	0.77680	<0.0001
Age + Sampling Milk-weight	0.66895	<0.0001
Age + Breeding Milk-weight	0.67582	<0.0001
Sampling Milk-weight + Breeding Milk-weight	0.92156	<0.0001

AMH Concentration

Lactation number, age and seven-day average milk-weight on sampling and breeding day were weakly correlated with AMH concentration ($P<0.0001$). AFC was also weakly correlated with AMH concentration ($P<0.0006$) as seen in Table 3.3. When all

variables were incorporated into a single predictive model, AFC (P=0.0008), seven-day average milk-weight on sampling day (p=0.0474) and an interaction between lactation number and seven-day average milk-weight on sampling day (P=0.0253) all positively impacted AMH concentration. Their p-values and regression coefficients are displayed in Table 3.4 while the means for the parameters by lactation are displayed in Table 3.5. Together, this data indicates AMH concentration is higher in older, higher lactation number and animals producing more milk at the sampling day milk-weight.

Table 3.3: Correlation coefficients and p-values for parameters correlated with AMH concentration.

Parameter	Correlation Coefficient	P-value
Lactation Number	0.26121	<0.0001
Age	0.22836	<0.0001
Sampling Milk-weight	0.29751	<0.0001
Breeding Milk-weight	0.27077	<0.0001
AFC	0.19339	0.0006

Table 3.4: Regression coefficients and p-values for parameters impacting AMH concentration.

Parameter	Regression Coefficient	P-value
AFC	0.02698	0.0008
Sampling Milk-weight	0.01319	0.0474
Lactation Number*Sampling Milk-weight	0.01572	0.0253

Table 3.5: Means for various parameters by lactation number.

		Lactation 1		Lactation 2		Lactation 3+
Parameter	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
AFC	140	7.96 ± 3.7	95	8.34 ± 3.7	76	9.24 ± 5.6
AMH in pg/mL	140	196.61 ±132.0	95	273.14 ± 155.6	76	298.99 ± 207.5
Breeding Protocol	107	2.43 + 0.9	93	2.39 ± 1.0	76	1.96 ± 0.9
Sampling Milk-weight in lbs.	140	92.72 ± 11.2	95	120.22 ± 13.9	76	134.39 ± 18.0
Breeding Milk-weight in lbs.	112	94.86 ± 8.9	95	118.25 ± 12.2	76	133.12 ± 16.9

AFC

As lactation number (P=0.0403), age (0.0039) and seven-day average milk-weight on breeding day (0.0078) increased, so did AFC. There tended to be a weak but positive correlation (P=0.0593) between breeding protocol and AFC. Based on breeding protocol numerical assignment, this indicates that as AFC increases, animals were bred to one of the TAI protocols (3 and 4) compared to the estrous detection protocols (1 and 2). The correlation coefficients and p-values are displayed in Table 3.6.

Table 3.6: Correlation coefficients and p-values for parameters correlated with AFC.

Parameter	Correlation Coefficient	P-value
Lactation Number	0.11636	0.0403
Age	0.16343	0.0039
Breeding Milk-weight	0.15775	0.0078
Breeding Protocol	0.11369	0.0593

Cyclicity

As lactation number (P<0.0001), age (P<0.0001) and seven-day average milk-weight on sampling day (P<0.0001) and seven-day average milk-weight on breeding day (P=0.0044) increased, so did the likelihood of cyclicity. Higher seven-day average milk-

weight on sampling day caused cyclicity (P=0.0060) and AFC trended towards a positive impact on cyclicity (P=0.0536). The interaction of AFC and milk-weight on sampling day together had a very slight negative impact on cyclicity (P=0.0060). Based on regression coefficients, AFC is more likely to cause cyclicity than milk-weight. A moderate AFC and average milk-weight cow would be less likely to cycle than a cow with high AFC and the same milk-weight, due to the greater impact of AFC on cyclicity. An additional interaction was seen between AFC and lactation (P=0.0010), which had a greater impact to cause cyclicity than the interaction between AFC and milk-weight or milk-weight alone. Correlation coefficients and p-values are displayed in Table 3.7 while regression coefficients and p-values for these parameters are displayed in Table 3.8.

Table 3.7: Correlation coefficients and p-values for parameters correlated with cyclicity.

Parameter	Correlation Coefficient	P-value
Lactation Number	0.28387	<0.0001
Age	0.24340	<0.0001
Sampling Milk-weight	0.23794	<0.0001
Breeding Milk-weight	0.16900	0.0044

Table 3.8: Regression coefficients and p-values for parameters impacting cyclicity.

