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REGULATION AND ROLE OF ANTI-MÜLLERIAN HORMONE IN BOVINE REPRODUCTION

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REGULATION AND ROLE OF ANTI-MÜLLERIAN HORMONE IN BOVINE REPRODUCTION

By

Danielle Marie Scheetz

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ABSTRACT

REGULATION AND ROLE OF ANTI-MÜLLERIAN HORMONE IN BOVINE REPRODUCTION

By

Danielle Marie Scheetz

The hypothesis that size of the ovarian reserve (total number of healthy oocytes/follicle in ovaries) is positively associated fertility has never been directly tested because it requires histological procedures to count follicles and large numbers of individuals to reliably assess fertility. Anti-Müllerian hormone (AMH) is produced in granulosal cells, and AMH concentrations are positively associated with number of healthy follicles. Thus, AMH may be a diagnostic marker to evaluate size of the ovarian reserve. However, the role of AMH in females and the factors that regulate AMH production are poorly understood. Results of present studies in cattle showed that: AMH concentrations are static during estrous cycles of individuals; a single AMH measurement is predictive of serum AMH concentrations during estrous cycles, ovary size and number of follicles; pregnant mothers with high somatic cell counts in milk have daughters with low AMH concentrations; low doses of FSH increase capacity of bovine granulosal cells to produce AMH while higher doses decrease AMH production and promote luteinization of granulosal cells; FSH action is diminished in cattle with low follicle numbers; and AMH inhibits FSH action in granulosal cells from cattle with high versus a low number of follicles. Results support the conclusions that a single AMH measurement is a reliable marker for the relative size of the ovarian reserve, and granulosal cells from cattle with low follicle numbers and correspondingly a diminished ovarian reserve and chronically high circulating FSH concentrations may be refractory to FSH and AMH action.

To my parents, Janet and Patrick Scheetz,

for all their love, encouragement, and patience.

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I would like to thank Dr. Joseph Folger, Mrs. Janet Ireland, and Dr. Fermin Jimenez-Krassel for their assistance with various aspects of my research, insights, and friendship.

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I would like to thank the Department of Animal Science at Michigan State University for research assistantship and graduate students from the department for their support.

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LIST OF ABBREVIATIONS

AFC	antral follicle count
ALKs	anaplastic lymphoma receptor tyrosine kinases
AMH	anti-Müllerian hormone
AMHRII	AMH type II receptor
ANOVA	analysis of variance
bFF	bovine follicular fluid
BMP	bone morphogenetic proteins
C	Celsius
CL	corpus luteum
CO_2	carbon dioxide
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline solution
E2	estadiol-17β
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FF	follicular fluid
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
High Group	cattle with a high number of antral follicles

LH	luteinizing hormone
LHCGR	luteinizing hormone/chorionic gonadotropin receptor
Low Group	cattle with a low number of antral follicles
ME	mature equivalent
MEM-α	medium essential medium-alpha
MHz	megahertz
MIS	Müllerian Inhibiting Substance
mRNA	messenger ribonucleic acid
n	number
oFSH	ovine follicle-stimulating hormone
Ovex	ovariectomized cows
OXT	oxytocin-neurophysin I precursor
PCR	polymerase chain reaction
PG	prostaglandin F 2 alpha
r	correlation coefficient
r^2	coefficient of determination
RNA	ribonucleic acid
SCC	somatic cell count
SEM	standard error of the mean
Std	standard
TGF-β	transforming growth factor beta

INTRODUCTION

It is a long held hypothesis in reproductive biology that the inherently high variation in total number of morphologically healthy follicles and oocytes in ovaries of mammals is correlated with fertility [2-4, 6, 7, 20-26]. However, this idea has never been directly tested primarily because tedious histological procedures are required to count number of follicles and oocytes in ovaries and large numbers of individuals are required to reliably assess fertility. Therefore, simpler diagnostic procedures are needed to determine number of follicles and oocytes in ovaries. Such procedures could be used to develop new breeding schemes to select for reproductively superior farm animals, to assess impact of environmental factors such as nutrition, disease and stress on number of healthy follicles and oocytes, and to monitor alterations in number of high-quality oocytes remaining in ovaries of women to assist with family planning. Moreover, the factors that cause the high variation in follicle and oocyte numbers in ovaries of individuals have yet to be identified, and the mechanisms whereby the variation in number of follicles and oocytes *per se* may negatively impact fertility are unknown.

Previous research demonstrating that number of follicles in ovaries of single-ovulating species like cattle and women may be associated with fertility [2-4, 6, 7, 21-26] can be criticized because most follicle number measurements were taken at a single-point-in-time, at unknown stages of follicular waves, and fertility was assessed indirectly. Therefore, our laboratory utilized the well characterized bovine dominant follicle model

[27, 28] to determine: i) the extent of the variation in number of follicles growing during ovarian follicular waves of estrous cycles, and ii) if the variation in number of follicles growing during waves was associated with alterations in ovarian function and fertility in cattle.

The ovaries of young adult cattle contain ~ 18,000 to 32,000 morphologically healthy primordial follicles [3, 11]. A primordial follicle is an immature oocyte arrested in meiosis I surrounded by a single squamous layer of pregranulosal cells [29]. While mechanisms are unclear, initiation of follicular growth or recruitment occurs as the layer of granulosal cells surrounding each immature oocyte of a primordial follicle hypertrophies thus forming a primary follicle. As groups of unknown sizes of "recruited" primary follicles continue growth, they form secondary or preantral follicles. Each preantral follicle is comprised of a growing oocyte surrounded by two or more layers of granulosal cells [29]. Henceforth, preantral follicles depend on secretion of the pituitary hormone, follicle stimulating hormone (FSH), to mature into antral follicles. Antral follicles contain fluid filled cavities rich in hormones, cytokines and growth factors [30-36].

Growth and development of antral follicles occur in two or three distinct "wave-like" patterns during the 21-day bovine estrous cycle [27, 37-41]. Each follicular wave coincides with a transient rise in serum FSH concentrations in cattle and growth of dozens of very small (~ 0.5 mm in diameter) antral follicles [42]. Once antral follicles reach ~ 3 mm in diameter their growth can be monitored accurately by serial ovarian ultrasonography. Emergence is defined by ultrasonography as the first day of the estrous cycle a new growing follicle $\geq$ 4 mm in diameter is observed [28]. Emergence of

follicular waves typically begin on Days 2 and 11 of estrous cycles for cattle with two follicular waves and on Days 2, 9, and 16 for cattle with three follicular waves [see Figure 1; 43, 44]. Thus, each follicular wave is 7 to 10 days in length during the bovine estrous cycle.

About two days after emergence, a single follicle becomes the largest growing follicle of the wave [usually ~ 8 mm in diameter; 28, 45] while all other smaller follicles undergo atresia (follicle death). Hereafter, the largest growing follicle of a wave is referred to as a dominant follicle [37, 46] while all other smaller growing antral follicles destined to become atretic are referred to as subordinate follicles [43, 44, 46, 47].

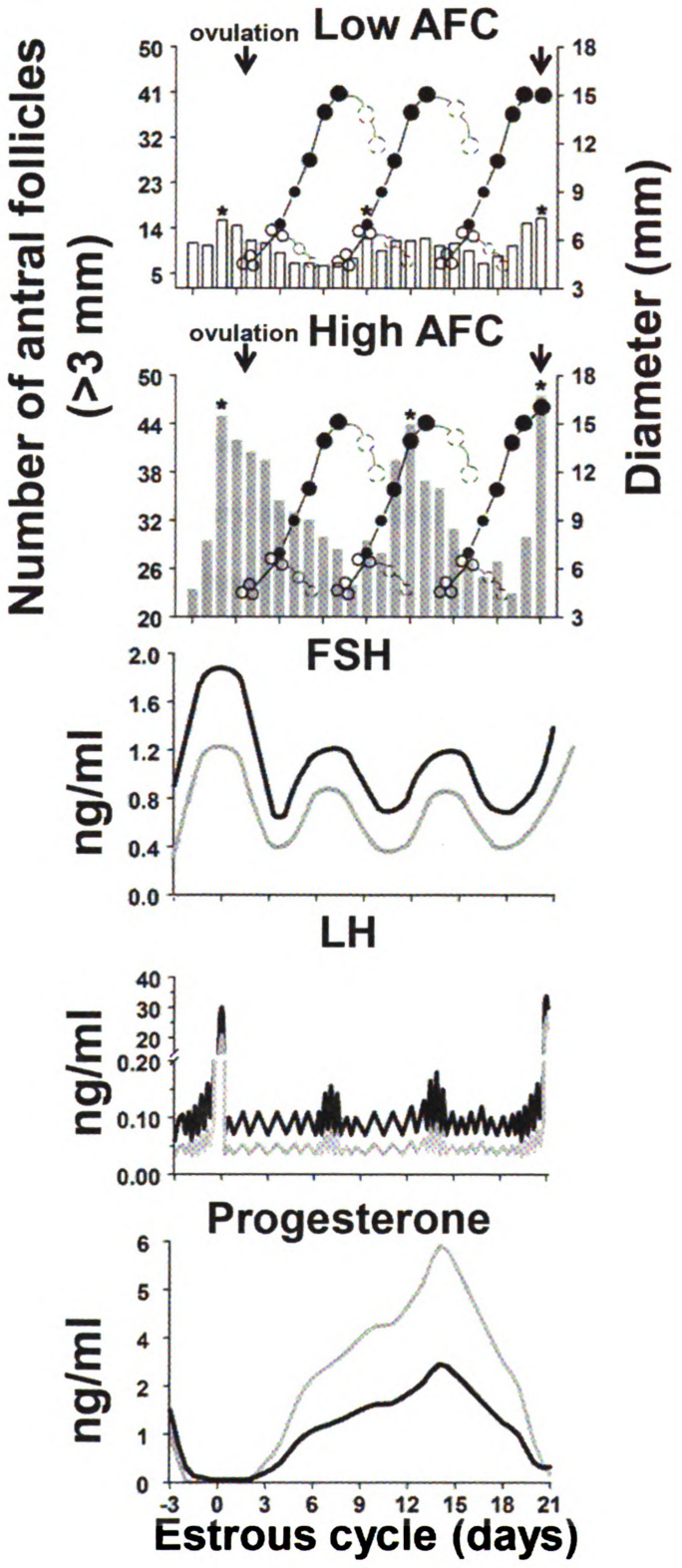
The process whereby a single growing follicle (post recruitment) becomes dominant while all other growing follicles undergo atresia is referred to as selection [28]. After selection ends during a follicular wave in cattle, the dominant follicle continues to grow from ~8 mm to ~ 15 to 20 mm in diameter then it either ovulates or undergoes atresia [28]. Dominant follicles ovulate if they develop during the follicular phase or undergo atresia if they develop during the luteal phase of the estrous cycle [48-51]. Once the dominant follicle ovulates or undergoes atresia, a new follicular wave begins. Thus, follicular waves, which begin shortly after birth, are recurrent throughout the reproductive lifespan of cattle [28].

The dominant follicle is not only distinguishable from subordinate follicles by its size, but also by the proportion of hormones in follicular fluid (FF). Estradiol concentrations are higher than progesterone in FF of estrogen-active dominant follicles [17, 49-51]. In contrast, estrogen-inactive subordinate follicles and atretic dominant follicles have higher progesterone than estradiol concentrations in FF [17, 49-51].

Figure 1. Model depicting dominant follicle development and alterations in circulating concentrations of FSH, LH and progesterone during estrous cycles of cattle with consistently low versus a high number of antral follicles growing during follicular waves.

Model depicts dominant follicle development during estrous cycles in cattle with a relatively low (open bars or black lines, $\leq$ 15 follicles $\geq$ 3 mm in diameter) versus high (gray bars or lines, $\geq$ 25 follicles) number of follicles growing during ovarian follicular waves (hereafter referred to as antral follicle count, AFC). Solid circles (●) represent the dominant follicle for each wave and open circles (○) represent subordinate follicles. Dashed circles (◌) represent atretic follicles. Arrows indicate times of ovulation for each animal. The model illustrates that despite the high variation in AFC during follicular waves among cattle, peak number of follicles growing during each follicular wave (*) is highly repeatable within individuals. Thus, cattle can be phenotyped reliably based on AFC. Model in top panel is based on studies by [9, 10]. The lower panel illustrates that circulating FSH and LH concentrations are chronically higher while progesterone is lower during estrous cycles of cattle with a low versus high AFC [Based on data from 9, 10, 13, 14, 15-18].

Figure 1


ovulation
Low AFC
ovulation
High AFC
Number of antral follicles (>3 mm)
Diameter (mm)
FSH
ng/ml
LH
ng/ml
Progesterone
ng/ml
Estrous cycle (days)

To determine the extent of the variation in number of antral follicles growing during follicular waves in cattle, our laboratory performed twice daily ovarian ultrasonography throughout two consecutive estrous cycles to determine peak number of growing follicles ≥ 3 mm in diameter during each follicular wave (hereafter referred to as antral follicle count, AFC). As depicted in Figure 1, results show that AFC is highly variable among cattle of similar ages but very highly repeatable within individuals [9, 10]. For example, AFC can be consistently as low as 8 during each follicular wave of the estrous cycle in one animal but as high as 56 in another animal [9]. Because AFC is highly variable among cattle but very highly repeatable (0.85 to 0.95) within individuals, cattle can be phenotyped reliably based on AFC.

To determine if the variation in AFC was associated with alterations in ovarian function, cattle were classified into arbitrary groups based on AFC as follows: low (≤ 15 follicles ≥ 3 mm in diameter), intermediate (16 to 24 follicles) or high [≥ 25 follicles; 9, 10]. Results show that the dynamics of dominant follicle development (e.g., emergence, length of dominance, size of dominant follicle), size of the corpus luteum (CL), and circulating estradiol concentrations during the estrous cycle are similar between cattle with low versus a high AFC. Thus, the high variation in AFC among cattle does not impact dominant follicle or CL size, or serum estradiol concentrations. However, as shown in Figure 1, cattle with low versus a high AFC have chronically higher serum FSH and LH, but much lower progesterone concentrations during estrous cycles [9, 10, 13]. In addition, cattle with low versus a high AFC have smaller ovaries, markedly reduced total number of morphologically healthy follicles and oocytes in ovaries, reduced responsiveness to superovulation, and decreased number of transferable embryos [data

not shown; 9, 10-13]. Surprisingly, the aforementioned traits in young adult cattle with low versus a high AFC are phenotypic characteristics usually associated with ovarian aging and infertility in old compared with younger cattle [1-8] and women [21, 52-63]. Thus, these findings support the overall hypothesis that variation in number of follicles and oocytes in ovaries is positively associated with fertility. However, direct studies to determine if fertility is suboptimal in young adult cattle with low versus a high AFC have not been completed, primarily because very large numbers of animals are needed to conduct statistically valid fertility trials [64]. For example, 1360 total or 660 animals per group would be needed to determine if a 10% difference in fertility exists between cattle with a low versus high AFC with 95% confidence.

Serial ovarian ultrasonography can be used to accurately determine AFC and thus phenotype cattle based on follicle numbers (Figure 1). However, this procedure is too time-consuming to routinely use to accurately phenotype cattle for a fertility trial, especially because it would involve handling each animal daily for several weeks to count number of follicles growing during the multiple follicular waves of an estrous cycle [9, 10]. In addition, although concentrations of FSH, LH, and progesterone differ between cattle with a low versus high AFC [9, 10, 12, 13, 65], as shown in Figure 1, daily blood sampling for at least a week during the same stage of an estrous cycle would be necessary to accurately determine differences in hormonal patterns for each animal. Consequently, a much simpler diagnostic method than determination of AFC is needed to phenotype cattle based on follicle numbers in ovaries.

