ANTI-MULLERIAN HORMONE CONCENTRATIONS AS A PREDICTOR OF SUPEROVULATION RESPONSE AND CONCEPTION RATE IN BEEF FEMALES

A Thesis

by

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ABSTRACT

The primary objective of this study was to determine the relationship of anti-Müllerian hormone (AMH) to ova production in various breeds of cattle in an embryo transfer program. Factors evaluated included breed type, age, weight, body condition, and previous response to flush history. Cow superovulation regimen included insertion of a CIDR (Controlled Internal Drug Release) and a 2 cc injection (IM) of Combo (25 mg and 1.25 mg/mL injectable) on Day 0. On Day 4, FSH treatments were initiated in both morning and afternoon with decreasing amounts over the next three days (five injections). Day 6, prostaglandin was also administered (IM) in both the AM and PM. On Day 7, AM final FSH injection and CIDR removal were performed. This resulted in estrus and AI on day 8 and collection of ova on day 15. Results were based on analysis of 369 animals; Angus (n = 25), Black Brangus (n = 43), Red Brangus (n = 53), , Brahman (n = 103), Beefmaster (n = 112), and Wagyu (n = 33). Age of donors ranged from 1.6 years to 15.4 years at collection with an average age of 7.2. Age had a significant ($P<0.05$) effect on total ova production, but total ova production did not differ among breeds ($P>0.05$). Total ova production was positively associated ($P<0.05$) with AMH concentration (the greater the AMH concentration, the greater number of ova per flush). Secondary studies evaluated the use of AMH as a predictor of conception rate following timed artificial insemination. and tracked the concentration both over 9 consecutive months in heifers and a superovulatory regimen in donors.

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I can do all things through Christ who strengthens me. Phil. 4:13.First, a special thanks to my family for the years of continuous support and guidance, teaching me to be a man of Christ and a man that people respect. Thanks to Dr. Charles and Mrs. Cathryn Looney and the entire staff of Ovagenix for teaching me invaluable years of experience of how to be successful in the field of assisted reproduction. Lastly, thanks for the wisdom and understanding from all of my professors as we worked together towards this degree.

TABLE OF CONTENTS

Page

ABSTRACT ii

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF FIGURES vi

LIST OF TABLES vii

CHAPTER I INTRODUCTION 1

Introduction 1
Review of Literature 1

CHAPTER II USING ANTI-MULLERIAN HORMONE (AMH) AS A PREDICTOR OF TOTAL OVA PRODUCTION FOR BOVINE EMBRYO TRANSFER 5

Introduction 5
Materials and Methods 5
Results and Discussion 8

CHAPTER III THE USE OF AMH IN PREDICTING FERTILITY OF HEIFERS 19

Introduction 19
Materials and Methods 20
Results and Discussion 21

CHAPTER IV AMH CONCENTRATION IN RELATION TO AGE AND ITS VARIABILITY OVER TIME 25

Introduction 25
Materials and Methods 25
Results and Discussion 26

CHAPTER V AMH LEVEL THROUGHOUT A SUPEROVULATORY REGIMEN 30

Introduction 30

Materials and Methods 30
Results and Discussion 31

CHAPTER VI SUMMARY 33

LITERATURE CITED 35

LIST OF FIGURES

Page

Figure 2.1 AMH concentration in relation to total ova - Brahman 13

Figure 2.2 AMH concentration in relation to total ova – Angus..... 13

Figure 2.3 AMH concentration in relation to total ova – Wagyu..... 14

Figure 2.4 AMH concentration in relation to total ova – Brangus..... 15

Figure 3.1 Visual representation of timed AI protocol 21

Figure 4.1 Progression of Average AMH by quartile 27

Figure 4.2 AMH by average concentration of each month 28

Figure 4.3 Linear Regression model of AMH over time 29

Figure 5.1 AMH concentration throughout superovulation 32

LIST OF TABLES

Page

Table 2.1 Representation of a FSH superovulation regimen 7

Table 2.2 ANCOVA of age, breed, and AMH 8

Table 2.3 Negative binomial model 9

Table 2.4 Negative binomial model excluding age. 10

Table 2.5 Negative binomial model using Akaike's Information Criterion and focusing only on Brahman and Beefmaster 11

Table 2.6 Mean age of donor by breed 15

Table 3.1 Reproductive tract score guidelines 20

Table 3.2 Mean conception rate by AMH concentration 22

Table 3.3 Mean conception rate by RTS 22

Table 4.1 Comparison of AMH concentrations between individuals and averages 26

CHAPTER I

INTRODUCTION

Introduction

In cattle and various other farm animal species, embryo transfer is a method of replicating valuable genetics at an accelerated rate of production. While the national average of ova collected per flush in cattle is cited at 10.1, results can range from 0 to more than 70 (5). Prostaglandins, progestins, and multiple FSH injections (porcine pituitary extracts) are all hormones that are commonly used in protocols to stimulate superovulatory responses. Multiple injections over several days, semen, and labor are all factors that go into the overall production of ova in a superovulation regimen. With 20% of donors producing 0 ova per flush (14), it would be beneficial to be able to predict which donor females will perform more efficiently in an embryo transfer program prior to trial and error, saving time and money. Variability can be attributed to the cow, season, follicle stimulating hormone regimen, and status of ovarian follicles at the time of initiation of FSH treatment, technical expertise, among other factor not yet identified (8, 13, 14, 22).

Review of Literature

In cattle, development of a follicular wave is characterized by the recruitment and synchronous growth of a large number of antral follicles, followed by selection and

growth of a dominant follicle and regression of subordinates. (1, 12, 17, 19, 32, 34). Presence of a dominant follicle not only suppresses the the next follicular wave but regresses subordinate follicles (17, 20). A typical estrous cycle consists of either one or two waves, with wave emergence detected on Day 0 (day of ovulation) and Day 10, or days 0, 9, and 16. (1, 12, 17). Follicular waves are not exclusive to cyclicity, but also occur prior to puberty, during pregnancy, and anestrus. However, these waves do have dominant follicles to produce enough estradiol (for various reasons) for ovulation and estrus (33).

Folliculogenesis begins in prenatal life with the production of the smallest of four types of follicles, primordial follicles. Females are born with a finite number of primordial follicles that are characterized by a small, non-growing oocyte, without a zona pellucida, surrounded by a single layer of flattened pre-granulosa cells that are at a state of meiotic arrest.(10, 30, 33, 38). Once recruited, the granulosa cells become cuboidal and begin replicating, and the primordial follicle develops into a slightly more advanced primary follicle (30, 38). Primary follicles that continue to be recruited develop into a preantral secondary follicle with two or more layers of follicle cells. Next, a tertiary or antral follicle is formed at a diameter of 200-300 μm, consisting of three layers: theca external, theca interna, and the granulosa cell layer (24, 33). The follicles are now responsive to FSH through the receptor development on granulosa cells. After antral formation, cattle and human follicles become gonadotropin dependent at about 3-5 mm. Antral follicles below this diameter comprises the pool of small, gonadotropin responsive, Anti-Müllerian hormone (AMH) producing follicles. (24,33). Although this

growing pool of small antral follicles is what most clinicians and scientists reference to as the ovarian reserve, this is not to be confused with the initial number of finite primordial follicles. To assist in inhibiting over-recruitment of follicles, AMH is secreted in highest concentration by preantral and early antral follicles. It slightly decreases once the follicle is selected for dominance and surpasses 5mm. This also gives circulating concentrations a low amplitude pattern. (27,37). Antral follicle count (AFC) is used extensively in both human and animal assisted reproductive technologies to establish infertility and predict ovarian response to gonatdotropin based treatments (24). When a superovulatory regimen is initiated, the number of small antral follicles is highly correlated to the number of transferrable embryos, and while AFC is variable among animals, it is highly consistent within an animal (4, 8, 15, 18, 23, 28). Anti-Müllerian hormone, also referred to as Müllerian inhibiting substance (MIS), is produced by ovarian granulosa cells of small antral follicles and therefore may be a useful marker of the antral follicle pool in the early follicular phase (7, 26, 28, 35).