Parameter	Regression Coefficient	P-value
AFC	0.3179	0.0536
AFC*Lactation Number	0.1015	0.0010
Sampling Milk-weight	0.03617	0.0060
AFC*Sampling Milk-weight	-0.00429	0.0092

Breeding Protocol

Age (P=0.0021), lactation number (P=0.0018) and seven-day average milk-weight for sampling day (P=0.0288) were higher for animals bred on the estrous detection

protocol. Lactation number also tended to determine breeding protocol, with higher lactation animals bred more frequently to an estrous detection protocol (P=0.0602; r=0.2353). Correlation and regression coefficients as well as p-values for these parameters are displayed in Table 3.9 and Table 3.10.

Table 3.9: Correlation coefficient and p-value for parameters correlated with breeding protocol.

Parameter	Correlation Coefficient	P-value
Age	-0.18404	0.0021
Lactation	-0.18704	0.0018
Sampling Milk-weight	-0.13161	0.0288
Breeding Milk-weight	-0.14655	0.0152

Conception Risk

Seven-day average milk-weight on sampling day tended to have a negative relationship with conception risk (P=0.0309). Age trended towards having a negative impact on conception risk (P=0.0802) as well. AMH trended towards a positive interaction with conception risk (P=0.0607). These data indicate older, higher milk producing animals had a lower conception risk, while high AMH animals had a higher conception risk. Regression coefficients and p-values are displayed in Table 3.10.

Table 3.10: Regression coefficient and p-values for parameters impacting conception risk.

Parameter	Regression Coefficient	P-value
Sampling Milk-weight	-0.02396	0.0309
Age	-0.03524	0.0802
AMH	0.001588	0.0607

Discussion

The data on AFC and AMH both demonstrating a positive relationship with lactation number and age, is consistent with previous works (Ribeiro et al., 2014; Koizumi et al., 2017). Likewise, the data on AFC and circulating AMH is also consistent with previous works (Ireland et al., 2011; Rico et al., 2011; Monniaux et al., 2013; Souza et al., 2015). This study also saw a positive relationship between seven-day average milk-weight on sampling and breeding day with AMH concentration, which was to be expected as age and lactation number were both highly correlated with milk-weight as well as AMH concentration. Of the animals in the herd, the older, higher lactation number and higher milk-producing animals were more likely to have higher AFC and AMH.

While no significant difference was seen between AMH level and conception risk by breeding protocol, the data indicating that more of the 1st lactation animals were bred to a TAI vs. an estrous detection protocol is interesting. Knowing that AFC, AMH and probability of cyclicity are higher in higher lactation number animals, it would make sense that are higher lactation animals are showing more visible signs of estrus and subsequently being bred to an estrous detection protocol vs a TAI protocol. However, because these greater lactation number animals are also producing more milk, they have greater clearance of steroid hormones which as previously described would negatively impact conception rates. This would explain how the higher lactation number animals were more likely to be bred on an estrous detection protocol but were also more likely to have a lower conception risk. Detection of estrous via different methods may have played a factor as well. This farm utilizes a DelPro activity monitoring system which identifies

animals in heat based off increased activity. Estrus signs and duration is reduced in higher milk producing animals due to elevated steroid clearance of estradiol from the blood. Therefore, the higher lactation, higher milking animals that were bred on an estrous detection protocol, may have been detected in heat due to increased locomotion identified by DelPro versus a true standing heat and identification via tail-chalk.

The negative impact of age and seven-day average milk-weight on sampling day on conception risk follow the negative association seen in the industry between older animals (who are generally producing more milk compared to lower lactation number animals) and fertility (Grohn and Rajala-Schultz, 2000). However, the reduced likelihood for cyclicity seen in the first lactation animals compared to the rest of the herd indicate other factors at play that could be negatively affecting normal cyclicity of the first lactation animals.

The trend for higher AMH concentration with pregnancy to 1st service breeding leads us to believe that there may be a difference in fertility between different AMH concentration animals, however that was not elucidated in this study perhaps due to sample size of the various breeding groups. This finding is similar to results seen by Ribeiro et al (2014) where a numerical difference in AMH concentration at the end of the breeding season was seen for pregnant animals and open animals, although not statistically significant. For open and pregnant animals, AMH concentration averaged 228.3 pg/mL and 278.4 pg/mL respectively, which is similar to the numbers found in this study for open and pregnant animals (245.25 pg/mL and 276.82 pg/mL respectively).