Anti-Müllerian hormone (AMH), a member of transforming growth factor-beta (TGF-β) superfamily, is primarily produced by small growing follicles in the ovary, and

serum AMH concentrations are positively associated with number of healthy growing follicles in mice and number of antral follicles in humans [19, 63, 66, 67]. In addition, AMH concentrations remain relatively static during menstrual cycles in women [68, 69], but decrease during aging in mice and women [67, 70, 71]. Thus, serum AMH concentrations may be a marker not only for number of healthy growing follicles in ovaries, but also fertility in individuals. However, the relationship between the inherently high variation in follicle numbers and AMH concentrations in young adult cattle of similar ages has not been examined. Moreover, little is known about the regulation and role of AMH during reproductive cycles. Therefore, the bovine dominant follicle model was used in my thesis research to address the following questions:

1. Do AMH concentrations change during the bovine estrous cycle?
2. Are AMH concentrations correlated with AFC and do AMH concentrations differ between animals with low versus a high number of follicles?
3. Is a single serum AMH measurement predictive of AMH concentrations during the estrous cycle, number of antral follicles, and ovary size in cattle?
4. Is the variation in somatic cell count of dams correlated with AMH concentration in their female offspring?
5. Does follicle-stimulating hormone (FSH), which regulates follicular development and estradiol production, also regulate AMH production in granulosal cells?

6. Is FSH-induced AMH production and FSH action in granulosal cells similar for cattle with high versus a low number of antral follicles?
7. Does AMH alter FSH-induced estradiol and progesterone production in granulosal cells from cattle with high versus a low number of follicles?

CHAPTER 1

A. Do AMH concentrations change during the bovine estrous cycle?

Introduction

Anti-Müllerian hormone (AMH) is primarily produced by healthy growing preantral and antral follicles in the ovary [19]. Serum AMH concentrations are positively associated with number of primordial and growing follicles in mice [67] and number of antral follicles in humans [19, 52, 63, 66]. However, the day-to-day fluctuations in AMH concentrations are minor during estrous cycles of mice [67]; and, even though antral follicles grow in multiple "waves" during menstrual cycles of women [72, 73], serum AMH concentrations remain static in women [19, 68]. These findings support the possibility that number of healthy growing preantral and small antral follicles may be relatively constant in *individuals* during each day of their estrous or menstrual cycle, as previously reported by others for women [53, 56, 74-76] and cattle [3]. Therefore, AMH concentrations are hypothesized to remain static during the bovine estrous cycle despite occurrence of multiple waves of growth of antral follicles [28]. To test this hypothesis, the objective of the present study was to determine whether daily AMH concentrations were altered during the bovine estrous cycle.

Methods and Materials

Animals and Blood Collection

Cattle used in experiments were located at the Lyons Research Farm, University College Dublin, Ireland. All experiments were performed in compliance with protocols approved by the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876) or the European Union Directive 86/609/EC. Beef heifers (18.9 ± 0.6 months of age; n = 16 animals) were synchronized with prostaglandin $F_{2\alpha}$ (PG) injections and the ovaries were subjected to daily ultrasonography to monitor follicular development and determine day of ovulation. Jugular blood samples were taken at 11:00 AM via venipuncture at 24-hour intervals beginning on Day 6 of the estrous cycle and continuing until 1 day post ovulation (Day 2 of estrous cycle). Blood samples that corresponded with ovulatory follicular waves (6 to 8 days preceding ovulation through the day of ovulation) were analyzed for serum AMH concentrations.

AMH assay

The commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc.) was used to measure serum AMH concentrations in cattle per kit instructions [11]. The two-site AMH assay does not cross react with other members of the TGF-β superfamily including TGF-β, bone morphogenetic protein-4 (BMP-4), or activin [67]. Because AMH concentrations are relatively low in cattle in comparison with humans [69], the only modification to the assay was to measure duplicate 40 µl rather than 20 µl volumes of serum during assays. To validate use of the commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc.) with

bovine serum, parallelism of different doses of the following sources of bovine sera or follicular fluid with the AMH standard curve were evaluated: ovariectomized Holstein cows (n = 4 animals), beef steers (castrated bulls, n = 2), old Holstein cows (6.8, 10 years old, n = 2), beef heifers with a low (n = 1) or high (n = 1) antral follicle count during follicular waves, fetal calf serum, and a pool of bovine follicular fluid obtained from small antral follicles (< 5 mm in diameter). The inter- and intra-assay coefficients of variation were <16% [n = 10 assays; 11].

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Results were analyzed by linear regression analysis ($P < 0.05$) [77].

Results

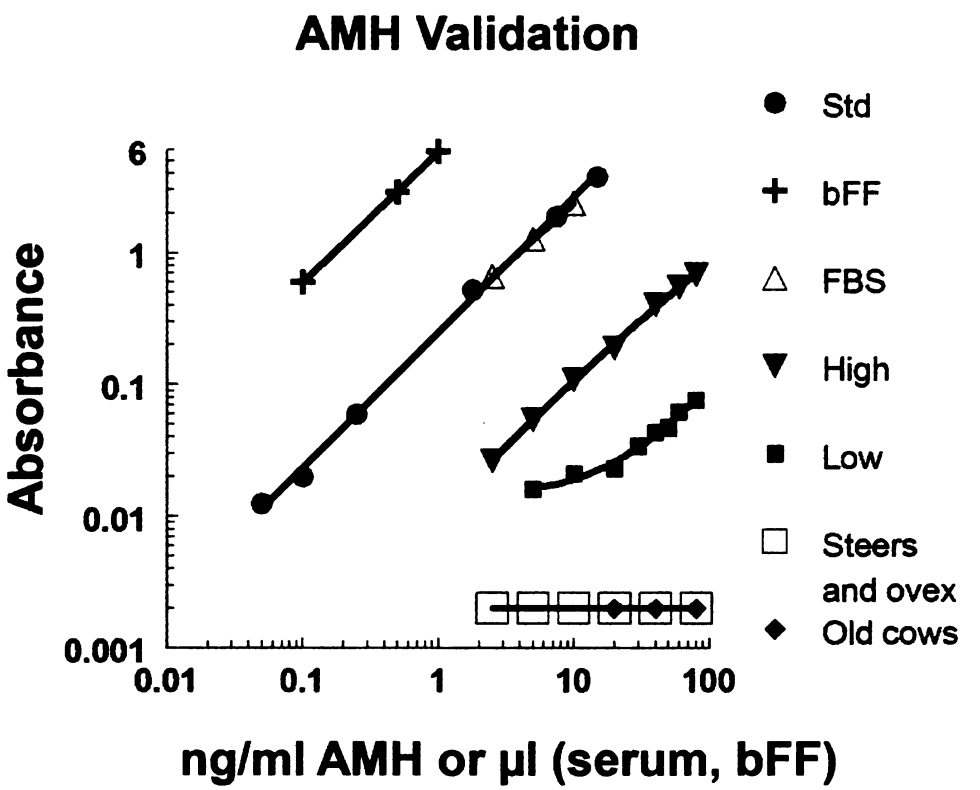
Validation of the human AMH kit to measure AMH concentrations in the bovine

AMH concentrations in the different volumes of bovine sera from nulliparous young adults, fetuses, and follicular fluids were parallel with the AMH standard curve (Figure 2). In addition, AMH was undetectable in sera from castrated male and female cattle, and older cows (6.8 and 10 years of age). These findings, coupled with previous validations of the AMH assay for humans [78, 79], confirmed that the commercial human

Figure 2: Parallelism of bovine sera and follicular fluid with the human AMH standard curve.

Anti-Müllerian hormone concentrations were determined in different volumes of bovine sera or follicular fluid using the commercial human MIS/AMH ELISA Assay Kit. Std, AMH standard curve; High, animal 8–190 with a high AFC during follicle waves (≥25 follicles); Low, animal 3–350 with a low (≤ 15 follicles) AFC during follicle waves; bFF, bovine follicular fluid, pooled sample obtained from small (> 5 mm in diameter) antral follicles; FBS, fetal bovine serum; Steers, castrated bulls (n = 2 animals); Ovex, ovariectomized cows (n = 4 animals); Old cows, sera from 6.8- and 10-year-old dairy cows. Note: AMH levels were undetectable in sera from all steers, ovariectomized cows, and old cows

Figure 2


AMH Validation
Absorbance
6
1
0.1
0.01
0.001
0.01
0.1
1
10
100
ng/ml AMH or µl (serum, bFF)
Std
bFF
FBS
High
Low
Steers and ovex
Old cows

MIS/AMH ELISA could be used reliably to measure AMH concentrations in serum of cattle [11].

Alterations in serum AMH concentrations during different days of estrous cycle

Serum AMH concentrations from 8 days prior to ovulation to the day of ovulation remained unchanged ($P > 0.72$) during the ovulatory follicular wave (Figure 3). In addition, examination of the alterations in AMH concentrations in individuals (Figure 4) demonstrated that the relatively large standard errors at each time point depicted in Figure 3 resulted primarily from the high variation in AMH concentrations among animals rather than within individuals. Nevertheless, linear regression analysis indicated that 3 of 16 individual animals had a significant ($P < 0.05$) albeit minor increase or decrease in AMH concentrations during the 8-day interval before ovulation (Figure 4).

Discussion

The most significant finding of this study demonstrated that serum AMH concentrations are highly variable among nulliparous young adult cattle, but remain relatively static in individuals during the last 6 to 8 days of their estrous cycles [11]. These results support previous findings demonstrating that daily AMH concentrations are relatively unchanged during reproductive cycles in mice [67], women [19, 63, 66, 68] and older Holstein dairy cows [80]. In contrast, although AMH concentrations are slightly lower during the luteal compared with the follicular phase of the menstrual cycle in women, overall alterations in

Figure 3. Mean concentrations of circulating AMH during ovulatory follicular waves in cattle.

Prostaglandin $F_{2\alpha}$ (PG) was used to synchronize estrus, and ovarian ultrasonography was used to map follicular development and determine day of ovulation (Day 0). Blood samples were taken daily at 11:00 AM beginning on Day 6 of the estrous cycle and ending 1 day after ovulation. Serum samples that corresponded with ovulatory follicular waves (obtained 6–8 days preceding ovulation through the day of ovulation) were analyzed for AMH concentrations. Data for each animal were aligned relative to day of ovulation. Results of linear regression analysis ($P > 0.72$) indicated there were no day to day alterations in AMH concentrations. Each point represents the mean ± standard error for 16 animals.

Figure 3

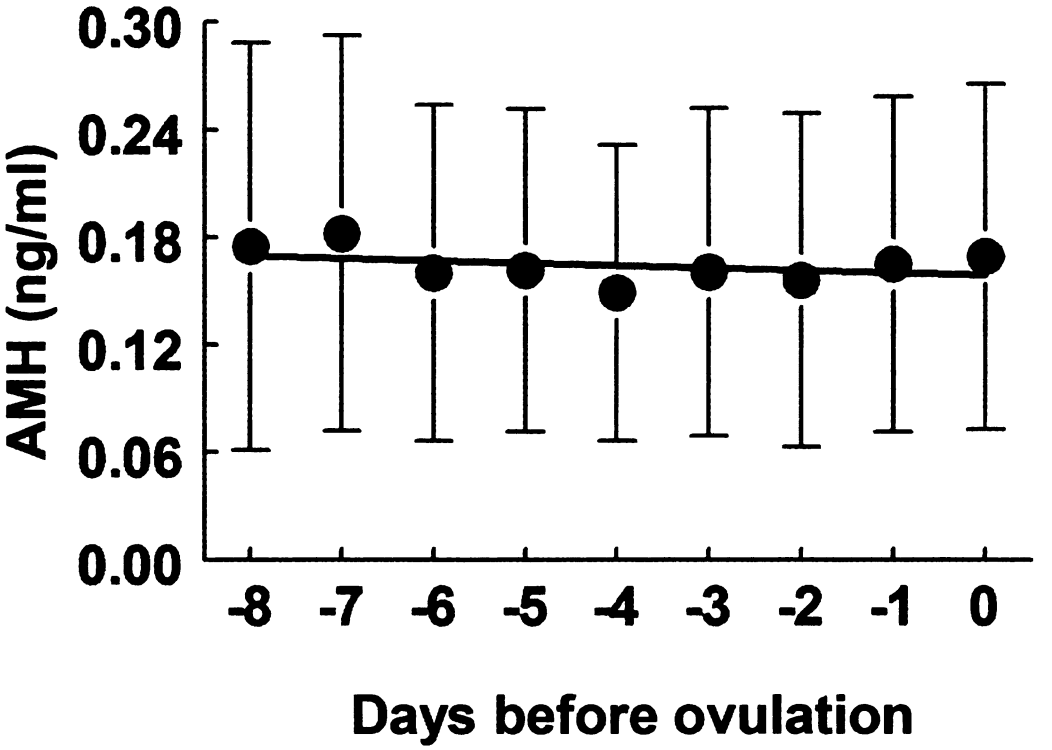
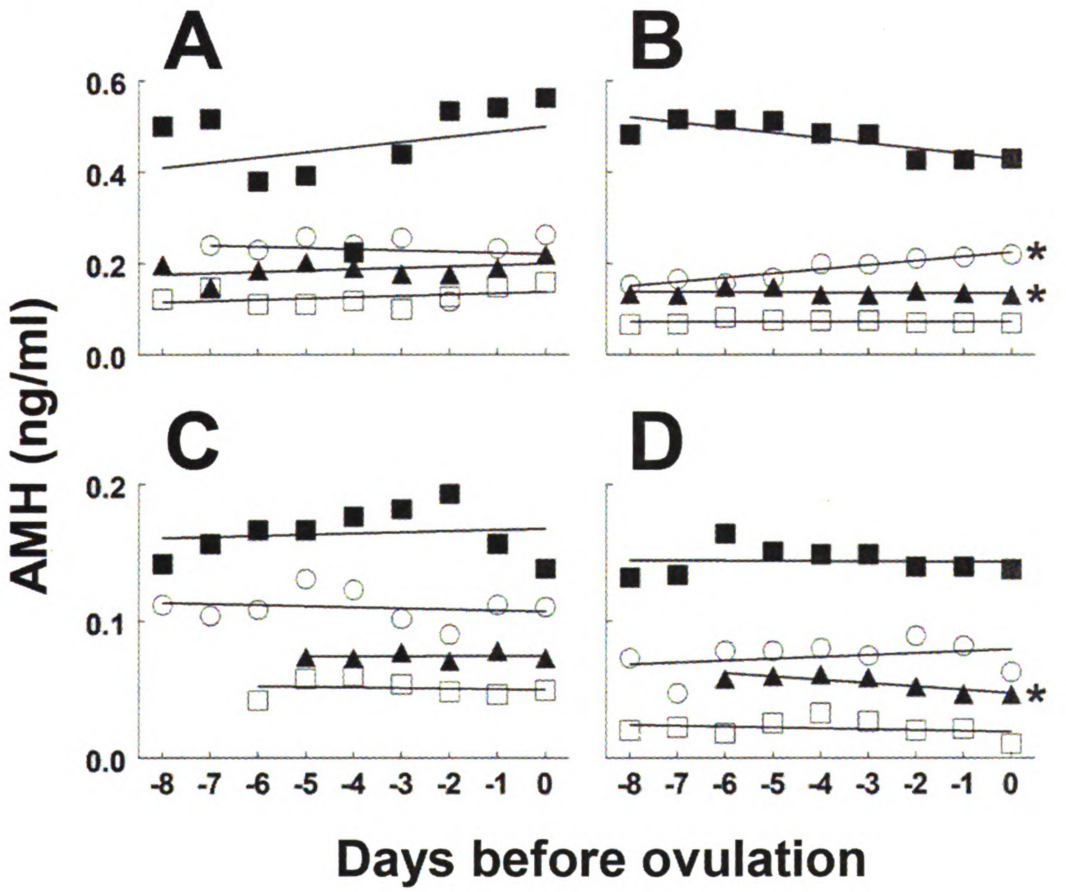

0.30
0.24
0.18
0.12
0.06
0.00
AMH (ng/ml)
-8
-7
-6
-5
-4
-3
-2
-1
0
Days before ovulation

Figure 4. Circulating AMH concentrations in individual animals during ovulatory follicular waves in cattle.