In embryonic development, regardless of gender, amniotes form two separate and distinct genital ducts, the Wolffian and Müllerian. In mammals, the Wolffian duct differentiates into the male tubular reproductive tract, the vas deferens, epididymides and seminal vesicles. The Müllerian duct develops into the female reproductive tract which consists of the oviducts, uterus, cervix and upper third of the vagina (27).

Anti-Müllerian hormone is a 140 kd protein and a member of the transforming growth factor (TGF) beta superfamily of growth and differentiation factors (3, 6, 28).

Contrary to other members of the family, which exert a broad range of functions in multiple tissues, the main function of prenatal AMH is to induce regression of Müllerian ducts during male sex differentiation (7, 16). This occurs in the bovine fetus between 50 and 80 days (36). Regulated by the SRY gene on the Y chromosome, the gonads in the male fetus differentiate into testes. While the leydig cells produce testosterone and insulin-like-factor-3 (Insl3), both of which are important for sexual differentiation, the Sertoli cells secrete AMH (37). Though the role of AMH in the adult male remains uncertain, the Sertoli cells' secrete AMH over the lifespan of the animal (11).

During female sexual differentiation, AMH is not expressed in the ovary (36). Production of AMH in the female is first observed in the postnatal granulosa cells of the recruited primordial follicles. Both quality and quantity of follicles decrease, and as a result, serum AMH concentrations decrease over time until it becomes undetectable at or around menopause in primates (11, 21, 28, 30). In the case of a twin pregnancy in cattle resulting in heterosexual births, the female fetus is exposed to AMH during gestation, (produced by the testes of its male twin and circulating through placental vascular anastomoses), resulting in regression of the Müllerian duct (also between day 50 and 80) (36) in the female, a disorder called bovine freemartinism, which can be detected using AMH to assess potential fertility (31).

CHAPTER II

USING ANTI-MULLERIAN HORMONE (AMH) AS A PREDICTOR OF TOTAL OVA PRODUCTION FOR BOVINE EMBRYO TRANSFER

Introduction

The present study aims to define the relationship of serum AMH concentration and ova production from cattle exposed to a superovulation regimen. Objectives of this study were to evaluate the influence of age and breed on serum AMH concentration, and to determine the utility of AMH as a predictor of ova production in beef cows. With the possibility of predicting ovarian (ova collection) response using serum AMH as a predictor, we aspire to use this knowledge to better formulate FSH regimens to maximize a donor's ova production which should translate into more efficient production and more profitability for the producer. Animals that overstimulate could be minimized by knowing AMH concentrations (elevated) prior to stimulation, and therefor reducing the FSH dose before trial and error. Donors that do not respond to stimulation well (lower AMH concentration) could have their FSH dose increased to maximize ova production and thus embryo numbers.

Materials and Methods

Six breeds of cattle (Angus, Beefmaster, Brahman, Black Brangus, Red Brangus and Wagyu) were used for determining the relationship of serum AMH concentrations to total ova production. All donor females were enrolled in an embryo transfer program by

Ovagenix (Bryan, Tx). Once enrolled, animals were assigned an embryo collection date, females were synchronized with a CIDR protocol and stimulated with commercially available follicle stimulating hormone (FSH, Pluset or Follitropin V). Breed type, age, weight, body condition score, flush history (if available), and ovarian ultrasonography were all used in formulating a FSH regimen, with total dosage ranging from 7.6-15.6cc. Table 2.1 below represents a typical FSH stimulation regimen

All ova/embryos in this study resulted from the nonsurgical recovery 6.5-7.5 days after estrus (Day 0). All flushes were collected into sterile, disposable filters and ova/embryos were identified in the filtrate by stereo microscopy with illumination from underneath at a magnification of at least 50X by Ovagenix (Bryan, Tx) staff.

Commercially available Heat Watch was used to monitor estrus behavior (heat). Collectively, 3 to 4 units of semen were used for artificial insemination (AI) at 12 and 24 hours post onset of estrus (heat). Blood samples were obtained from donor cows through puncture of a tail vessel at the time of embryo collection (day 7). Samples were centrifuged at 11180 RCF for 10 minutes and the serum collected with disposable pipettes. The serum was aliquotted into two 1.5 mL microtubes and frozen (-20^{O} +/- 2^{O} C). Assays were performed by enzyme linked immunosorbant assay (ELISA) with a bovine specific AMH assay supplied from ANSH Labs. (Webster, Tx) (Lowest detectable dose 0.07 ng/ml, intra-assay variation 4.3 % and inter-assay variation 6.2%). Results were obtained using a Micromedics Vmax Plate Reader (Sunnyvale,CA) at 450 angstrom wave length.

Analysis included effect of age, breed, and AMH on the total ova production. We treated the total ova production as a continuous variable, and used an analysis of covariance (ANCOVA) for our methodology to determine significance. For this analysis we excluded all the Wagyu cattle due to not having enough variability of age within this breed.

Day	**Hour**	**Procedure**
0	A.M.	CIDR+Combo
4-5	A.M.	FSH
4-5	P.M.	FSH
6	A.M.	FSH, PGF2α
6	P.M.	FSH, PGF2α
7	A.M.	FSH, CIDR-out
8	Expect heat	Cystorelin at onset of estrus
8-9	+12 &24 hrs post estrus	AI

Table 2.1. Representation of a typical FSH regimen used in this study.

Results and Discussion

	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Age at Collection	1	885	884.5	7.398	0.0068
Breed	4	874	218.5	1.828	0.12313
AMH	1	269	269.1	2.251	0.13452

Table 2.2. ANCOVA of age, breed, and AMH.

The results, listed in Table 2.2, indicate that age is clearly one important predictor for total ova production (22). The limitations of ANCOVA analysis include 1) treating total ova production as a continuous variable and 2) the observed distribution of age or AMH concentration differs across breed. For the actual model fitting to the count data, one may initially think of the Poisson model. However, due to the presence of over dispersion in the data, we fit the negative binomial model and report the results in Table 2.3.

	Estimate	Std. Error	Z Value	Pr(>z)
(Intercept)	2.68913	0.17654	15.233	<2e-16
Age	-0.03979	0.01524	-2.61	0.00905
Breed 4	2.1459	0.19153	1.12	0.26254
Breed 1	-0.05409	0.20936	-0.258	0.79615
Breed 3	0.0475	0.19871	0.239	0.81077
Breed 0	0.18326	0.21084	0.869	0.38473
AMH	0.14808	0.05939	2.493	0.01265

Table 2.3. Negative Binomial Model

The results clearly indicate that both age and AMH concentration have significant effect at the ($P<0.05$) level on the total ova production. Explanatory variable breed is presented in the model via the dummy variables $z0$, $z1$, $z3$, $z4$. However, breed does not have a measurable effect on the ova production. This analysis excluded all Wagyu subjects as age was not available for all Wagyu cattle and there was not enough variability in the age when they were observed. Next, we analyzed the data using all breeds and consequently excluding age from the model. Results are listed in Table 2.4.