The combined impact of AFC and seven-day average milk-weight on sampling day has a negative effect on cyclicity compared to these factors individually. This may be

explained by the degree to which each variable impacts cyclicity. AFC, with a larger regression coefficient, impacts cyclicity to a greater degree compared to seven-day average milk-weight on sampling day. AFC with its stronger correlation than milk-weight, explains how a cow with high AFC and high milk-weight may be less likely to cycle, but that a cow with high AFC coupled with a moderate milk-weights would be more likely to cycle.

Although data from this study did not entirely match results from previous works, the variety of parameters that impact cyclicity and conception risk to first service as well as difference in herd dynamics from farm to farm demonstrate that no one factor will determine ability to achieve a pregnancy for an animal. Rather the interaction of numerous variables previously described creates an environment to impact conception risk.

CHAPTER 4

IMPLICATIONS

The advantages of utilizing AMH as a tool for making reproductive decisions tailored to cows has not yet been translated to the industry. However, results of recent works such as this suggest AMH could be valuable tool for making management decisions. This study has further validated that AMH concentration is directly correlated with AFC, lactation number and age. The heifer component of this study has outlined the impact of calving for 1st lactation animals on circulating AMH concentration as a depression. However, the duration of this depression is unknown, and the depression was seen in 95% of the heifers despite an incidence rate of only 12.3% for negative health events. Further research tracking AMH concentrations in pre and post-calving multiparous animals would assist in determining whether the drop in AMH in these first lactation animals is normal, or whether this drop is extreme for them as a result of increased metabolic and physical stress associated with parturition and onset of lactation for the first time. Based on AMH concentrations by lactation number and age, the latter seems a likely culprit. If this association were found, this would alter the way that breeding programs are tailored to animals. Multiparous animals may recover reproductively more quickly post-calving and could then be bred after a normal 45-60 day voluntary wait period. Getting these animals serviced in a timely manner would be essential as depressed conception risks associated with them would likely mean more breedings are necessary to achieve a conception. The first lactation animals on the other

hand which struggle to recoup as quickly after calving, may require a longer voluntary wait period to return to normal cyclicity. With higher conception rates in these animals, pushing back the voluntary wait period would not be a large concern for a negative impact on overall herd reproduction.

Looking at heifers over time, more of the LOW AMH animals maintained their AMH categorization than the other groups. Combined with information from Jimenez-Krassel (2015) showing AMH concentration as heifers predicts productive life of that animal, this data would allow producers to identify heifers who will have a shorter productive life with high reliability, allowing them to make informed culling decision. The inconsistency in AMH categorization over time for the MID and HIGH AMH groups may indicate that the LOW group is more easily and reliably targeted as a true poor performing group in regard to productive life.

Data from this study shows no differences in 1st service conception risk by AMH level for animals placed on estrous detection vs. TAI breeding protocols. However, the positive relationship between 1) cyclicity and lactation number/age and 2) AMH concentration and age coupled with the negative relationship between animals being bred on a TAI protocol and lactation number/age indicate that the older, higher lactation number animals were more likely to be cycling and had a greater AMH concentration. Most likely as a result of this, these animals were bred on an estrous detection protocol due to them being more likely to exhibit estrus as previously described. The activity monitoring system could be responsible for identifying these animals in estrus, without physical signs of estrus visible

There are some recommendations for future study. First and foremost, a larger sample size on a research farm would not only increase the power of the results, but also significantly decrease the number of animals that were forced to leave the study for various culling reasons. In addition, tracking cyclicity of these animals leading up to breeding would be interesting to ascertain if TAI protocols are in fact optimizing ovarian performance through forced follicular development, luteal regression and ovulation in low AMH animals as theorized by other researchers. Future research tracking the animals through gestation to determine the effects of gestation on circulating AMH, as well as conducting a comprehensive, long-term study to determine the productive life of animals based on AMH categorization would lend more knowledge to AMH as a tool for making management decisions.

From this project, it is evident that relying on a singular biomarker to serve as a predictor of on an animal's performance is unrealistic. This study shows that there are a number of different variables playing a direct role in an animals' reproductive performance. Instead, future research should expand upon how AMH could play a part in reproductive programs. However, to date it cannot be used as a sole predictor of performance, nor should it be used as a sole agent for determination of culling.

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