Blood samples were obtained as explained in the legend for Figure 3. Each panel represents AMH concentrations for 4 individual animals aligned relative to the day of ovulation. Lines in each figure were generated by linear regression analysis and * at the end of the line denotes significance at $P \leq 0.05$.

Figure 4


A
B
C
D
AMH (ng/ml)
0.6
0.4
0.2
0.0
0.2
0.1
0.0
-8 -7 -6 -5 -4 -3 -2 -1 0
*
*
*
Days before ovulation

serum AMH during the cycle in this study were minimal [81].

The precise reason serum AMH concentrations are static during reproductive cycles of women and cattle, despite "waves" of growth of antral follicles, is unknown. Nevertheless, unlike numbers of *large* antral follicles (≥ 3 mm), which vary greatly during follicular waves [9, 72, 73], other studies report that total number of growing preantral and smaller antral follicles remains relatively constant in cattle until 7–9 years of age [3, 74] and in women until 35–40 years of age [56, 75, 76]. This finding implies that as preantral and antral follicles in the growing pool become atretic, they are rapidly replaced by a similar number of healthy growing follicles. As already mentioned, AMH is primarily produced by granulosal cells of healthy growing preantral and small antral follicles in mice [67, 82-84], and number of these types of follicles remain relatively constant during reproductive cycles [3, 56, 74-76]. Consequently, a relatively constant number of healthy growing preantral and small antral follicles during each day of the reproductive cycle would likely explain why daily AMH concentrations also remain static during reproductive cycles of individual cattle despite follicular waves, as observed in the present study.

Analysis of alterations in daily AMH concentrations during the ovulatory wave in individual cattle showed that one animal (Figure 4, Panel A, solid squares) had an AMH value 4 days before ovulation that was much lower than her other AMH values. In addition, results of ovarian ultrasonography showed a corresponding decline in number of small (3-5 mm) growing follicles from 24 to 14. The reason for an occasional transient alteration in AMH concentration in individuals is unknown, but may potentially be explained by unexpected fluctuations in number of growing follicles, as already

mentioned, and (or) a transient suppression in AMH production, perhaps as a result of environmental factors such as acute stress or abrupt changes in diet or temperature. For example, heat stress reduces estradiol secretion [85, 86], prolongs follicular dominance, delays ovulation, and results in development of a greater number of relatively large follicles, but a reduction in number of smaller antral follicles in cattle [87, 88], which could also result in a decrease in AMH production. While it has yet to be determined if environmental changes can affect AMH production in cattle, serum AMH concentrations are reduced as follicle numbers decline during aging in humans [63, 70, 71] and mice [67]. Thus, environmental factors that reduce follicle numbers could also reduce AMH concentrations in cattle.

In summary, serum AMH concentrations are highly variable among nulliparous young adult beef heifers, but remain relatively constant in individuals during the last 6 to 8 days of their estrous cycle [11]. These results support the conclusion that, like women [19, 63, 66, 68], mice [67] and older dairy cows [80], AMH concentrations are static during estrous cycles of young adult beef heifers. In addition, these findings, coupled with histological observations that number of preantral and antral follicles remain relatively constant in women [56, 75, 76] and cattle [3, 74] during most of their reproductive lifespan, strongly support the possibility that size of the growing pool of preantral and small antral follicles remains relatively constant during reproductive cycles in cattle.

CHAPTER 2

B. Are AMH concentrations correlated with AFC and do AMH concentrations differ between cattle with low, intermediate or a high AFC?

Introduction

Circulating AMH concentrations are static during reproductive cycles of women [68, 69], mice [67], and cattle [11, 80, Figure 3, Chapter 1], but positively associated with the high variation in total number of healthy follicles and oocytes in the ovaries of mice [67] and number of antral follicles in women [52, 63, 66, 69]. However, it has yet to be determined if the high variation in AFC in cattle [9, 10], which comprises < 1% of the total number of morphologically healthy follicles in ovaries [11], are positively associated with the high variation in AMH concentrations among cattle (as shown in Figure 4, Chapter 1). Based on previous results in women [72, 73] and mice [67], AMH concentrations are, therefore, hypothesized to be positively associated with AFC and greater in cattle with high versus a lower AFC during follicular waves. To test these hypotheses, the present study determined whether AFC was also positively correlated with AMH concentrations during the bovine estrous cycle and whether AMH concentrations were higher in cattle with a high compared with a low or intermediate AFC.

Methods and Materials

Animals

Cattle used in experiments were located at the Lyons Research Farm, University College Dublin, Ireland. All experiments were performed in compliance with protocols approved by the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876), and the European Union Directive 86/609/EC, or the Institutional Animal Care & Use Committee at Michigan State University (04/08-064-00).

Identification of cattle with low, intermediate, or high AFC during follicular waves

The ovaries of each animal were monitored with an Aloka SSD-900 linear array trans-rectal probe (7.5-MHz transducer) and follicles were counted as previously described [9, 10]. Each ovary was scanned from end to end to identify the positions of the corpus luteum and antral follicles. Images of different ovarian sections were captured on the ultrasound monitor, and the locations of the corpus luteum and each antral follicle ≥ 3 mm in diameter were drawn on an ovarian map. The diameter and total number of follicles ≥ 3 mm in diameter per pair of ovaries was recorded for each animal. Two separate perpendicular measurements of diameter were averaged for each follicle. Animals were injected twice with PG spaced 11 days apart to initiate luteolysis. Ovaries were then subjected once or twice daily to ultrasonography to determine AFC beginning 1 to 2 days after the last PG injection and continuing for each animal until the completion of the study. AFC was determined for 3 to 5 follicular waves, and the average peak value

for AFC per wave was used to classify cattle arbitrarily into the Low (≤ 15 follicles), Intermediate (16 to 24 follicles) or High (≥25 follicles) Groups [9, 10].

Association of AFC with AMH concentrations

Beef heifers (18.9 ± 0.6 mo of age; n = 16 animals) were synchronized with PG injections and ovaries were subjected to daily ultrasonography to classify cattle into Low (n = 4), Intermediate (n = 8), or High Groups (n = 4) [9, 10] and to determine day of ovulation. Jugular blood samples were taken at 11:00 AM via venipuncture at 24-hour intervals beginning on Day 6 of the estrous cycle and continuing until 1 day post ovulation (Day 2 of estrous cycle). Blood samples corresponded with ovulatory follicular waves (6–8 days preceding ovulation through the day of ovulation) were analyzed for serum AMH concentrations.

AMH assay

The commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc.) was used to measure serum AMH concentrations in cattle per kit instructions [11]. Validation of the kit to measure AMH in serum of cattle is reported in Chapter 1. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, BMP4, or activin [67]. Because AMH concentrations are relatively low in cattle in comparison with humans [69], the only modification to the assay was to measure duplicate 40 µl rather than 20 µl volumes of serum during assays. The inter-assay and intra-assay coefficients of variation were <16% [n = 10 assays; 11].

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Results were analyzed by linear regression analysis ($P < 0.05$) [77].

Results

Association of AFC with AMH concentrations

Overall average serum AMH concentrations during ovulatory follicular waves per animal were highly correlated with both the average AFC (peak follicle number during each wave) during the two or three waves of an estrous cycle (Figure 5, $r = 0.88$, $P < 0.01$) and with the overall average for daily follicle counts during estrous cycles for each animal [$r = 0.92$, $P < 0.01$, data not shown; 11].

Serum AMH concentrations in animals with low, intermediate, or high AFC

Circulating AMH concentrations did not change within AFC groups ($P > 0.20$) during the 6- to 8-day bleeding period prior to ovulation, but were ~ 6- and 2-fold greater ($P < 0.01$) in animals with high or intermediate compared with low AFC during follicular waves [Figure 6; 11].

Figure 5. Correlation of AFC with AMH concentrations during the ovulatory follicular wave in cattle.

Serial ovarian ultrasonography was used to identify cattle with a consistently low ($\leq$ 15 follicles $\geq$ 3 mm in diameter per wave, n = 4 animals), intermediate (16–24 follicles, n = 8 animals), or high ($\geq$ 25 follicles, n = 4 animals) AFC during ovarian follicular waves. PG was then used to synchronize estrus, and ovarian ultrasonography was used to map follicular development and determine the day of ovulation (Day 0). Blood samples were taken daily at 11:00 AM beginning on Day 6 of the estrous cycle and ending 1 day after ovulation. Serum samples that corresponded with ovulatory follicular waves (6–8 days preceding ovulation through the day of ovulation) were analyzed for AMH concentrations. The average AMH concentration per animal was plotted relative to the average peak number of antral follicles per wave during the estrous cycle for each animal (r = correlation coefficient, n = total number of animals).

Figure 5

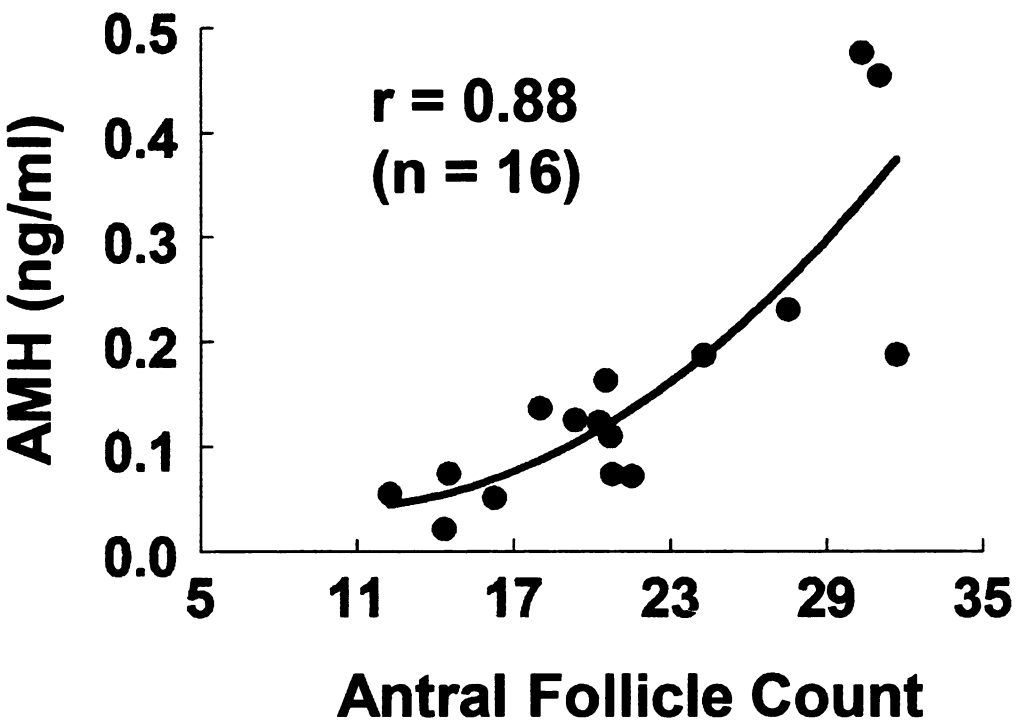
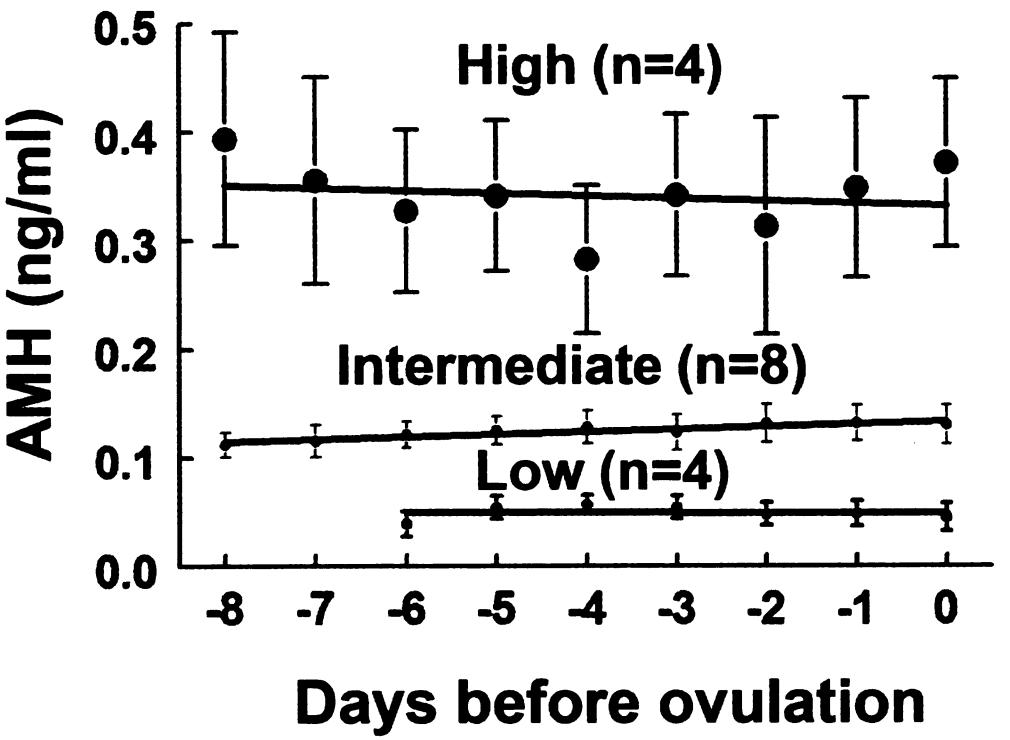

0.5
0.4
0.3
0.2
0.1
0.0
AMH (ng/ml)
r = 0.88
(n = 16)
5
11
17
23
29
35
Antral Follicle Count

Figure 6. Serum AMH concentrations in cattle with low, intermediate, or high AFC.

Serial ovarian ultrasonography was used to identify cattle with a consistently low ($\leq$ 15 follicles $\geq$ 3 mm in diameter per wave, n = 4 animals), intermediate (16–24 follicles, n = 8), or high ($\geq$ 25 follicles, n = 4 animals) AFC during ovarian follicular waves. PG was used to synchronize estrus, and ovarian ultrasonography was used to map follicular development and determine the day of ovulation (Day 0). Blood samples were taken daily at 11:00 AM beginning on Day 6 of the estrous cycle and ending 1 day after ovulation. Serum samples that corresponded with ovulatory follicular waves (6–8 days preceding ovulation through day of ovulation) were analyzed for AMH concentrations. Data for each animal were aligned relative to the day of ovulation and data were plotted based on the results of a linear regression analysis ($P > 0.20$) for each group. Each point represents the mean $\pm$ SEM for 4 or 8 animals and n = total number of animals in each group.