	Estimate	Std. Error	Z Value	Pr(>z)
(Intercept)	2.50695	0.16502	15.192	<2e-16
Breed 4	0.11402	0.18477	0.617	0.53717
Breed 1	-0.06476	0.20903	-0.31	0.75672
Breed 3	-0.11324	0.18507	-0.612	0.54064
Breed 0	0.05542	0.20362	0.272	0.78549
Breed 2	-0.32661	0.21952	-1.488	0.1368
AMH	0.17983	0.05768	3.118	0.00182

Table 2.4. Negative Binomial Model excluding age.

Serum AMH ($P<0.05$), but not breed was a significant predictor for the total ova production. Of course, there are several limitations of the analysis. First, the distribution of age within the breeds is not the same creating an imbalance in the analysis. Second, the distribution of AMH across the breed is not homogeneous. Thus, the results should be interpreted cautiously. Since the data contained 112 Beefmaster and 103 Brahman cattle, a sizeable proportion compared to the other breeds and both of these breeds contain sufficient variability in terms of age, we ran a further analysis using only these two breeds. In this analysis, we investigated if there was any change-point in the AMH concentration. We created two predictors out of AMH concentrations as follows: $X1 = \text{AMH} \times I(\text{AMH} < r)$ and $X2 = \text{AMH} \times \text{I}(\text{AMH} > \text{r})$. We took different values of r and chose the best r based on the minimum Akaike's Information Criterion (AIC)

criteria. The optimum *r* came out to be 1.9 and the results of the analysis are given Table 2.5.

	Estimate	Std. Error	z value	pr (>z)
Intercept	2.82331	0.18796	15.021	<2e-16
AMH<1.9	0.40037	0.12905	3.103	0.001919
AMH>1.9	0.02359	0.09588	-0.246	0.805681
Age at Collection	0.0621	0.01697	-3.66	0.000252

Table 2.5. Negative Binomial Model using Akaike's Information Criterion and focusing on Brahman and Beefmaster.

Analyzing the data showed a cutoff AMH concentrations within each breed where donors above the given concentration can be deemed better ova producers than females below a certain concentration of AMH. For the 25 Angus donors, those that were below 0.25 ng/ml had an average of 10.07 ova per flush, while those above 0.25 ng/ml gave 17.2 ova per flush, as shown in figure 2.2 Out of the 103 Brahman donors, the 50 females with AMH concentrations less than 0.70 ng/ml averaged 8.28 ova per flush, while the 53 cows above averaged 16.96 ova per flush, as shown in Figure 2.1. Of the 111 Beefmaster donors, those less than 0.30 ng/ml averaged 9.0 total ova per flush, while those greater than 0.30 ng/ml gave 17.2 ova per flush, as shown in Figure 2.4. We viewed the Brangus together and separate (Red Brangus and Black Brangus). Since their breed makeup is closely related, it was to our expectation that results would show

the same cutoff AMH concentration. Combining the red and black brangus, there were 94 animals. Donors below 0.70 ng/ml of AMH produced 10.89 ova per flush while the females whose concentration for AMH was above 0.70 ng/ml gave 17.18 ova per flush, as shown in Figure 2.5. Separately, the numbers were 9.11, 17.7, 13.39, and 17.39 for black Brangus below 0.70 ng/ml, black Brangus above 0.70 ng/ml, red Brangus below 0.70 ng/ml and red Brangus above 0.70 ng/ml, respectively. The 32 Wagyu collections resulted in a cutoff concentration for AMH of 0.30 ng/ml for donors that were arbitrarily assigned as good versus poor ova donors. Donors with AMH concentrations that were below 0.30 ng/ml averaged 7.39 total ova per flush while the females greater than 0.30 ng/ml produced 12.14 ova per flush, as shown in figure 2.1. Results from other breeds are shown in Figures 2.2-2.5. Mean age per breed was also computed and listed in Table 2.6.

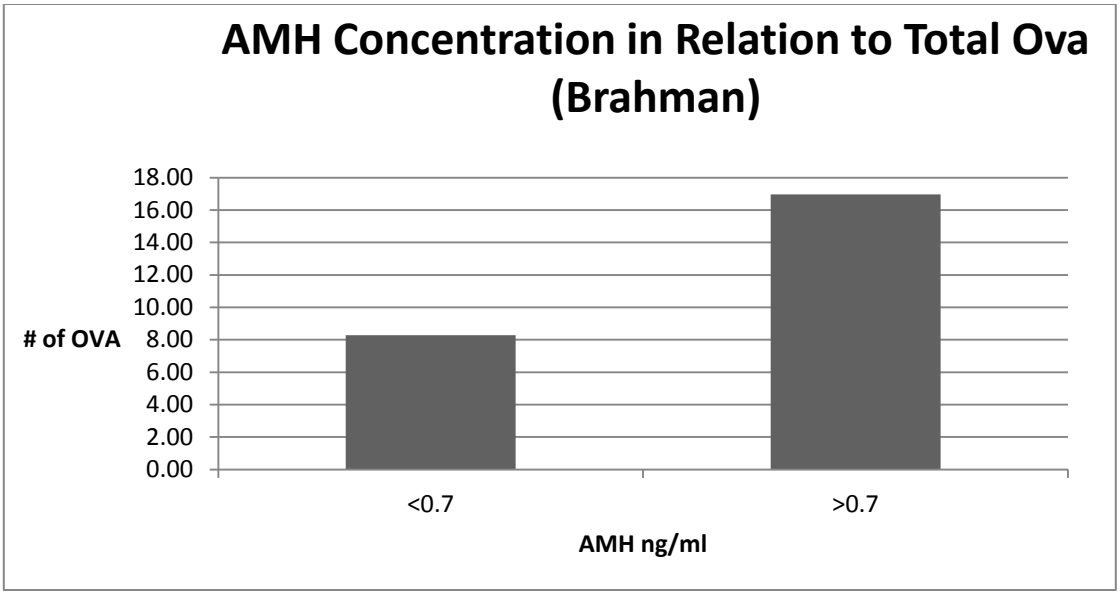

Figure 2.1. Total ova produced by Brahman donors, separated by those below and above the AMH cutoff concentration.