Figure 6


0.5
0.4
0.3
0.2
0.1
0.0
AMH (ng/ml)
High (n=4)
Intermediate (n=8)
Low (n=4)
-8
-7
-6
-5
-4
-3
-2
-1
0
Days before ovulation

Discussion

Cattle with low versus a high AFC also have higher serum FSH and LH but lower progesterone concentrations during estrous cycles [9, 10, 12, 13]. However, unlike FSH, LH, and progesterone, which vary considerably during estrous cycles of individual cattle [9, 10, 13-18], AMH concentrations remain static during reproductive cycles [19, 63, 66-68, 80]. Moreover, the variation in AMH concentrations among individuals is positively associated with number of healthy growing follicles in ovaries [19, 52, 63, 66, 67, 71]. The most significant findings of the present study demonstrated that: 1) circulating AMH concentrations are highly positively associated with average daily follicle counts during all follicular waves and with the average AFC (peak follicle number per wave), and 2) AMH concentrations are static, but much greater during estrous cycles of cattle with high or intermediate versus a low AFC. AFC is also highly positively associated with ovary size and total number of morphologically healthy follicles and oocytes in the ovarian reserve of cattle [11]. Taken together, these findings illustrate that the high variation in AMH concentrations observed among individual cattle in the present study is positively linked not only to the high variation in AFC, but also to the inherently high variation in the ovarian reserve [11]. This important observation, coupled with results showing that AMH concentrations are static during reproductive cycles[Chapter 1; 19, 63, 66-68, 80], strongly indicate that measurement of AMH concentrations on any day of the estrous cycle could be a simple, non-invasive method to predict reliably the relative size of ovarian reserves in young adult cattle. This finding has practical importance, as already mentioned, because future studies can take advantage of this discovery to conduct large field trials with sufficient numbers of animals to firmly establish if the variation in AMH

concentrations and correspondingly AFC and size of the ovarian reserve are positively linked with fertility in cattle.

CHAPTER 3

C. Is a single serum AMH measurement predictive of AMH concentrations during the estrous cycle, number of antral follicles, and ovary size in cattle?

Introduction

Cattle can be phenotyped reliably based on AFC [10], and AFC is highly positively associated with ovary size, total number of morphologically healthy follicles and oocytes in ovaries, and circulating concentrations of AMH in cattle [11]. Although AMH concentrations are highly variable among cattle, like AFC, alterations in daily serum AMH concentrations during estrous cycles are static in individuals [Chapter 1, 11, 80] similar to results for mice [67] and women [52, 68, 69]. These important findings support the hypothesis that a single AMH measurement taken on any day of the estrous cycle is representative of AMH concentrations throughout the estrous cycle and positively associated with AFC, ovary size, and total number of morphologically healthy follicles in ovaries of cattle. To test this hypothesis, the present study examined whether a single AMH measurement was correlated with the average of multiple AMH measurements made on different days of the estrous cycle, number of antral follicles, and ovary size in cattle.

Methods and Materials

Animals

Cattle used in experiments were at one of three different locations: 1) the Lyons Research Farm, University College Dublin, Ireland; 2) Michigan State University Beef Cattle Teaching and Research Centers, East Lansing, Michigan; or 3) Green Meadow Farms Inc., Elsie, Michigan. All experiments were performed in compliance with protocols approved by the Animal Research Ethics Subcommittee, University College Dublin, the Cruelty to Animal Act (Ireland, 1876), and the European Union Directive 86/609/EC, or the Institutional Animal Care & Use Committee at Michigan State University (04/08-064-00).

Association of a single AMH concentration with the average of multiple AMH concentrations on different days of an estrous cycle in beef heifers

Cattle used for this experiment were from two different studies [11, 13]. In Study 1, conducted at the Lyons Research Farm in Ireland, the estrous cycles of beef heifers (18.9 ± 0.6 months of age; n = 18 animals) were synchronized with PG injections spaced 11 days apart, and the ovaries were subjected to daily ultrasonography to determine the day of ovulation. Jugular blood samples were taken at 11:00 AM via venipuncture at 24-hour intervals beginning on Day 6 of the estrous cycle and continuing until 1 day post ovulation (Day 2 of estrous cycle). In Study 2, conducted at Michigan State University Beef Cattle Teaching and Research Center, blood samples were collected from the coccygeal vein of beef heifers (12–14 months of age, n = 7 animals) via venipuncture. The first blood sample was taken immediately before first PG injection, whereas

additional samples were taken 48 hours after the last injection of PG and daily until 4 days after ovulation. A total of 4 to 8 daily samples from the 25 animals in the two studies were analyzed for serum AMH concentrations. To examine the relationship between a single versus the average for multiple AMH measurements during the estrous cycle, each single AMH measurement was compared with the overall mean of the 4 to 8 daily AMH measurements in the same individual.

Association of a single AMH concentration with the average of multiple AMH concentrations on different days of an estrous cycle in dairy heifers

Blood samples were collected via venipuncture from the coccygeal vein of Holstein dairy heifers (12–15 months of age; n = 23 animals) located at Green Meadow Farms Inc. Elsie, Michigan. The first blood sample was taken on an unknown day during the estrous cycle, followed immediately by a PG injection to induce luteolysis. The second blood sample was taken 11 days after the first blood sample and immediately before the second PG injection. The last blood sample was taken 4 days after the second blood sample (and PG injection). The three blood samples were analyzed for serum AMH concentrations. Each single AMH measurement was compared with the overall mean for the three AMH measurements in the same individual.

Association of a single AMH concentration with follicle number and ovary size in dairy cattle

Purebred Holstein heifers from Green Meadow Farms Inc. (12–15 months of age; n = 275 animals) were administered 2 PG injections 11 days apart. A single coccygeal

vein blood sample was removed from each animal 96 hours after the second PG injection (~ 0 to 2 days after ovulation) and analyzed for serum AMH concentrations. Immediately before blood sampling, ovarian ultrasonography with an Aloka SSD-900 linear array trans-rectal probe (7.5-MHz transducer) was used to determine total number of follicles ≥ 3 mm in diameter as previously described [9, 10]. Because of the large number of animals in this study, AFC was not determined to avoid significant disruption of routine management practices at Green Meadow Farms Inc., caused by the handling of each animal daily for several weeks to reliably determine AFC [10]. After each ovary was scanned from end to end, the largest ovary image was "frozen" on the ultrasound monitor and the height and length of each ovary image were determined with an internal calibrator and recorded [11]. Total ovary area (hereafter referred to as ovary size) was calculated by combining the surface area (3.1416 x (length/2) x (height/2)) for each ovary.

AMH concentrations were highly variable among cattle ranging from 6.25 to 432.5 pg/ml in the present study. Thus, to evaluate the relationship between AMH concentrations, follicle number and ovary size, frequency distribution analysis was used to determine number of animals at each 20-pg/ml increment of AMH concentration from 5 to > 85 pg/ml (e.g., 5-25, 26-45, 46-65, 66-85, > 85 pg/ml). AMH increments stopped at > 85 pg/ml because of the sparse distribution of the remaining 66 animals that had AMH concentrations > 85 pg/ml within each subsequent 20-pg/ml interval. Frequency distribution was utilized to determine if mean follicle number and ovary size differed between groups of animals at the different 20-pg/ml AMH intervals.

AMH assay

The commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc.) was validated previously (Chapter 1) and used to measure serum AMH concentrations in cattle per kit instructions. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, bone morphogenetic protein-4 (BMP-4), or activin [67]. Because AMH concentrations are relatively low in cattle in comparison to humans [69], the only modification to the assay was to measure duplicate 40 µl rather than 20 µl volumes of serum from beef heifers and 80 µl rather than 40 µl or 20 µl volumes of serum from dairy heifers during assays. The inter- and intra-assay coefficients of variation for beef and dairy cattle were <15% (n = 11 assays) and <23% (n = 11 assays), respectively.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Linear regression analysis ($P < 0.05$) was used to compare single AMH measurement with the overall mean for the 3-8 AMH measurements in the same individual [77]. Pearson correlation analysis was used to determine the association between AMH measurements, number of follicles ≥ 3 mm in diameter, and ovary size. A frequency distribution analysis was used to further evaluate the relationship between AMH concentrations, follicle number and ovary size by grouping animals at each 20-pg/ml increment of AMH concentration from 5 to > 85 pg/ml (e.g., 5-25, 26-45, 46-65, 66-85, > 85 pg/ml).

Results

Association of a single AMH concentration with the average of multiple AMH concentrations on different days of an estrous cycle in beef and dairy heifers

Each single AMH measurement from an individual beef heifer, regardless of which day during the estrous cycle the measurement was made, was very highly correlated ($r = 0.98$, $P < 0.01$; Figure 7) with the overall mean of 4 to 8 daily AMH measurements in the same individual. Similarly, results from dairy heifers confirm findings from beef heifers and show that a single AMH measurement at a random stage of the estrous cycle and before PG injections was very highly correlated ($r = 0.97$, $P < 0.01$; Figure 8) with the overall mean for the three daily AMH measurements made before and on several different days after PG.

Association of a single AMH concentration with follicle number and ovary size in dairy cattle

The results of a Pearson correlation analysis indicated that a single AMH measurement was moderately associated with number of follicles ($r = 0.54$, $P < 0.01$) and ovary size ($r = 0.32$, $P < 0.01$) in dairy cattle. Frequency distribution analysis showed that the proportion of the total number of animals ($n = 275$) within each 20-pg/ml increment of AMH from 5 to > 85 pg/ml ranged from 15 to 24%. The average number of follicles was greater ($P < 0.1$ to $P < 0.01$) for animals that had AMH concentrations >45 pg/ml compared with the animals with AMH concentrations between 5-25 pg/ml.

Figure 7. Association of a single AMH measurement with the average of multiple AMH measurements on different days of an estrous cycle in beef heifers.

Blood samples were obtained from beef heifers (18.9 ± 0.6 months of age, n = 18 animals, 12-14 months of age, n = 7 animals) on different days of the estrous cycle from animals in Study 1 and Study 2 as explained in Materials and Methods. Data from Studies 1 and 2 were combined for statistical analysis. Each symbol represents a single AMH measurement for each animal versus the average of 4 to 8 AMH measurements for that animal. The line depicts the results of linear regression analysis (n = 25 animals; r = correlation coefficient = 0.98, r^2 = coefficient of determination = 0.97; $P < 0.01$).

Figure 7

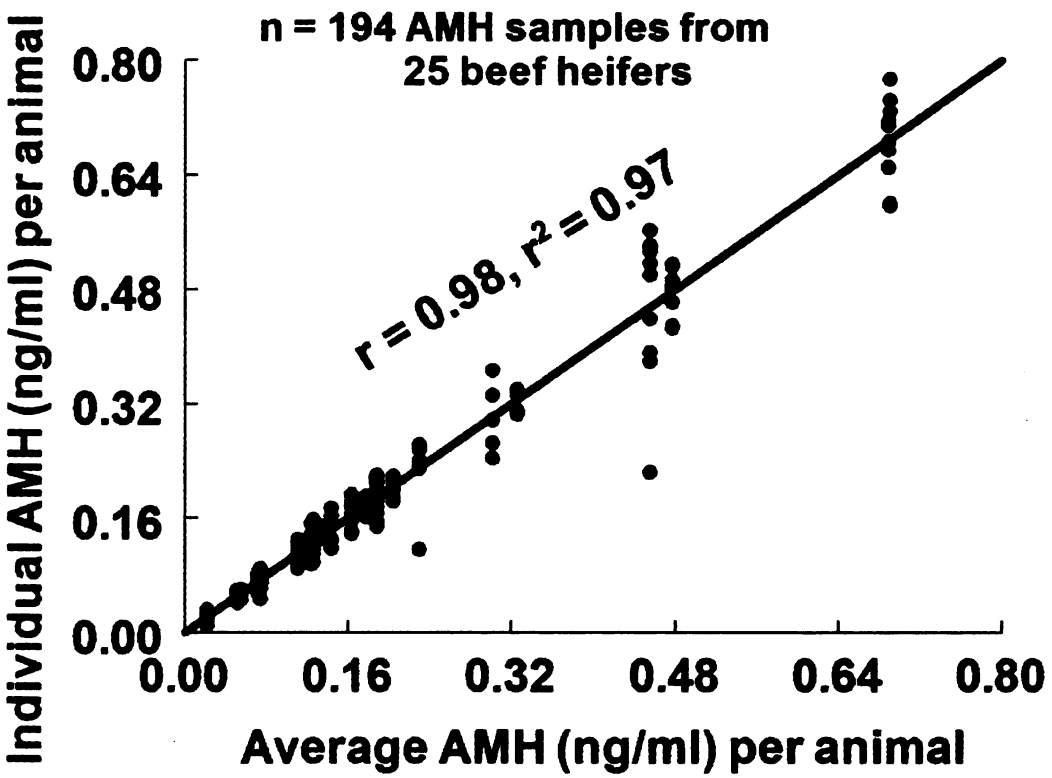
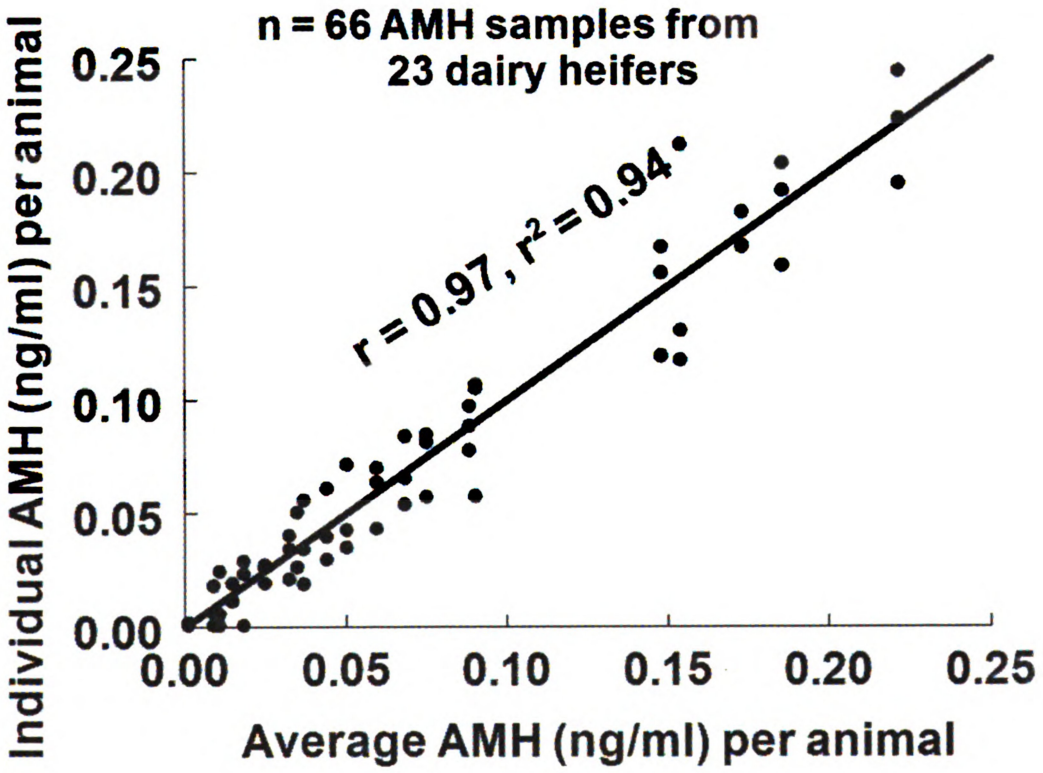

n = 194 AMH samples from
25 beef heifers
r = 0.98, r² = 0.97
Individual AMH (ng/ml) per animal
Average AMH (ng/ml) per animal
0.00
0.16
0.32
0.48
0.64
0.80

Figure 8. Association of a single AMH measurement with the average of multiple AMH measurements on different days of an estrous cycle in dairy heifers.

Blood samples were obtained from purebred Holstein heifers (12-15 months of age; n = 23 animals) located at Green Meadow Inc. in Elsie, Michigan. The first blood sample was taken on an unknown day during the estrous cycle, followed immediately by a PG injection to induce luteolysis. The second blood sample was taken 11 days after the first blood sample and immediately before the second PG injection. The last blood sample was taken 4 days after the second blood sample (and PG injection). Each symbol represents a single AMH measurement for each animal versus the average of 3 AMH measurements for that animal. The line depicts the results of linear regression analysis (r = correlation coefficient = 0.97, r^2 = coefficient of determination = 0.94; $P < 0.01$).

Figure 8


n = 66 AMH samples from
23 dairy heifers
r = 0.97 , r² = 0.94
Individual AMH (ng/ml) per animal
Average AMH (ng/ml) per animal
0.00
0.05
0.10
0.15
0.20
0.25

The average ovary size was greater ($P < 0.1$ to $P < 0.01$) for animals with AMH concentrations > 65 pg/ml when compared with animals with AMH concentrations between 5-25 pg/ml (Figure 9).

Discussion

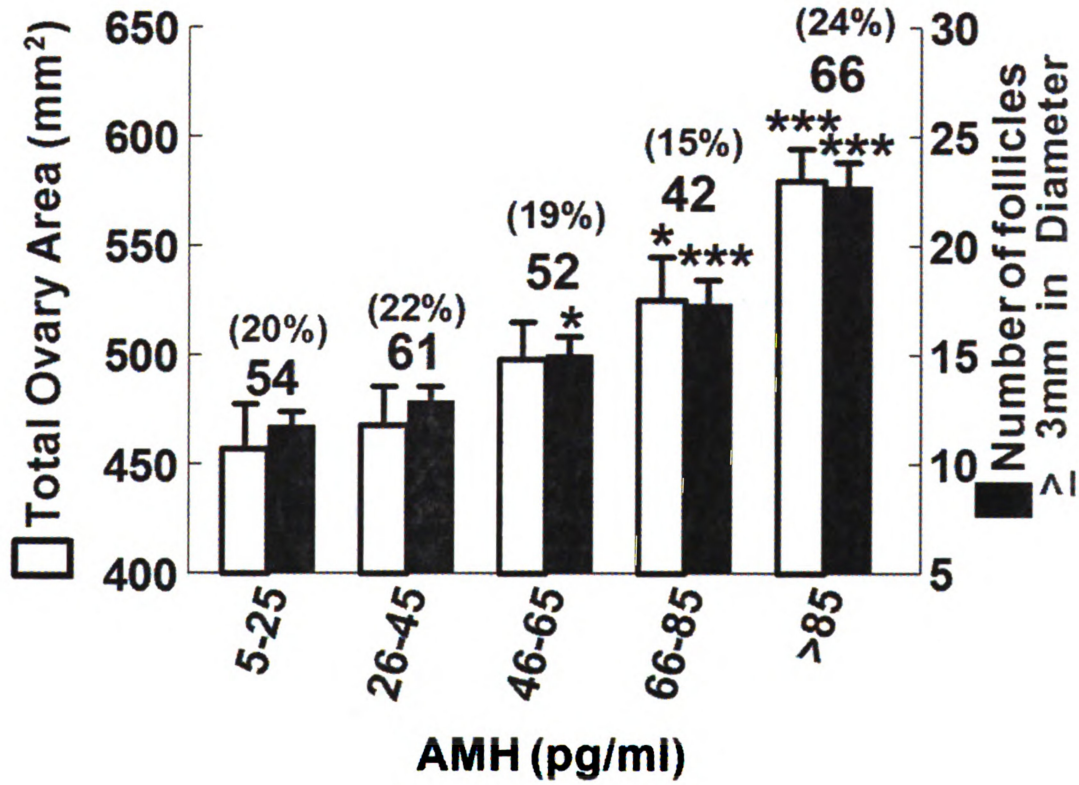
The most significant finding of the present study demonstrated that a *single* AMH measurement was nearly identical to the average for multiple daily AMH measurements on different days of an estrous cycle and was correlated with number of follicles and ovary size in cattle. These findings, coupled with the high degree of association between AFC and size of the ovarian reserve in cattle [11], indicate that a single AMH measurement during the estrous cycle is a reliable diagnostic marker to predict relative AFC, ovary size, and total number of morphologically healthy follicles and oocytes in ovaries of age-matched, young adult cattle.

The present study rigorously tested whether a single AMH measurement was reflective of multiple AMH measurements on different days of the estrous cycle. AMH measurements were made on randomly chosen days of an estrous cycle and before and after PG-induced luteolysis in beef and dairy heifers. The results showed that any single AMH measurement was very highly correlated with the average for all AMH measurements during the estrous cycle. Indeed, only 3 of 260 AMH measurements differed markedly from the linear regression lines (see Figure 7 and Figure 8). Moreover, our previous report shows that only 1 of 16 animals had daily AMH concentrations that varied significantly during an estrous cycle (see Chapter 1, Figure 4). Taken together, these findings provide strong evidence that, in the vast majority of cattle, a single AMH

Figure 9. Distribution of cattle and the corresponding average for number of follicles and ovary size at each 20-pg/ml interval of AMH.

Purebred Holstein heifers (12-15 months of age; n = 275 animals) were given two PG injections 11 days apart. A single coccygeal vein sample was removed from each animal 96 hours after the second PG injection (~ 0 to 2 days after ovulation) and analyzed for serum AMH concentrations. Immediately before blood sampling, ovarian ultrasonography was used to determine the total number of follicles ≥ 3 mm in diameter and length and height of each ovary as previously described [9, 10]. Total ovary area was calculated by combining the surface area (3.1416 x (length/2) x (height/2)) for each ovary. A frequency distribution plot shows proportion of the total number of animals (n = 275) ranged from 15 to 24% at each 20-pg/ml interval of AMH from 5 to > 85 pg/ml, as explained in Materials and Methods. Results also depict the number of follicles ≥ 3 mm in diameter and ovary size for groups of animals at each 20 pg/ml increment of AMH. Asterisk indicates means differ significantly (* = $P < 0.1$, *** = $P < 0.01$) from the mean for animals in the 5-25 pg/ml of AMH group.

Figure 9


Total Ovary Area (mm²)
650
600
550
500
450
400
30
25
20
15
10
5
Number of follicles ≥ 3mm in Diameter
(20%)
54
(22%)
61
(19%)
52
*
(15%)
42
*
***
(24%)
66
***
***
5-25
26-45
46-65
66-85
>85
AMH (pg/ml)

measurement, regardless of stage of the estrous cycle or breed, is very highly correlated with the overall average for multiple AMH measurements on different days of the estrous cycle. This important finding supports previous studies showing that AMH concentrations are static not only during estrous cycles in cattle [11, 80], but also during reproductive cycles in mice [67] and women [52, 68, 69].

Number of follicles growing during each day of a follicular wave in individual cattle vary 200 to 400%, whereas the peak number of growing follicles during each of the two or three waves of an estrous cycle (AFC) is highly repeatable ($r = 0.85$ to 0.95) within individuals and typically varies only ~ 10 to 30% [9, 10]. Moreover, as already mentioned, AFC is highly correlated with AMH concentrations in cattle [11] and with ovary size and total number of morphologically healthy follicles in the ovarian reserve of cattle [11]. Because a single determination of follicle number at an unknown stage of a follicular wave rather than AFC was determined for cattle in the present study, this may explain why AMH concentrations were not as highly correlated with follicle number ($r = 0.54$) or ovary size ($r = 0.32$) in dairy heifers as previously reported for beef heifers [$r = > 0.88$; 11]. Nevertheless, even though AFC was not determined in the present study, AMH concentrations, follicle numbers and ovary size were moderately correlated. This finding, coupled with results of previous studies demonstrating a positive association between AMH concentration and number of follicles in women [63, 66, 69], mice [67], and cattle [11], strongly supports the conclusion that a single AMH measurement is a reliable diagnostic marker to predict relative number of follicles, ovary size, and correspondingly the total number of morphologically healthy follicles and oocytes in the ovarian reserve in cattle.

CHAPTER 4

D. Is the variation in somatic cell count of dams correlated with AMH concentration in their female offspring?

Introduction

The factors that cause or contribute to the inherently high variation in number of follicles and oocytes, AMH concentrations, and ovary size [11], which may negatively impact ovarian function and fertility in cattle [2-4, 6, 7, 11, 24-26] are unknown. Nevertheless, numerous environmental factors during pregnancy such as exposure to toxicants [89], excessive hormones [90], disease [91-93], or inadequate nutrition [94, 95] negatively impact embryo development, and reduce follicle numbers and ovary size in human and bovine embryos [94, 96, 97]. Based on these findings, a chronic mammary gland infection or inflammation in dairy cows is hypothesized to negatively impact ovarian development in offspring. To test this hypothesis, the present study examined whether somatic cell count (SCC) during pregnancy of dairy cows was associated with circulating AMH concentrations in their young adult daughters. AMH was measured because it is a reliable biomarker for total number of morphologically healthy follicles and oocytes in ovaries and ovary size and function in cattle [9-13]. SCC was measured because maternal infection or inflammation during gestation, such as a mastitis, which results in a high SCC in milk [$\geq$ 200,000 cells/ml; 98, 99-103], could potentially

negatively impact embryo development and in turn ovary development and function in the embryo.

Methods and Materials

Animals

Purebred Holstein heifers (12-15 months of age; n = 275 animals) from Green Meadow Farms Inc. (Elsie, MI) were administered two PG injections 11 days apart. A single coccygeal vein sample was removed from each animal 96 hours after the second PG injection (~ 0 to 2 days after ovulation) and analyzed for serum AMH concentrations. All experiments were performed in compliance with protocols approved by the Institutional Animal Care & Use Committee at Michigan State University (04/08-063-00).

Dairy Cow Records

Dairy Comp 305 software [104] was used to access records for each heifer's dam at Green Meadow Farms Inc. Records were used to determine the age of the dam at birth of each heifer, level of milk production, as determined by the 305 mature equivalent (ME, average milk production for 305 days adjusted for dam age and season of calving) during gestation of the daughter, and the somatic cell count (SCC) in milk from two months prior to pregnancy and during pregnancy (5 to 7 measurements recorded). Records for milk production and average SCC were only available for dams with one or more calves (n = 192 of 275 dams).

Association of number of SCC measurements ≥ 200,000 in individual dairy cows with AMH concentrations in their daughters and age and level of milk production for each heifer's dam

The SCC measurements (n = 5 to 7 per cow) from two months prior to pregnancy and during pregnancy were very highly variable both within (13,000 to 9,052,000 cells/ml) and among (13,000 to 9,701,000 cells/ml) dairy cows in the present study. Thus, a single very high SCC measurement could distort the overall mean SCC for an individual and not be indicative of a chronic mammary gland infection. Moreover, a portion of the high variation in the 5 to 7 SCC measurements for each animal may be representative of a normal physiological rather than pathological range. Consequently, use of the overall mean SCC two months before and during pregnancy is probably not the best method to identify cows with potential chronic mammary gland infections or inflammation. Alternatively, SCC measurements ≥ 200,000 are routinely used as an index of recurrent udder infections or inflammation in dairy cattle [98-103].

To evaluate the potential impact of a chronic mammary gland infection or inflammation on reproductive function in their daughters, each dam was grouped based on the number of times her SCC measurements were ≥ 200,000 cells/ml of milk. Whether number of SCC measurements ≥ 200,000 in individual dairy cows was associated with AMH concentrations in their daughters and with the dam's age and level of milk production was then determined.

AMH assay

The commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc.) was previously validated (Chapter 1) and used to measure serum AMH concentrations in cattle per kit instructions. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, bone morphogenetic protein-4 (BMP4), or activin [67]. Because AMH concentrations are relatively low in cattle in comparison to humans [69], the only modification to the assay was to measure duplicate 80 µl rather than 20 µl volumes of serum from dairy heifers during assays. The inter- and intra-assay coefficients of variation were <23% (n = 11 assays).

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). AMH concentrations in heifers and age and milk production were grouped based on number of SCC measurements ≥ 200,000 for each heifer's dam. Results were analyzed by quadratic or linear regression analysis, and a t-test was used to determine if means differed ($P < 0.05$) among the SCC groups [77].

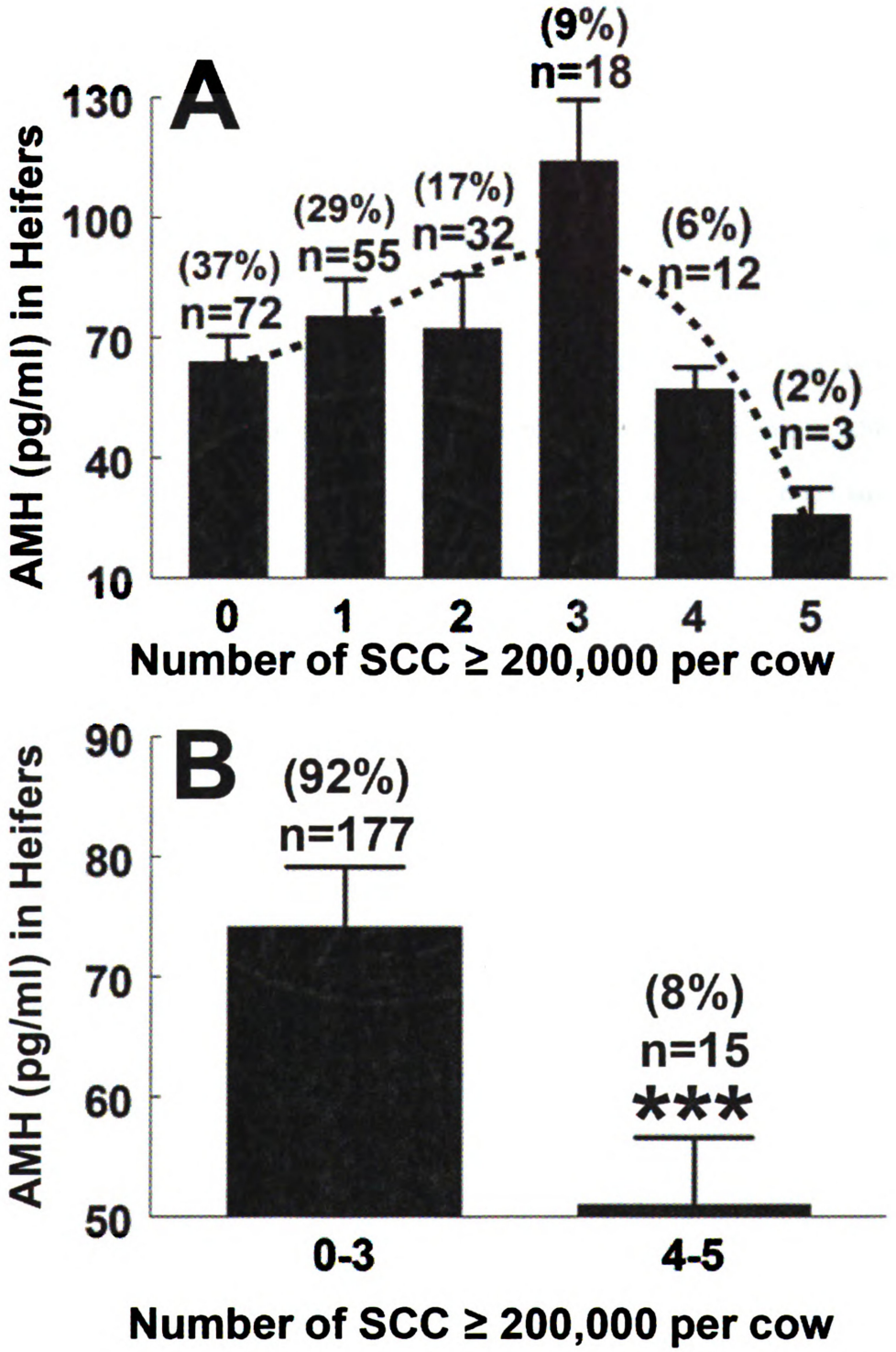
Results

Number of SCC measurements ≥ 200,000 ranged from 0 to 5 per cow, and AMH concentrations in heifers decreased ($P < 0.02$) as number of SCC measurements ≥200,000 per cow increased from 0 to 5 in their dams (Figure 10A). Although the average AMH

Figure 10. Association of number of SCC measurements ≥ 200,000 in cows with alterations in AMH concentrations in their daughters.