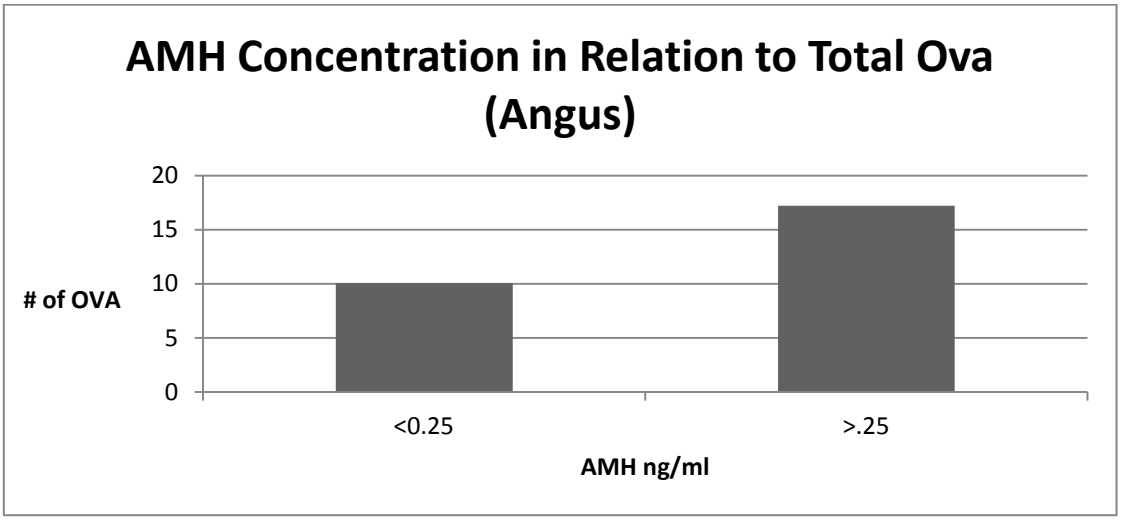

Figure 2.2. Total ova produced by Angus donors, separated by those below and above the AMH cutoff concentration.

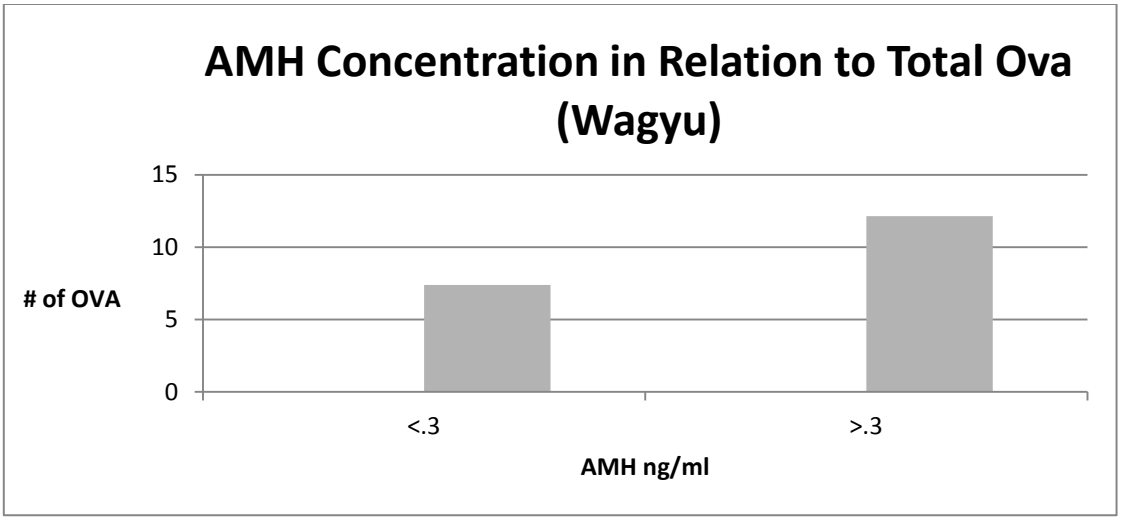

Figure 2.3. Total ova produced by Wagyu donors, separated by those below and above the AMH cutoff concentration.

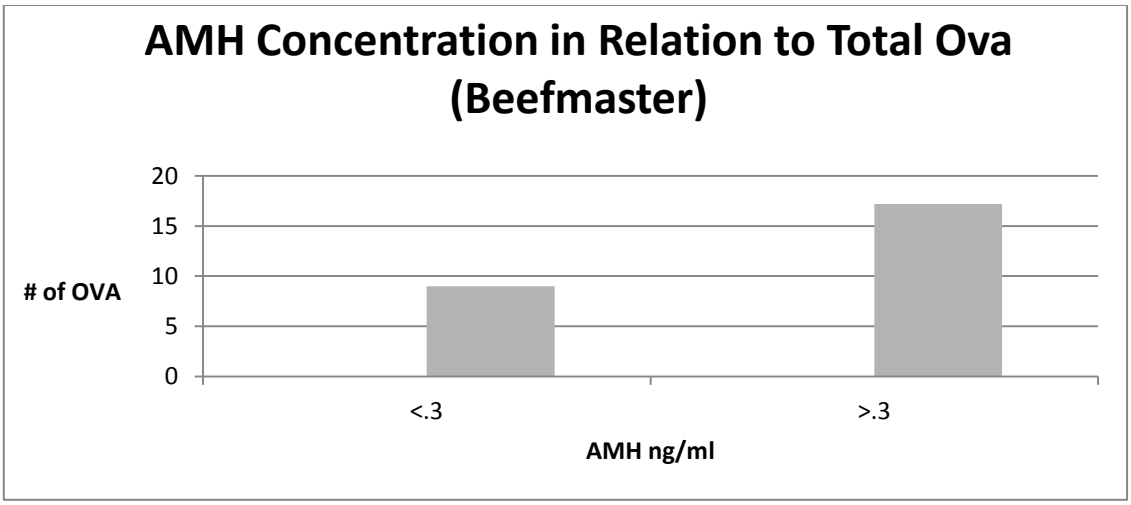

Figure 2.4. Total ova produced by Beefmaster donors, separated by those below and above the AMH cutoff concentration.

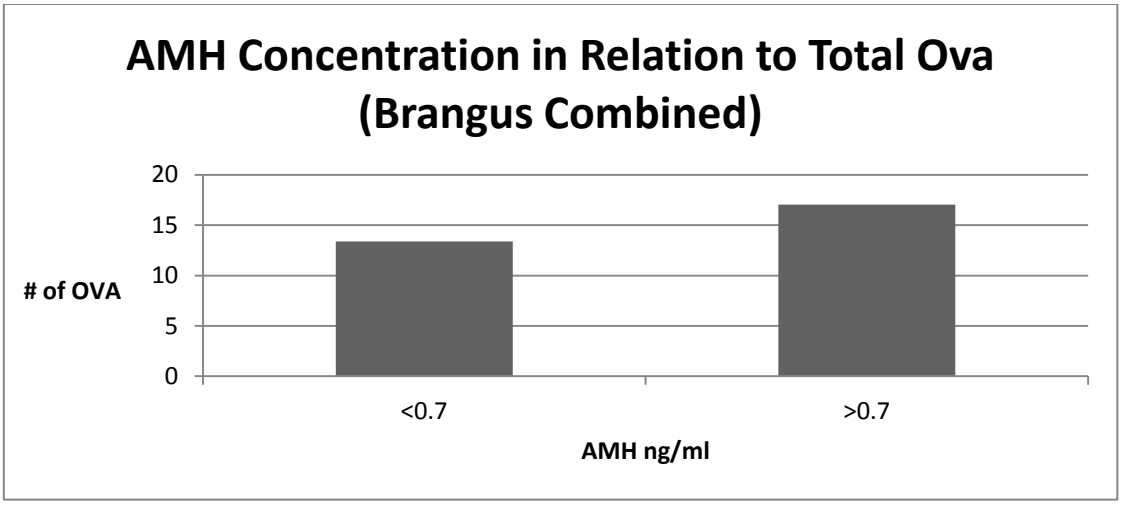

Figure 2.5. Total ova produced by Brangus donors, separated by those below and above the AMH cutoff concentration.