AMH concentrations were determined in young adult Holstein heifers (n = 192). Each heifer was grouped based on number of SCC measurements 2 months before and during pregnancy (n = 5 to 7 measurements per cow) that were ≥ 200,000 in their dams (range = 0 to 5 measurements ≥ 200,000 per cow). Number of cows and percentage of the herd for each SCC group are depicted above each bar. In Panel A, the dotted regression line shows that AMH concentrations in heifers decrease ($P < 0.05$) as number of SCC measurements ≥ 200,000 increases in their dams. Panel B shows the combined average for AMH concentration for daughter's of cows with 4 or 5 SCC ≥ 200,000 compared with the combined average for AMH concentration for daughters of cows with 0 to 3 SCC ≥ 200,000. Asterisk indicates a significant difference (*** = $P < 0.01$) between groups.

Figure 10


A
AMH (pg/ml) in Heifers
130
100
70
40
10
(37%)
n=72
(29%)
n=55
(17%)
n=32
(9%)
n=18
(6%)
n=12
(2%)
n=3
0
1
2
3
4
5
Number of SCC ≥ 200,000 per cow
B
AMH (pg/ml) in Heifers
90
80
70
60
50
(92%)
n=177
(8%)
n=15
***
0-3
4-5
Number of SCC ≥ 200,000 per cow

concentration was similar ($P > 0.10$) among SCC groups, the combined average for AMH concentration for daughter's of cows with 4 or 5 SCC $\geq$ 200,000 was much lower ($P < 0.01$) compared with the combined average for AMH concentration for daughters of cows with 0 to 3 SCC $\geq$ 200,000 (Figure 10B).

Number of SCC measurements $\geq$ 200,000 in dams was also positively associated ($P < 0.01$; Figure 11A) with the dam's age. Although individual means were similar ($P > 0.10$), the combined average age of cows with 4 or 5 SCC measurements $\geq$ 200,000 was 1.3 years greater ($P < 0.01$) compared with the combined average age for cows with 0 to 3 SCC measurements $\geq$ 200,000 (Figure 11B). Number of SCC measurements $\geq$200,000 also tended ($P < 0.10$) to be inversely associated with level of milk production (data not shown).

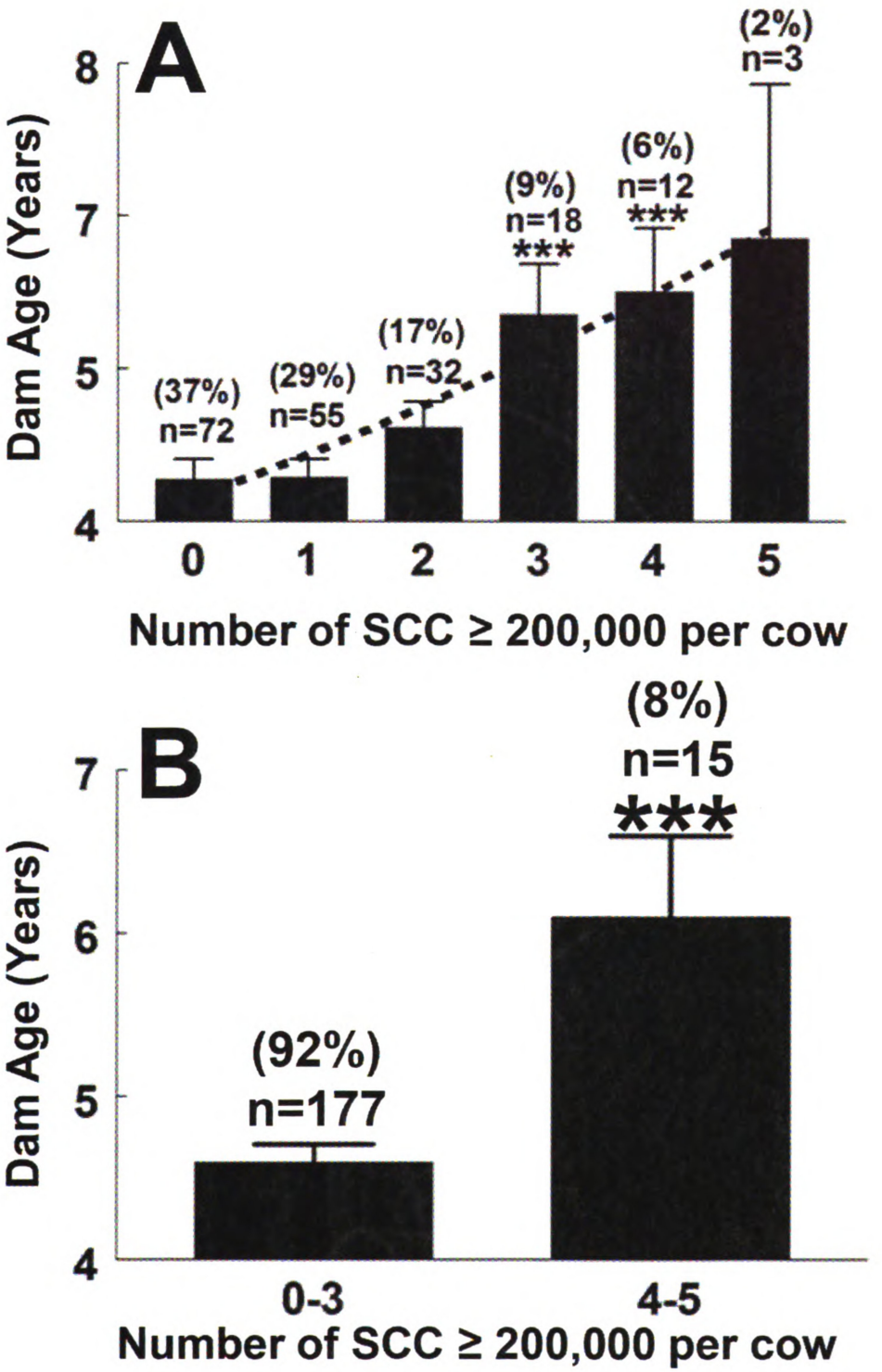
Discussion

The most significant finding of the present study indicated that dairy cows with the highest number of SCC measurement $\geq$ 200,000 were older, tended to have lower milk production, and had daughters with much lower serum AMH concentrations as nulliparous young adults compared with the dairy cows with a lower number of SCC measurements $\geq$ 200,000. Others also report that older dairy cattle have a higher incidence of mastitis [105-107] and lower milk production [108-110]. However, our study is the first to link a potential chronic mammary gland infection/inflammation in dairy cows with potentially diminished ovarian development and function in their daughters.

Figure 11. Association of age with number of SCC measurements ≥ 200,000 in cows.

Holstein cows (n = 192) were grouped based on number of SCC measurements (n = 5 to 7 total measurements per cow) that were ≥ 200,000 (range = 0 to 5 measurements ≥ 200,000 per cow). Number of cows and percentage of the herd for each SCC group are depicted above each bar. In Panel A, the dotted regression line shows that number of SCC measurements ≥ 200,000 per individual increased as age of cows increased ($P < 0.01$). Panel B shows the combined average age of cows with 4 or 5 SCC ≥ 200,000 compared with the combined average age of cows with 0 to 3 SCC ≥ 200,000. Asterisk indicates a significant difference (*** = $P < 0.01$) between groups.

Figure 11


A
Dam Age (Years)
8
7
5
4
(37%)
n=72
(29%)
n=55
(17%)
n=32
(9%)
n=18
***
(6%)
n=12
***
(2%)
n=3
0
1
2
3
4
5
Number of SCC ≥ 200,000 per cow
B
Dam Age (Years)
7
6
5
4
(92%)
n=177
(8%)
n=15
***
0-3
4-5
Number of SCC ≥ 200,000 per cow

While the mechanism whereby a chronic mammary gland infection during pregnancy may negatively impact ovary development in offspring is unclear, mastitis enhances maternal secretion of cytokines such as interleukin-1β and interferon-α [111-114]. Interleukin-1β reduces proliferation of endometrial stromal cells [115] while interferon-α decreases LH secretion and progesterone production [116, 117]. Consequently, significant alterations in secretion of these maternal factors would be expected to alter uterine function [118], which in turn, may have a negative impact on embryo development and thus also potentially compromise ovary development and function in embryos. In addition, several studies show that maternal environment in otherwise healthy individuals can have a negative impact on ovarian development and function of offspring. For example, pregnant sheep exposed to environmental factors such as excessive androgens have smaller offspring at birth, and these offspring have a reduced ovarian reserve and earlier onset of infertility as adults compared with controls [90]. In addition, nutrition restriction during pregnancy in sheep results in fewer follicles developing beyond the primordial stages in offspring [119], and calves born to nutritionally restricted mothers have 60% lower AFC compared with calves born to control mothers [94]. Moreover, girls born with low gestational birth weights, potentially caused by the undernourishment of the mother [95], have reduced ovulation rates [120] and reduced uterine and ovary size [96, 97]. Taken together, it is possible that potentially inadequate nutrition of the older pregnant dairy cows with relatively low milk production and chronic mammary gland infection in the present study could also have a negative impact on ovarian development during fetal life and potentially explain why AMH concentrations were diminished in their daughters as nulliparous young adults.

In conclusion, although mechanisms are unclear, our results imply that a chronic mammary infection or inflammation during pregnancy of cows (as predicted by a high number of SCC measurements $\geq$ 200,000) is associated with lower AMH concentrations, and correspondingly diminished ovarian development and function and perhaps reduced fertility of their daughters.

CHAPTER 5

E. Does FSH, which regulates follicular development and estradiol production, also regulate AMH production in granulosal cells?

Introduction

Granulosal cells produce AMH [19], and circulating AMH concentrations are positively associated with number of healthy growing follicles in several species [52, 63, 66, 67, 71, 121] including cattle [11, 80]. Thus, the high variation in number of antral follicles [9-11] may explain why AMH concentrations are also highly variable among cattle (e.g., up to ~72-fold among young adult Holstein heifers, Chapter 3). However, the factors (e.g., hormones, growth factors) that regulate AMH production by granulosal cells have not been thoroughly investigated.

FSH is a key hormone that regulates granulosal cell differentiation and function, follicle growth and survival, and estradiol production [122]. Because AMH is produced exclusively in females by granulosal cells of healthy growing follicles [19], FSH may also regulate AMH production. For example, treatment of adult rats with human recombinant FSH or estradiol benzoate decreases abundance of AMH and AMH receptor type 2 (AMHRII) mRNAs in granulosal cells of preantral and small antral follicles [84]. In addition, serum AMH concentrations decrease in women treated with FSH during ovarian stimulation protocols while estradiol concentrations increase [121, 123-125].

Similarly, as women age and number of follicles decline, serum AMH concentrations decrease [63, 71] coincident with an increase in circulating FSH concentrations [56, 63, 121, 126-130]. Moreover, our laboratory has recently demonstrated that serum AMH concentrations are inversely correlated with circulating concentrations of FSH in age-matched heifers [11]. Based on these findings, FSH is hypothesized to have a negative impact on AMH production by bovine granulosal cells. To test this hypothesis, the present study determined if: 1) FSH decreased capacity of granulosal cells to produce AMH, and 2) removal of androgen substrate, which is required for estradiol production, modulated FSH-induced alterations in AMH production. Unlike previous studies, the present study utilized a long-term serum-free bovine granulosal cell culture system [13, 131, 132] to *directly* test the effect of FSH on granulosal cell AMH production.

Methods and Materials

Long-term bovine granulosal cell culture

Granulosal cells were cultured in serum-free media as described previously [131] with some modifications. Briefly, ovaries from beef and dairy cows at random stages of the estrous cycle were obtained from JBS Packing Company Inc. (Plainwell, MI), placed into a bottle containing ice-cold supplemented Dulbecco's phosphate buffered saline solution (DPBS), and transported to the laboratory. Granulosal cells from 3 to 5 mm follicles were collected and pooled in a 15-ml centrifuge tube containing MEM-α culture media supplemented with sodium bicarbonate (10mM), HEPES (20mM), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), Fungizone-amphotericin B (0.625μl/ml), nonessential amino acids (1.1mM), bovine insulin (1 ng/ml), long R3-IGF-I (2 ng/ml),

sodium selenite (4 ng/ml), apo-transferrin (5μg/ml), and androstenedione (10^{-6} M). After harvest from follicles, the cells were washed with culture media three times and resuspended in 2 ml media. Cell number was estimated by a Coulter Counter Particle Z1 (Beckman Coulter, Inc., Fullerton, CA) while cell viability was estimated using Trypan Blue dye exclusion [133]. Cells (100,000 live cells per well) were plated in 96-well Falcon Primaria plates and cultured at 37°C in a humidified atmosphere (5% CO_2 and 95% air). During culture, 75% of media was removed and replaced with fresh media on days 2 and 4 of culture, and cultures were terminated after 6 days of culture.

Estradiol and progesterone assays

Commercially available RIA kits (Diagnostic Products Corp., Los Angeles, CA) previously validated by our laboratory [13, 134, 135] were used to measure concentrations of estradiol and progesterone in spent media. Estradiol assay sensitivity was 0.5 pg/ml and progesterone assay sensitivity was 0.05 ng/ml [13, 131]. Intra- and interassay coefficients of variation for both RIA assays were < 8%. Results were expressed as pg or ng per 30,000 cells.

AMH assay

A commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc., Brea, CA), which was validated for use in cattle [Chapter 1; 11], was used to measure AMH concentrations in spent media per kit instructions. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, bone morphogenetic protein-4 (BMP-4), or activin [67]. The inter- and

intra-assay coefficients of variation were <7%. AMH concentrations in different volumes of spent media collected from granulosal cells treated with 0 or 25 ng/ml ovine FSH (oFSH) were parallel with the AMH standard curve, and AMH concentrations were undetectable in media (data not shown).

Messenger RNA Analyses

Total RNA was isolated from granulosal cells using the RNeasy mini kit (Qiagen, Valencia, CA) per kit instructions. RNA was treated with DNase to remove genomic DNA contamination and reverse transcribed [136]. Expression of AMH and oxytocin mRNAs was analyzed by real-time quantitative PCR [137]. Primers were designed using either Primer Express (Applied Biosystems, Foster City, CA) or PerlPrimer [138] for bovine sequences in Genbank, and the amplicon sizes ranged from 73 to 269 bp (Table 1). Copies of AMH and oxytocin mRNAs were quantified using the standard curve method for absolute quantification [139] and expressed as copies of target gene per 10,000 copies of β-actin.

Study 1: Effect of FSH and removal of androgen substrate on AMH production and abundance of AMH mRNA in bovine granulosal cells.

Granulosal cells were treated with 0, 0.01, 0.05, 0.1, 0.5, 1, 10, or 25 ng/ml oFSH (AFP7558C; National Hormone and Pituitary Program, Baltimore, MD) for 6 days. Each FSH dose was replicated 12 times. On day 4 of culture, 75% media was removed as explained earlier. However, cells were washed twice with fresh media with or without androstenedione (10^{-6} M), and cells were then cultured

Table 1: Gene name, accession number, and forward (F) and reverse (R) primer sequences used for quantitative real-time PCR analysis of AMH and OXT mRNA in bovine granulosal cells.

Gene	Primer Sequence	GenBank accession no.
anti-Müllerian hormone (AMH)	F: 5'-CAGGGAAGAAGTCTTCAGCA-3'	M13151
	R: 5'-AAGGTGGTCAAGTCACTCAG-3'	
oxytocin-neurophysin I precursor (OXT)	F: 5'-CCGGCCCCCGATACC-3'	V00114
	R: 5'-TCATTGTCATAATTCCTAGGGATGATT-3'	

for two additional days with or without androstenedione and the different doses of oFSH. To terminate cultures on day 6, 75% of the media was removed from each well and stored at -20°C until subsequent measurements of estradiol and AMH. Wells were then washed with 150 µl DPBS twice, incubated with trypsin-EDTA (0.125mg/well), and cells were removed from each well by trituration. Cell numbers were determined from 3 pools of granulosal cells (2 wells per pool x 3 pools) per FSH dose. Estradiol and AMH concentrations were expressed as ng or pg per 30,000 cells. Total RNA was isolated from 1 pool of granulosal cells (6 wells) per oFSH dose. Abundance of AMH mRNA was expressed as copies of AMH per 10,000 copies of β-actin. Each experiment was replicated three times.

Study 2: Effect of FSH on progesterone production and abundance of oxytocin mRNA in bovine granulosal cells.

Enhanced progesterone production and abundance of oxytocin mRNA are well established markers for luteinization of granulosal cells [140]. Previous studies demonstrated that relatively high doses of FSH increase progesterone and oxytocin production during culture of bovine granulosal cells [141-144]. Therefore, media and cells obtained from Study 1 were measured for progesterone production and abundance of oxytocin mRNA, respectively, as explained in Study 1, to determine if doses of FSH induced luteinization of granulosal cells. Progesterone concentrations were expressed as ng progesterone per 30,000 cells while abundance of oxytocin mRNA was expressed as copies of oxytocin per 10,000 copies of β-actin mRNA.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Data are presented as means ± SEM for all experiments. Results were analyzed statistically using a linear regression or a multivariate ANOVA followed by Tukey-Kramer test to determine if means differed. Data were log transformed when necessary to meet the assumptions of normality [77]. A value of $P \leq 0.05$ was considered significant [77].

Results

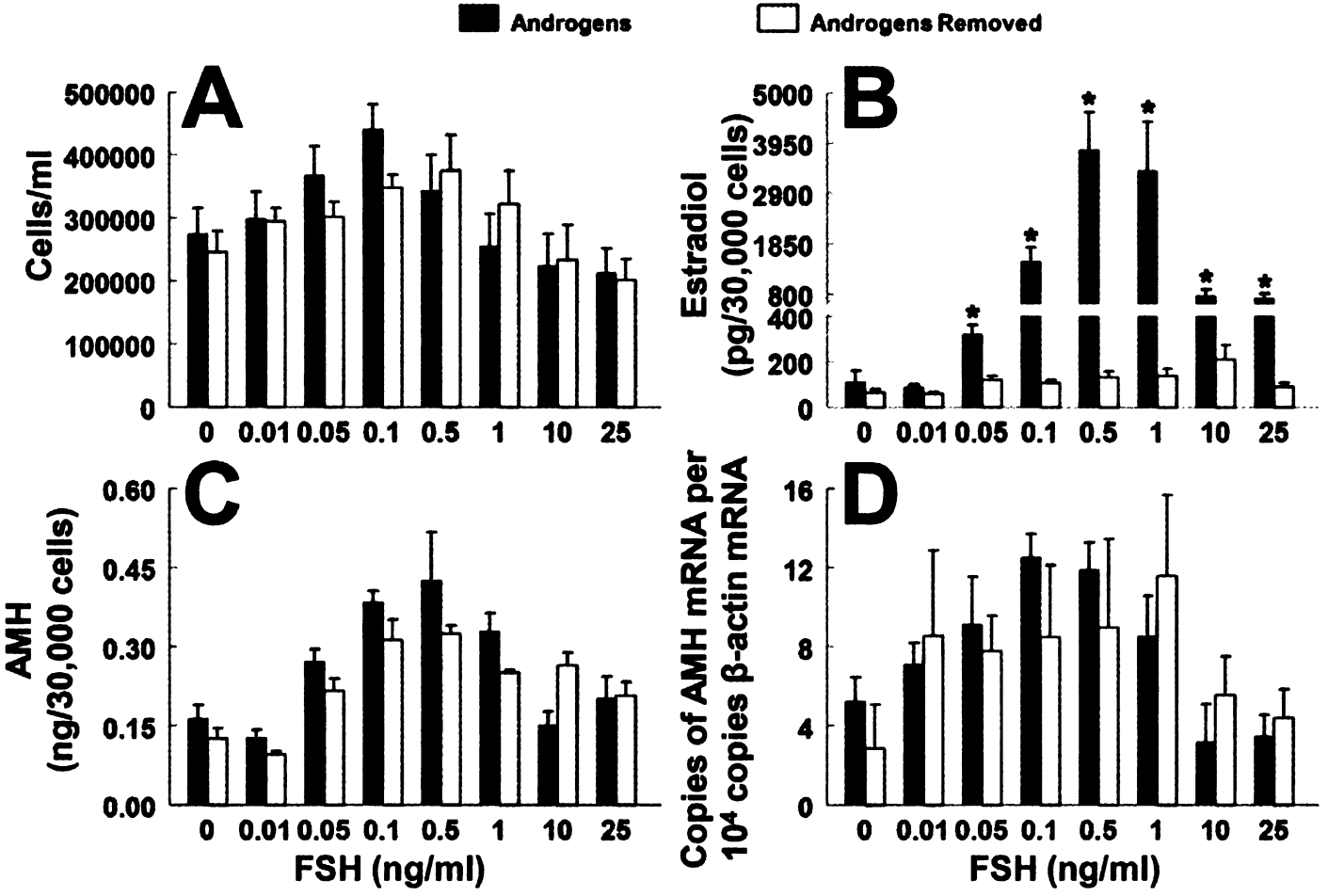
Study 1: Effect of FSH and removal of androgen substrate on AMH production and abundance of AMH mRNA.

Treatment of granulosal cells with doses of FSH up to 0.5ng/ml did not alter ($P > 0.60$) cell numbers, but resulted in a dose-dependent linear increase ($P < 0.01$) in estradiol and AMH concentrations in media and abundance of AMH mRNA (Figure 12 solid bars). In contrast, treatment of cells with doses of FSH > 0.5 ng/ml resulted in a linear decrease ($P < 0.01$) in estradiol and AMH concentrations and abundance of AMH mRNA. Removal of androgens decreased ($P < 0.01$) overall FSH-stimulated estradiol production 74% (Figure 12B), but did not alter ($P > 0.10$) AMH production or abundance of AMH mRNA (Figure 12C and 12D) compared with cells cultured with androstenedione. Removal of androstenedione from media also did not alter ($P > 0.93$) cell numbers (Figure 12A).

Figure 12. Effect of FSH and removal of androgen substrate during culture on bovine granulosal cell numbers, estradiol and AMH production, and abundance of AMH mRNA.

Granulosal cells were treated with various doses of FSH for 6 days. Cell numbers (Panel A), estradiol production (Panel B), AMH production (Panel C) and abundance of AMH mRNA (Panel D) were measured on Day 6 of culture as described in Materials and Methods. Estradiol and AMH production were normalized to 30,000 cells and abundance of AMH mRNA was expressed as copies of AMH mRNA per 10,000 copies β-actin mRNA in the same samples. Cell number and estradiol bars represent the mean $\pm$ SEM for 9 pools of granulosal cells (3 experiments x 3 pools per experiment) and AMH production and AMH mRNA bars represent the mean $\pm$ SEM for 3 pools of granulosal cells (3 experiments x 1 pool per experiment). Results of ANOVA and linear regression analysis are reported in Results. Asterisk indicates a significant difference ($P \leq 0.05$) between cells cultured with or without androstenedione for each FSH dose.

Figure 12


Androgens
Androgens Removed
A
Cells/ml
500000
400000
300000
200000
100000
0
B
Estradiol (pg/30,000 cells)
5000
3950
2900
1850
800
400
200
0
C
AMH (ng/30,000 cells)
0.60
0.45
0.30
0.15
0.00
D
Copies of AMH mRNA per 10^4 copies β-actin mRNA
16
12
8
4
0
0 0.01 0.05 0.1 0.5 1 10 25
FSH (ng/ml)

Study 2: Effect of FSH on progesterone production and abundance of oxytocin mRNA in culture bovine granulosal cell.

FSH doses < 1 ng/ml did not alter progesterone concentrations in media and FSH doses < 0.5 ng/ml did not alter abundance of oxytocin mRNA. However, FSH doses ≥1ng/ml increased ($P < 0.01$) progesterone concentrations and abundance of oxytocin mRNA in a linear fashion (Figure 13), which coincided with the decrease in estradiol (Figure 12B, solid bars) and AMH production (Figure 12C, solid bars) and abundance of AMH mRNA (Figure 12D, solid bars).

Discussion

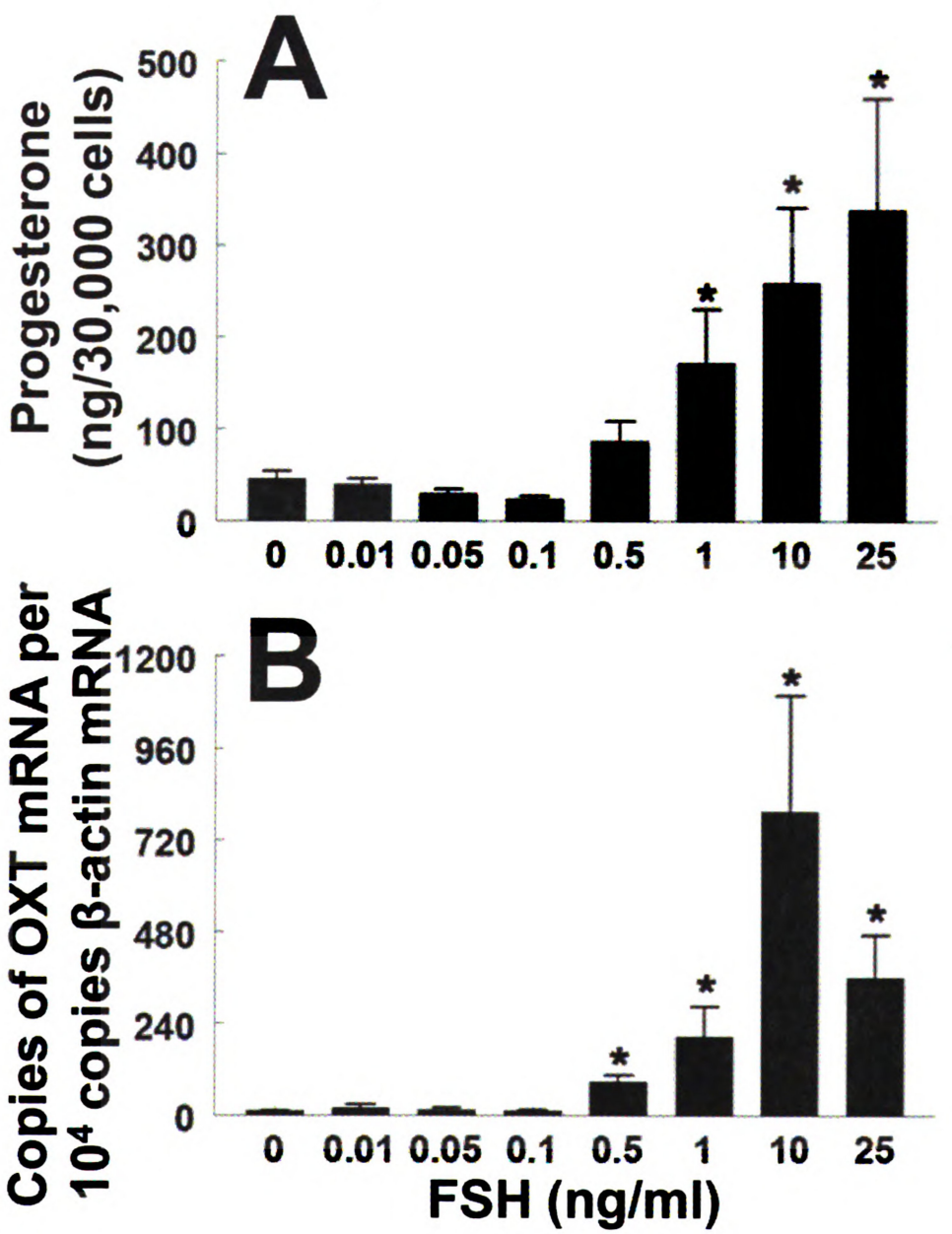
The most significant findings of the present study demonstrated that during culture of bovine granulosal cells: 1) relatively low doses of FSH increased AMH production and abundance of AMH mRNA concomitant with an increase in estradiol production, 2) the decline in AMH and estradiol production following treatment with relatively high doses of FSH may be caused by luteinization of granulosal cells, and 3) removal of androgen substrate and the corresponding marked reduction in estradiol concentrations had no effect on the FSH-induced capacity of granulosal cells to produce AMH. These results imply that FSH has a key role in modulation of the capacity of bovine granulosal cells to produce AMH.

AMH is produced primarily by granulosal cells of morphologically healthy small growing preantral and antral follicles in mice [19]. However, the major cell or follicle type that produces AMH in cattle is unknown. The present study used a serum-free culture system to determine the direct effect of FSH on AMH production by bovine

Figure 13. Effect of FSH on progesterone production and abundance of oxytocin mRNA in cultured bovine granulosal cells.

Granulosal cells were treated with various doses of FSH for 6 days as explained in the legend for Figure 12. Progesterone production was normalized to 30,000 cells and oxytocin mRNA expressed as copies of oxytocin mRNA per 10,000 copies β-actin mRNA. Bars for progesterone values represent the mean ± SEM for 9 pools of granulosal cells (3 experiments x 3 pools per experiment) and bars for oxytocin mRNA values represent the mean ± SEM for 3 pools of granulosal cells (3 experiments x 1 pool per experiment). Results of linear regression analysis are explained in Results. Asterisk indicates a significant difference ($P < 0.05$) compared with the untreated (0 ng/ml FSH) group.

Figure 13


A
Progesterone (ng/30,000 cells)
500
400
300
200
100
0
0
0.01
0.05
0.1
0.5
1
10
25
B
Copies of OXT mRNA per 10⁴ copies β-actin mRNA
1200
960
720
480
240
0
FSH (ng/ml)

granulosal cells isolated from relatively small [3 to 5 mm in diameter; for comparison ovulatory follicles = 15 to 20 mm in diameter in cattle; 145] antral follicles. Results demonstrated that treatment of bovine granulosal cells with concentrations of FSH ≤ 0.5 ng/ml, which are similar to circulating serum FSH concentrations in cattle [9, 10], increases AMH production and abundance of AMH mRNA. In contrast to the positive role of FSH on AMH production in granulosal cells in vitro, results of previous in vivo studies imply that FSH may have a negative role in regulation of AMH production. For example, FSH treatment during controlled ovarian hyperstimulation decreases circulating AMH concentrations in women [121, 123-125] and inhibits AMH mRNA expression in rats [84]. Age-matched cattle with a low AFC and relatively high circulating FSH concentrations also have lower serum AMH concentrations [11] and lower expression of AMH mRNA in granulosal cells [12] compared to cattle with a high AFC and lower circulating FSH concentrations. While the precise reason for the difference in FSH action on AMH production between the in vivo and in vitro studies is unknown, several explanations are plausible. For example, none of the in vivo studies established if the decrease in AMH production was caused by FSH treatment directly, or indirectly by FSH-induced alterations in growth of different follicle types with differing capacities to produce AMH, as previously suggested [123, 146, 147]. In women, for example, FSH treatment administered during controlled ovarian hyperstimulation protocols stimulates small antral follicles to develop into much larger antral follicles [148]. Studies in women show that preantral and small antral follicles (≤ 4 mm) have the highest levels of AMH expression [149], and small antral follicles have the highest follicular fluid AMH concentrations [150]. In contrast, large antral follicles (4-8 mm) have a reduced or no

AMH expression [149] and very low follicular fluid AMH concentrations [> 9 mm; 150]. Consequently, the decrease in serum AMH concentrations following exogenous FSH treatment in women may have been caused by a reduction in the number of small antral follicles, which have the highest AMH production.