	Angus	Brahman	Brangus	Beefmaster	Wagyu	All Breeds
Mean Age	3.94	8.57	5.71	7.97	<3.0	7.16
Std Dev	1.72	3.11	3.029	10.85	n/a	6.92

Table 2.6. Mean age of donors at time of collection and standard deviation.

Results demonstrate that AMH concentration is indicative of total ova collected in a superovulation program (greater the AMH, the greater number of ova collected), Cows with greater AMH concentrations had better ovulatory responses to FSH treatment (more ova collected) than cows with lower AMH concentrations. However, there are

many factors associated with success or failure in an embryo transfer program. Variability in an embryo transfer program is always a concern and what can be done to minimize that variability is subjective in most cases. Some of the inconsistency when the data is viewed as a whole is attributed to the fact that the AMH concentration chosen as a cutoff for above average ova producers cannot be considered the same across breeds, much like expected progeny differences (EPDs). In Brahman cattle, for example, the results show 0.70 ng/ml appears to be an appropriate boundary. Out of 103 females, there were 50 donors with an AMH concentration below 0.70 ng/ml and they averaged 8.28 total ova per flush. There were 53 donors with a value greater than 0.70 ng/ml that had an average of 16.96 ova per collection. In Wagyu cattle, there were six females that were above the 0.70 ng/ml out of 32. The cutoff AMH concentration results show the more appropriate cutoff concentration at 0.30 ng/ml providing evidence that one AMH concentration cannot be utilized across breeds. Breed specific ranges and possibly age related changes should be evaluated carefully.

The FSH regimen a donor receives is another major contributor towards the variability in ova collection. Currently, there is not a science in prescribing an exact dosage of FSH to maximize ova production in a donor cow. Overstimulation results in a large majority of the ova either unfertilized or degenerate, while “under-stimulation” does not maximize the donor’s ability to produce embryos or efficiently capitalize the embryo transfer superovulatory program. Many embryo transfer companies use ultrasonography to obtain an antral follicle count at time of CIDR insertion, along with breed type, age, and flush history to try and predict the correct dosages of FSH to

maximize stimulation of the ovary and recovery of viable embryos. There are many variables that cannot be accounted for and therefore lead to decrease superovulatory response and hence embryo recovery, especially for first time donors. However, the more a donor cow is flushed, the better a skilled embryologist can prescribe the correct FSH dosage and maximize embryo production. Another contributing factor would be the Lot # and or type (manufacturer) of FSH used for stimulation. Being that the current FSH available is a pituitary derived porcine product and different lots could have different potencies, make knowing animal history important and utilizing other tools to try and better formulate FSH regimens. At some point in time there will have to be a standard developed for potency, which currently can and does affect the follicular response. It is predicted the AMH concentration will be used in conjunction with breed type and flush history (if available) to take some of the guesswork out of assigning FSH regimens and maximize the production of ova, especially on animals enrolled in their first FSH regimen for superovulation.

As we become more familiar with AMH concentration in association with breed type, age, and ova production, this will be another tool to help determine ova production in response to superovulation. As mentioned previously, with each estrous cycle, the number of follicles in the antral follicle pool is reduced until follicular pool exhaustion (menopause in humans), and thus the AMH concentrations is slowly reduced as the animal ages (11). Can we optimize the FSH regimen knowing the AMH concentration prior to administration of FSH? In other reports, in both humans and animals (11, 28, 36) the decline in AMH is gradual up to a point. Where the break for good ova donors

vs poor ova donors will certainly be variable based on age and breed type, but where that break may occur and be most beneficial will be answered in future experiments in relation to AMH concentration. This decline in AMH concentration could be accountable for variance in cattle as it is in the human female with respect to ova production (2, 6, 28, 37). These results indicate that age clearly has a significant effect on the total ova production. More importantly, we see the total ova production is significantly ($P<0.05$) associated with the AMH concentration, the higher the AMH, the greater number of ova.

We believe with an increased number of data points across breeds, the results will shift towards more definitive results with respect to age and AMH concentration. There are simply too many variables at play when observing small numbers of animals. From this study, it appears that AMH concentration, even in the breeds with a limited number of animals, can be a useful tool in trying to predict ova production in response to a superovulatory regimen. This would allow a producer to save time and money by using AMH concentration as a predictor of ova production before beginning a superovulation regimen which should result in decreasing "over stimulation or under stimulating" the ovary of the donor animals and maximizing FSH use and limiting expense of FSH.

CHAPTER III

THE USE OF AMH IN PREDICTING FERTILITY OF HEIFERS

Introduction

Growth and development of replacement heifers until breeding, and furthermore, calving, is a slow and costly process. For most cattlemen, these females generate revenue by the sale of their weaned calves. It is typically 20-24 months from the time of the selection of which females will be kept as breeding stock until their first calf and 4 -6 months later before the sale of that first calf. The use of pregnancy detection can greatly cut down on cost by reducing the days on feed of open females, either by rebreeding or culling, but it is our intent to be able to give the industry another tool to become more efficient by being able to hedge your selection of females with the most reproductive potential compared to their contemporaries. The intent is to select females with the highest concentrations of AMH. This may also relate to longevity of reproductive success. However, that will not be evaluated as one of the experiments and is based on the human literature that older females that have greater concentrations than age match females are more successful in becoming pregnant and having offspring (11,26) By using AMH concentration to determine the most fertile females, producers can better select at a "normal working times for selection" which females to use as replacements without the cost of developing and the trial and error of the first breeding season.

Even though the industry in agreement that circulating AMH serves as a valuable predictor of follicular population and response to superovulation, the ability of AMH to

be correlated with conception rates following artificial insemination is still unknown in beef cattle. Being that there are many variables for successful pregnancy, AMH may at least serve as an indicator of "adequate" ovarian reserve. Since studies have indicated that females with lower antral follicle counts result in lower fertility (9,11), it would be reasonable to believe that lower AMH concentration can be correlated to reproductive success as compared to age matched, breed specific individuals.

Materials and Methods

155 brangus and red brangus, angus and red angus females were synchronized with a Co-Synch + CIDR protocol with timed AI at 72 hours after CIDR pull and prostaglandin injection. This synchronization method is outlined in Figure 3.1. At the time of CIDR insertion, a reproductive tract score (RTS)- a widely accepted form of measuring maturity and predicting immediate fertility in heifers, was recorded. Standards for RTS determination are outlined in Table 3.1

RTS	Uterine horn diameter (mm)	Ovarian Structures
1	<5	No palpable follicles
2	5-10	8mm follicles
3	10-15	8-10 mm follicles
4	15-20	>10 mm follicles
5	>20	>10 mm follicles and corpus luteum

Table 3.1. Reproductive tract score guidelines

Along with this classification, a blood sample was obtained through tail bleeding. Samples were then centrifuged down at 11180 RCF for 10 minutes and the serum collected with disposable pipettes. The serum was aliquotted into two 1.5 mL microtubes and frozen (-20O +/- 2O C). Assays were performed by enzyme linked immunosorbant assay (ELISA) with a bovine specific AMH assay supplied from ANSH Labs. Results were obtained using a Micromedics Vmax Plate Reader at 450 angstrom wave length. Females were inseminated by Ovagenix (Bryan, Tx) staff and clean up bulls turned out the following week. Pregnancy status was be determined 65 days post insemination by use of ultrasonography. Females were grouped into in one of three classes by fetal age, first breeding conception (AI conception), second breeding conception, or third or more/open.

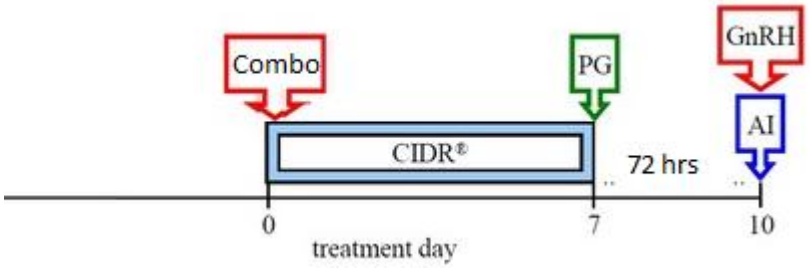

Figure 3.1: Visual representation of synchronization protocol used.

Results and Discussion

Of the 155 females, 138 were brangus and 17 were angus. As AMH appears to be breed specific, the two groups were divided for analysis of results. Since n=17 is not an appropriate sample size for statistical inference, the results focused on the 138 brangus

heifers. Using AMH concentrations, females were split into two groups, High and Low AMH. Using SAS, Least Squares means results were found and listed in Table 3.2. Results were also obtained with 6 groupings (Very high, High, Moderately High, Moderately low, Low, Very low) instead of two (High, Low), but with not as many animals per group, results should be interpreted with caution (more numbers are need to make inferences).

	Mean 1st Service Conception Rate	**Standard Error Mean**
High Concentration	43.56	.076
Low Concentration	44.35	.074

Table 3.2. Mean conception rate by AMH concentration.

RTS	**Mean 1st Service Conception Rate**	**Standard Error Mean**
2	25.07	.1536
3	41.97	.06696
4	52.71	.08355
5	58.36	.0822

Table 3.3. Mean conception rate by reproductive tract score.

In this study, AMH does not show a significance at the .05 as predictor of better conception rate following timed artificial insemination in beef heifers. This is supported

by work from Dr. Pietro Baruselli's lab (n=939), published at 2014 SBTE. There were not significant differences in conception rate between the 2 classes or the 6 classes of AMH for the 1st, 2nd, or 3rd service. Contradictory results showed positive correlations between circulating AMH and fertility (29). With females resulting into three groups after AMH sampling 8 days before synchronization, top 20% concentration, average, and bottom 20% concentration, conception rates showed that cows with greater circulating AMH had more pregnancies from the first AI service. Females that did not conceive to the first breeding became pregnant at a greater percentage than contemporaries with a lower AMH concentration. More research is needed with more appropriate design to clarify the relationship of pregnancy rates to AMH concentrations

Reproductive tract score measured confirmed its industry accepted reliability of predicting fertility in heifers. Conception rates sorted by RTS are listed in Table 3.3. Perhaps the RTS was a better predictor because it is a more direct measure of a female's immediate ability to conceive. We calculated to see if there was significance between RTS and AMH with the following results: RTS-2: Average AMH - .3495, RTS - 3: Average AMH - .3001, RTS - 4: Average AMH - .3106, RTS – 5: Average AMH - .3575, indicating no correlation between AMH and reproductive tract score. This is because AMH is relatively steady from birth until puberty and then slightly decreases until primordial follicle exhaustion. Since circulating AMH is produced by early antral follicles and is highly repeatable, a heifer classified as a RTS of 2 will have nearly the same AMH 6 months later classified as a RTS of 5. In comparison to RTS, AMH concentration is more robust at a young age as it is nondiscriminatory to cyclicity and

only reflects small antral follicle population. Perhaps AMH is viewed as a representative of the follicular pool, and it is agreed upon that this pool is an indicator of the primordial follicular reserve, and will serve as a better predictor of reproductive longevity rather than immediate reproductive fertility. Coupling RTS with AMH may prove to be a more beneficial route of predicting which animals to select. Measuring AMH and evaluating RTS at different time points to evaluate the relationship would be an obvious next step.

CHAPTER IV

AMH CONCENTRATION IN RELATION TO AGE AND ITS VARIABILITY OVER TIME

Introduction

It has been demonstrated that circulating AMH has relatively steady intraindividual long term concentration with the use of AMH as a predictor for several reproductive related strategies, it is important to know the appropriate sampling time. The preferred timing of obtaining serum samples to determine AMH concentrations are not the same time across cattle operations. The variability of timing (age) can be quite different. While some choose to process calves at birth, others wait as long as the yearling stage. This experiment was designed to evaluate samples at various times, regardless of age at collection. For example, females with the highest AMH concentrations at weaning should continue to have the highest concentrations in comparison to contemporaries over her lifetime.

Materials and Methods

24 Wagyu heifers were be bled by Marble Genetics staff once per month from a prepuberal age (30-150 days), through the next 9 months. The heifers were bled approximately once every 30 days, via tail bleeding, for health checks and determination of AMH concentrations over time. Samples were centrifuged down at 11180 RCF for 10 minutes and the serum collected with disposable pipettes. The serum was aliquotted into

two 1.5 mL micr8tubes and frozen (-20^{O} +/- 2^{O} C). Assays were performed by enzyme linked immunosorbant assay (ELISA) with a bovine specific AMH assay supplied from ANSH Labs (Webster, Tx). Results were obtained using a Micromedics Vmax Plate Reader (Sunnyvale, Ca) at 450 angstrom wave length.

Results and Discussion

With results ranging from.562 ng/ml to .06 ng/ml, concentrations were first looked as individual samples to determine the mean, upper quartile, lower quartile, and inner quartile range. Next, average concentration of each female was used and then divided into the upper quartile, inner quartile range, and lower quartile. Table 4.1 compares the two.

	Mean	Std. Dev.	Upper Quartile	Lower Quartile	Goodness of Fit	N	
Average Individual Concentration	.168	.058	.183	.127	.0035	24	
Average Group Concentration	.168	.078	.194	.12	.001	219	

Table 4.1. Comparison of AMH concentrations between individuals samples and the group average.

Once grouped into quartiles, the average of each quartile was plotted for each month to track the average AMH concentration over time for each group. As hoped, each line follows the same progression curve and did not cross. Displayed in Figure 4.1.

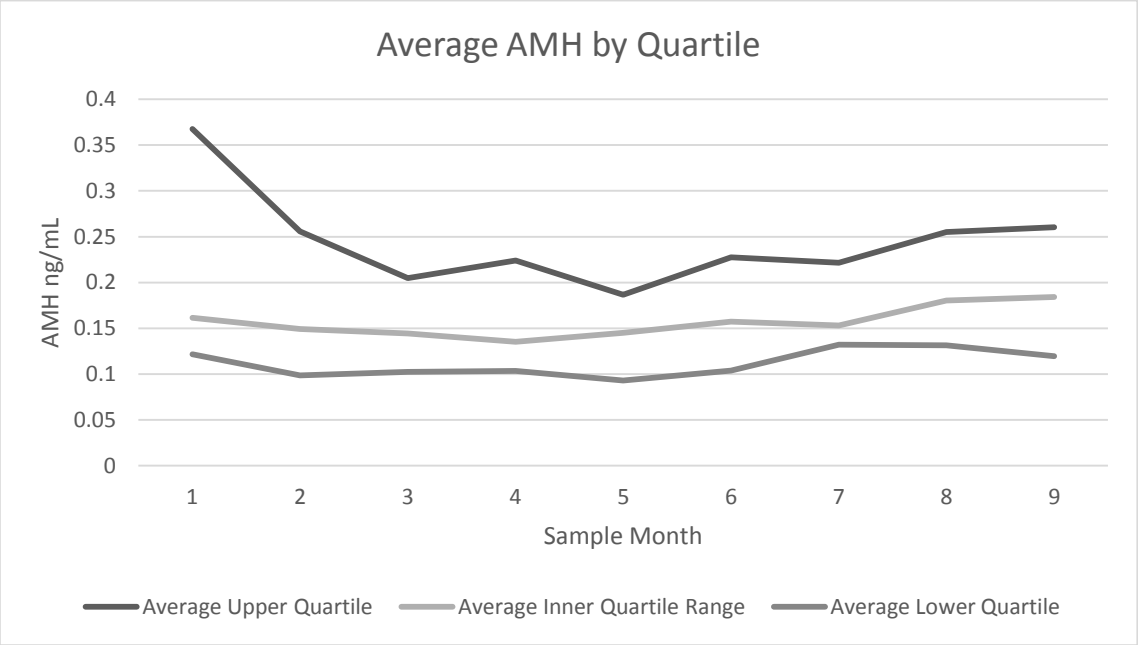

Figure 4.1. Progression of the average AMH by quartile.

Though the average AMH by quartile yields positive results that the groups never crossed, some individual results were variable. This raises the question of where did the variability come from. Was it related to the processing and storage of the blood, test, technician, lot # of reagents, among other variables, or the actual circulating concentration of AMH? Concentration over time was evaluated by the average of the

entire sample group by each month to help establish a model to follow. Results are displayed in Figure 4.2.

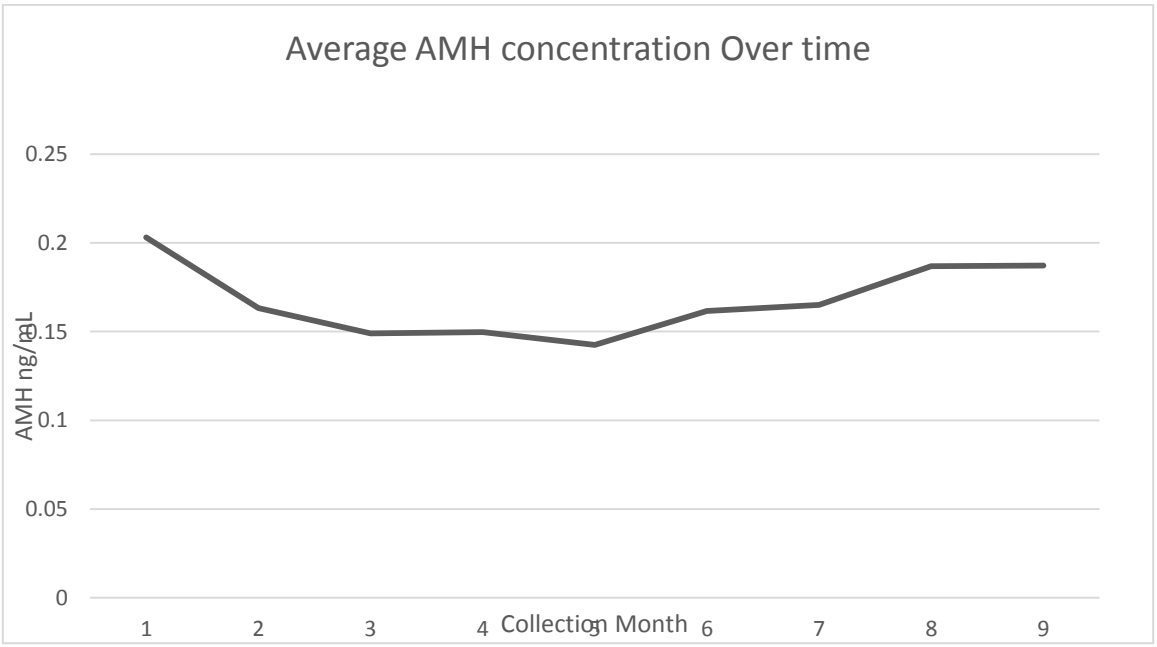

Figure 4.2. AMH by average concentration of each month.

An ANOVA table illustrated that time is not a significant predictor (P = 0.54) of AMH (R2 = 0.001). An analysis of lack of fit demonstrated that linear regression was not an appropriate model (p=.0794) for day as a predictor of AMH, indicating that AMH did not change over time, as shown in Figure 4.3

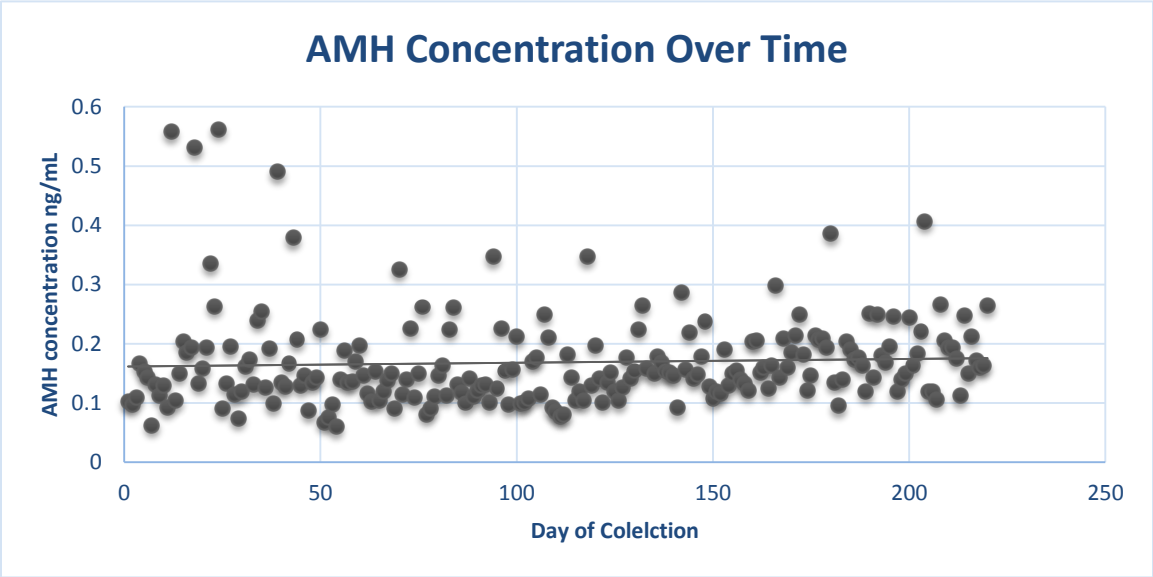

Figure 4.3. Linear regression model of AMH over time.

CHAPTER V

AMH LEVEL THROUGHOUT A SUPEROVULATORY REGIMEN

Introduction

With the use of AMH as a potential predictor for several reproductive sciences, it is important to know if the estrous cycle contributes to variability of AMH concentration. This experiment is designed to prove that the sample, regardless of the stage of estrus cycle at collection, is meaningful. To assist in inhibiting over-recruitment of follicles, AMH is secreted in highest concentration by preantral and early antral follicles. It slightly decreases once the follicle is selected for dominance and surpasses 5mm. This also gives circulating concentrations a slight wave-like pattern. (27,37). Though AMH has been proven to have high repeatability for each of the four different phases of the estrous cycle, days in milk, and levels of milk production by Ireland et all (2011), and Monniaux et al (2013), this study aims to continue to prove legitimacy of AMH concentration in beef cattle.

Materials and Methods

Donors in this study were enrolled in an embryo transfer program at Ovagenix (Bryan, Tx). At the initiation of the embryo transfer program, Day 0 (CIDR in), a blood sample was taken via tail bleeding. Samples were collected each day of FSH injections (days 4,5,6,7), at estrus (day 8), and at the time of embryo collection (day 15). Samples were centrifuged down at 11180 RCF for 10 minutes and the serum collected with

disposable pipettes. The serum was aliquotted into two 1.5 mL microtubes and frozen (-20O +/- 2O C). Assays were performed by enzyme linked immunosorbant assay (ELISA) with a bovine specific AMH assay supplied from ANSH Labs. Results will be obtained using a Micromedics Vmax Plate Reader at 450 angstrom wave length.

Results and Discussion

The superovulation regimen called for CIDR (Controlled Internal Drug Release) insertion and a 2 cc injection of Combo (25 mg and 1.25 mg/mL injectable) on day 0. Day 4, FSH treatments were initiated in both AM &PM with decreasing amounts over the next four days. Day 6, along with FSH and prostaglandin were given in both the AM and PM. On Day 7, AM final FSH injection and CIDR removal were done. This resulted in estrus and AI on day 8 and collection of ova on day 15. Previously to this, a reference heat was obtained on each donor on day -8. This is utilized to obtain a functional corpus luteum. The estradiol in the Combo injection causes regression of the dominant follicle from the first follicular wave and begins a new wave in four days. The initiation of exogenous FSH on Day 4-7 prevents atresia of recruited follicles and then grows them to ovulation at estrus. Since AMH is produced by the granulosa cells of antral follicles, it is expected to see an increasing concentration as follicles grow, with the sample taken at estrus being the highest. This is because it has the highest number and largest (mm) follicles, and in turn the most granulosa cells producing AMH. However, this differs from samples taken through an estrous cycle of a non-stimulated ovary. Reason being, with a non-stimulated ovary, the atresia of follicles not selected for

dominance results in a slight decrease of AMH and a wave like pattern. Figure 5.1 displays the 7 different samples in chronological order across 15 days for 15 different donor females.

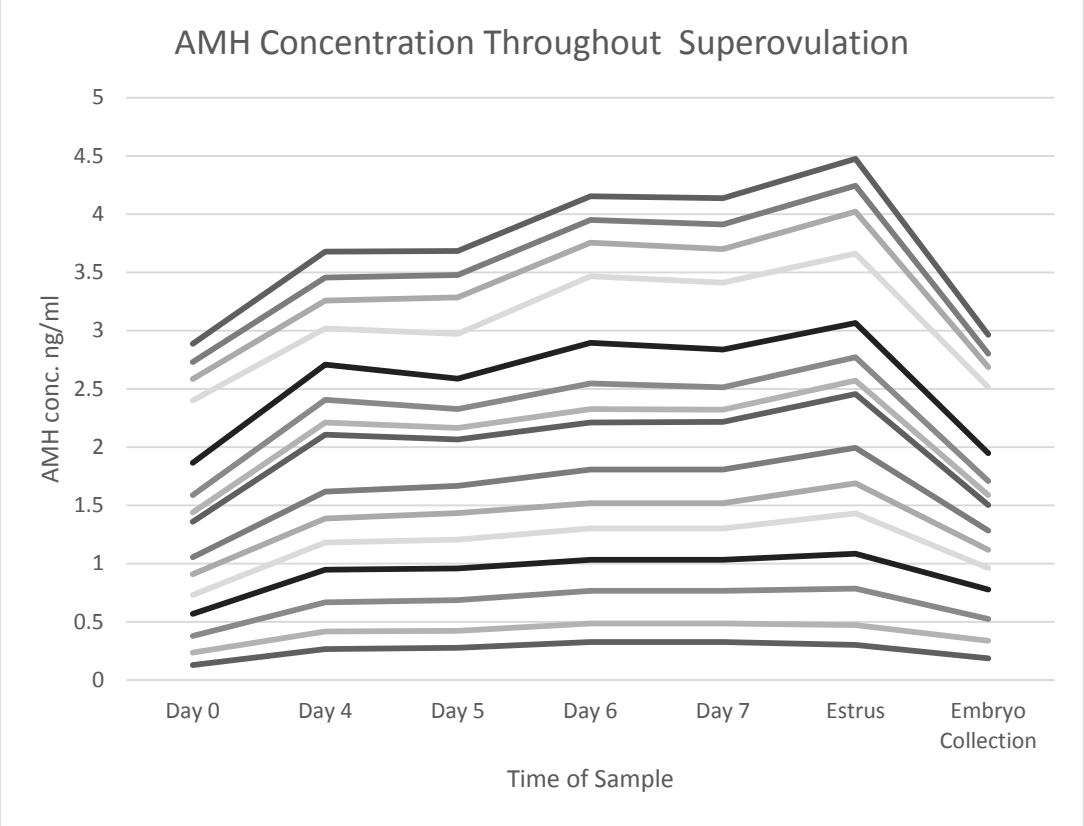

Figure 5.1 AMH concentration throughout superovulation..

CHAPTER VI

SUMMARY

Anti-Müllerian hormone (AMH) is exclusively produced by granulosa cells of the developing pre-antral and antral follicles in females and is being increasingly used to assess ovarian function. It has been highly utilized in human fertility clinics as a marker of ovarian aging, ovarian dysfunctions, and to predict assisted reproduction response. It is highly correlated to antral follicle counts and therefore viewed as a potential valuable predictor of ova production following superovulatory treatment in cattle. Since appears to be breed specific, further studies are needed to establish guidelines for high versus low AMH concentrations associated with each breed. Age also has to be a consideration. Once many of the direct variables have been evaluated and theories validated, will the embryo transfer industry begin to utilize AMH concentrations to assist in formulating FSH dosages for superovulation-in a more predictable fashion. Using AMH along with a more purified FSH preparation should help in lessening the guesswork of which dose to begin with other than experience. In a superovulation regimen, higher than normal increases in AMH were observed. One possible reason is because of the recruitment and continued growth of follicles that would otherwise be atretic are producing AMH. After ovulation, the circulating AMH concentration is returned to its basal concentrations. It is important to note that in both studies with multiple samples from the same subject, females with a high concentration continued to have samples with high concentrations, with the same results in regards to moderate and low concentrations, helping to prove

reliability of AMH samples. AMH is highly correlated to antral follicle count (AFC), and while AFC's are highly variable between cows, they are highly repeatable within an individuals. thus females classified as "high", "moderate", or "low" will remain in the respective "high", "moderate", or "low" classification for life.. With different studies using circulating AMH as a predictor of conception rate yielding contradictory results, further studies are needed for clarification. . Perhaps since AMH is viewed as a representative of the follicular pool, and it is agreed upon that this pool is an indicator of the primordial follicular reserve, it will serve as a better aid in predicting reproductive longevity rather than immediate reproductive fertility. It is because of the ease of collection and high intraindividual repeatability behind the AMH assay that makes it a practical method of improving the efficiency of reproductive technologies in cattle.

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