In further support of the possibility that FSH may have a differential impact on AMH production by granulosal cells from different follicle types, previous studies reported that serum AMH concentrations are positively correlated with number of small antral follicles (< 12 mm), but not with number of large growing antral follicles (≥ 12 mm) in patients following ovarian hyperstimulation [147]. Taken together, these findings imply that a FSH-induced decrease in number of small antral follicles, rather than a direct negative effect of FSH on AMH production by granulosal cells, could explain the decrease in circulating serum AMH concentrations following exogenous FSH treatments [123, 146, 147].

The decrease in circulating AMH concentrations following FSH treatments in vivo [121, 123-125] could also have been caused by use of potentially high physiological or pharmacological doses of FSH during ovarian stimulation. Indeed, results of our present in vitro study show that relatively high doses of FSH diminish AMH production in granulosal cells. While the reason AMH production is diminished by FSH is unknown, results of the present studies show that high FSH doses induce luteinization of granulosal cells. For example, doses of FSH > 0.5 ng/ml triggered a linear decrease in AMH and estradiol production while simultaneously enhancing progesterone production and abundance of oxytocin mRNA in granulosal cells. These divergent alterations in estradiol and progesterone production and abundance of oxytocin mRNA are the well

established hallmarks of luteinization of bovine granulosal cells [140]. In support of our results, previous studies using bovine granulosal cells also show that relatively high doses of FSH increase progesterone and oxytocin production [141-144]. Moreover, AMH expression is limited or undetectable in corpora lutea compared with non-luteinized follicles in rats, horses, and women [84, 151, 152]. Taken together, these findings imply that the decrease in AMH production following exogenous FSH treatment [84, 121, 123-125] may not only be caused by a decrease in number of small growing follicles, but also by FSH-induced suppression of AMH production in granulosal cells, as shown by the negative impact of relatively high doses of FSH on granulosal cell AMH production in the present study.

In contrast to results of the present study, results of several studies using granulosal cells from humans show that FSH has no effect on regulation of AMH production. For example, FSH does not alter AMH mRNA expression in cultured human granulosa-luteal cells retrieved from ovulatory follicles after gonadotropin stimulation [153], and a 5 ng/ml dose of human FSH does not alter AMH production by human granulosal cells [150]. However, as previously discussed, capacity of granulosal cells to produce AMH in response to FSH may depend not only on follicle type [e.g. small antral or dominant; 84, 149, 150] used as a source of granulosal cells for culture studies, but also dose of FSH.

FSH-induced production of AMH was paralleled by an increase in estradiol in the present study, implying that estradiol could also have a role in regulation of AMH production [84]. To evaluate this possibility, androgen substrate (androstenedione) was removed from culture media during the last two days of the 6-day culture period to

minimize estradiol production. Results showed that despite the significant reduction in estradiol there were no differences in AMH concentrations in media or abundance of AMH mRNA between granulosal cells treated with or without androgen substrate. These findings implied that FSH rather than estradiol may be the primary regulator of AMH production in granulosal cells. In support of the key role for FSH in AMH regulation, others report that use of a gonadotropin-releasing hormone antagonist to block FSH action also inhibits AMH production by human granulosal cells [154]. However, a potential role for estradiol in AMH production cannot be eliminated in our study because estradiol concentrations were not reduced to zero. Moreover, other factors regulated by FSH, such as inhibin, activin or BMPs [155], could have a role in regulation of AMH production in granulosal cells.

In summary, the present study demonstrated that physiological doses of FSH increase AMH production by bovine granulosal cells, while higher doses of FSH induce luteinization of granulosal cells and a corresponding decrease in AMH production. In addition, removal of androgen substrate during cell culture and the associated marked reduction in estradiol did not markedly alter FSH-induced AMH production. Based on these findings, FSH is concluded to have a key role in modulation of AMH production by bovine granulosal cells.

CHAPTER 6

F. Is FSH-induced AMH production and FSH action in granulosal cells similar for animals with high versus a low number of antral follicles?

Introduction

The factors that regulate capacity of granulosal cells to produce AMH and the biological role of AMH in reproduction are poorly understood. However, FSH is a key hormone that regulates granulosal cell differentiation and function [122] and thus may have an important role in regulation of AMH production in granulosal cells. Recent results from our laboratory show that young adult cattle with consistently a relatively low number of follicles growing during follicular waves also have a markedly diminished ovarian reserve and correspondingly very low circulating concentrations of AMH but higher circulating concentrations of FSH compared with their age-matched counterparts with higher follicle numbers [11]. Results of several studies in women [121, 123-125] and rats [84] show that FSH treatment decreases circulating AMH concentrations or AMH production in granulosal cells. However, our recent studies demonstrate that capacity of bovine granulosal cells to produce AMH is biphasic. For example, relatively low doses of FSH stimulate a dose-dependent increase in AMH production in granulosal cells while higher doses initiate luteinization of granulosal cells (as measured by increased progesterone production and oxytocin mRNA abundance coupled with decline

in estradiol production) and a corresponding dose dependent decrease in AMH production (Chapter 5). Taken together, these in vivo and in vitro findings lead to the hypothesis that the chronically high physiological concentrations of FSH in cattle with a low versus high number of follicles growing during follicular waves may diminish capacity of granulosal cells in growing follicles to produce AMH in response to FSH and inhibit FSH action in granulosal cells. To test this hypothesis, the objective of the present study was to determine if capacity of granulosal cells to produce AMH and FSH action differed between cattle with low versus high follicle numbers.

Methods and Materials

Long-term bovine granulosal cell culture

Pairs of ovaries from cattle (unknown ages and breeds) at random stages of the estrous cycle were obtained from JBS Packing Company Inc. (Plainwell, MI) and placed into one of two groups (High, Low) based on the number of antral follicles: High = ≥ 25 antral follicles ≥ 3 mm per pair of ovaries; Low = ≤ 15 follicles. Ovaries were placed in ice-cold supplemented Dulbecco's phosphate buffered saline solution (DPBS) and transported to the laboratory. Granulosal cells were then cultured in serum-free media as described previously [131] with some modifications. Briefly, granulosal cells from 3 to 5 mm follicles from High and Low Groups were collected and separately pooled in a 15-ml centrifuge tube containing MEM-α culture media supplemented with sodium bicarbonate (10 mM), HEPES (20 mM), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), Fungizone-amphotericin B (0.625 µl/ml), nonessential amino acids (1.1 mM), bovine insulin (1 ng/ml), long R3-IGF-I (2 ng/ml), sodium selenite (4 ng/ml), apo-

transferrin (5 μg/ml), and androstenedione (10^{-6} M). The cells were washed with culture media three times and resuspended in 2 ml media. Cell number was estimated by a Coulter Counter Particle Z1 (Beckman Coulter, Inc., Fullerton, CA) while cell viability was estimated using Trypan Blue dye exclusion [133]. Cells (50,000 live cells per well) were plated in 96-well Falcon Primaria plates and cultured at 37°C in a humidified atmosphere (5% CO_2 and 95% air) for 6 days. During culture, 75% of media was removed and replaced with fresh media on days 2 and 4 of culture, and cultures were terminated after 6 days.

We acknowledge that use of this abattoir model for phenotypically classifying animals with low versus high follicle numbers is likely less accurate than our *in vivo* approach using ultrasonography [9]. Although stages of follicular waves were unknown at time of collection, pairs of ovaries from the abattoir classified into the High Group (≥ 25 follicles) are reflective of animals with high follicle numbers during follicular waves [9]. However, because total number of follicles ≥ 3 mm in diameter may decline 50% from peak values during follicular waves of individuals [9], pairs of ovaries classified into the Low Group at the abattoir will not only contain cattle with low follicle numbers during waves, but also some cattle with intermediate and high follicle numbers during waves. Given this limitation, any observed differences between granulosal cells from cattle with low versus high follicle numbers are potentially even more significant.

Estradiol and progesterone assays

Commercially available RIA kits (Diagnostic Products Corp., Los Angeles, CA) previously validated by our laboratory [13, 134, 135] were used to measure

concentrations of estradiol and progesterone in spent media. Estradiol assay sensitivity was 0.5 pg/ml and progesterone assay sensitivity was 0.05 ng/ml [13, 131]. Intra- and interassay coefficients of variation for both RIA assays were < 10%. Results were expressed as ng or pg per 30,000 cells.

AMH assay

A commercially available human MIS/AMH ELISA kit (DSL-10-14400, Beckman Coulter, Inc., Brea, CA), which was validated for use in cattle [Chapter 1; 11], was used to measure AMH concentrations in spent media per kit instructions. The two-site AMH assay does not cross-react with other members of the TGFβ superfamily including TGFβ, BMP4, or activin [67]. The inter-assay and intra-assay coefficients of variation were < 6%. AMH concentrations in different volumes of spent media collected from granulosal cells treated with 0 or 25 ng/ml ovine FSH (oFSH) were parallel with the AMH standard curve, and AMH concentrations were undetectable in media (data not shown).

Messenger RNA Analyses

Total RNA was isolated from granulosal cells using the RNeasy mini kit (Qiagen, Valencia, CA) per kit instructions. RNA was treated with DNase to remove genomic DNA contamination and reverse transcribed [136]. Expression of all genes were analyzed by real-time quantitative PCR [137]. Primers were designed using either Primer Express (Applied Biosystems, Foster City, CA) or PerlPrimer [138] for bovine sequences in Genbank, and the amplicon sizes ranged from 73 to 269 bp (Table 2). Copies of

Table 2: Gene name, accession number, and forward (F) and reverse (R) primer sequences used for quantitative real-time PCR analysis of AMH, ALK2, ALK3, ALK6, AMHRII, LHCGR, FSHR, CPY19A1, and OXT mRNA in bovine granulosal cells.

Gene	Primer Sequence	GenBank accession no.
anti-Müllerian hormone (AMH)	F: 5'-CAGGGAAGAAGTCTTCAGCA-3' R: 5'-AAGGTGGTCAAGTCACTCAG-3'	M13151
Anaplastic lymphoma kinase type 2 (ALK2)	F: 5'-TCTCAGACCCGACATTAACC-3' R: 5'-GATCTTCCTTCTTGACACCA-3'	NM176663
Anaplastic lymphoma kinase type 3 (ALK3)	F: 5'-TTTGGCTTGATCATTTGGGA-3' R: 5'-GCTCGTAGACATTCATCACTG-3'	NM001076800
Anaplastic lymphoma kinase type 6 (ALK6)	F: 5'-GAATGAAGTTGACATACCACC-3' R: 5'-TTCCACTATACCTCCTGATACAC-3'	NM001105328
anti-Müllerian hormone receptor type 2 (AMHRII)	F: 5'-CCACATTGTCCGCTTTATCAC-3' R: 5'-GCGATACCTGGTTTGTACTG-3'	XR028119
Luteinizing hormone/chorionic gonadotropin receptor (LHCGR)	F: 5'-TGACCATGGCCCGTCTAAAA-3' R: 5'-TACTACCCAAAGCAATTTATAGATTCAATG-3'	NM174381
Follicle stimulating hormone receptor (FSHR)	F: 5'-TGGTCCTGTTCTACCCCATCA-3' R: 5'-GAAGAAATCCCTGCGGAAGTT-3'	L22319
Cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1)	F: 5'-CACCCATCTTTGCCAGGTAGTC-3' R: 5'-ACCCACAGGAGGTAAGCCTATAAA-3'	NM174305
oxytocin-neurophysin I precursor (OXT)	F: 5'-CCGGCCCCCGATACC-3' R: 5'-TCATTGTCATAATTCCTAGGGATGATT-3'	V00114

AMH

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AMH, LHCGR, FSHR, CYP19A1, and oxytocin were quantified using the standard curve method for absolute quantification [139] and expressed as copies of target gene per 10,000 copies of β-actin. AMHRII, ALK2, ALK3, and ALK6 were quantified using the delta-delta CT method [156] and normalized using β-actin as a calibrator.

Study 1: Effect of FSH on cell numbers, AMH production, and abundance of AMH mRNA in granulosal cells from cattle with high versus a low number of follicles.

Granulosal cells from cattle with high versus a low follicle number were treated with 0, 0.01, 0.05, and 0.1 ng/ml oFSH (AFP7558C; National Hormone and Pituitary Program, Baltimore, MD) for 6 days. Doses of FSH > 0.1 ng/ml decreased estradiol production but increased progesterone production and oxytocin mRNA abundance implying granulosal cells were undergoing luteinization as shown in Chapter 5 and reported by others [141-144]. Each FSH dose was replicated 12 times. On day 2 and 4 of culture, 75% media was removed as explained earlier. To terminate cultures (on day 6 of culture), 75% of the media was removed from 6 wells and stored at -20°C until subsequent measurement of AMH. Wells were then washed with 150 µl DPBS twice, incubated with trypsin-EDTA (0.125mg/well), and cells were removed from each well by trituration. Cell numbers were determined from 3 pools of granulosal cells (2 wells per pool x 3 pools) per FSH dose. AMH concentrations in media were expressed as ng per 30,000 cells. All media was removed from the remaining 6 wells, cells were lysed, and total RNA isolated from 1 pool of granulosal cells (6 wells) per oFSH dose. Abundance of AMH mRNA was expressed as copies of AMH per 10,000 copies of β-actin and AMH

type 1 and type 2 receptor mRNA abundance was normalized using β-actin as a calibrator. Each experiment was replicated three times.

Study 2: Effect of FSH on estradiol production and abundance of CYP19A1, LHCGR, and FSHR mRNA in granulosal cells from cattle with high versus a low follicle number.

Our recent granulosal cell culture studies (Chapter 5) demonstrated that relatively high doses of FSH decrease not only AMH, but also estradiol production. These findings imply that the chronically high circulating FSH concentrations observed in cattle with a low number of antral follicles [9, 10] may also inhibit FSH-induced estradiol production and potentially negatively impact granulosal cell differentiation and follicle development. To determine if FSH responsiveness differs between granulosal cells isolated from cattle with high versus a low number of antral follicles, estradiol production and abundance of CYP19A1, LHCGR, and FSHR mRNAs were measured as targets of FSH action using samples from Study 1. Estradiol concentrations were expressed as pg estradiol per 30,000 cells while abundance of CYP19A1, LHCGR, and FSHR mRNAs was expressed as copies of target gene per 10,000 copies of β-actin mRNA.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Data are presented as means ± SEM for all experiments. Results were analyzed statistically using linear regression analysis or a multivariate ANOVA followed by Tukey-Kramer test to determine if means differed. Data were log

transformed when necessary to meet the assumptions of normality [77]. A value of $P < 0.05$ was considered significant [77].

Results

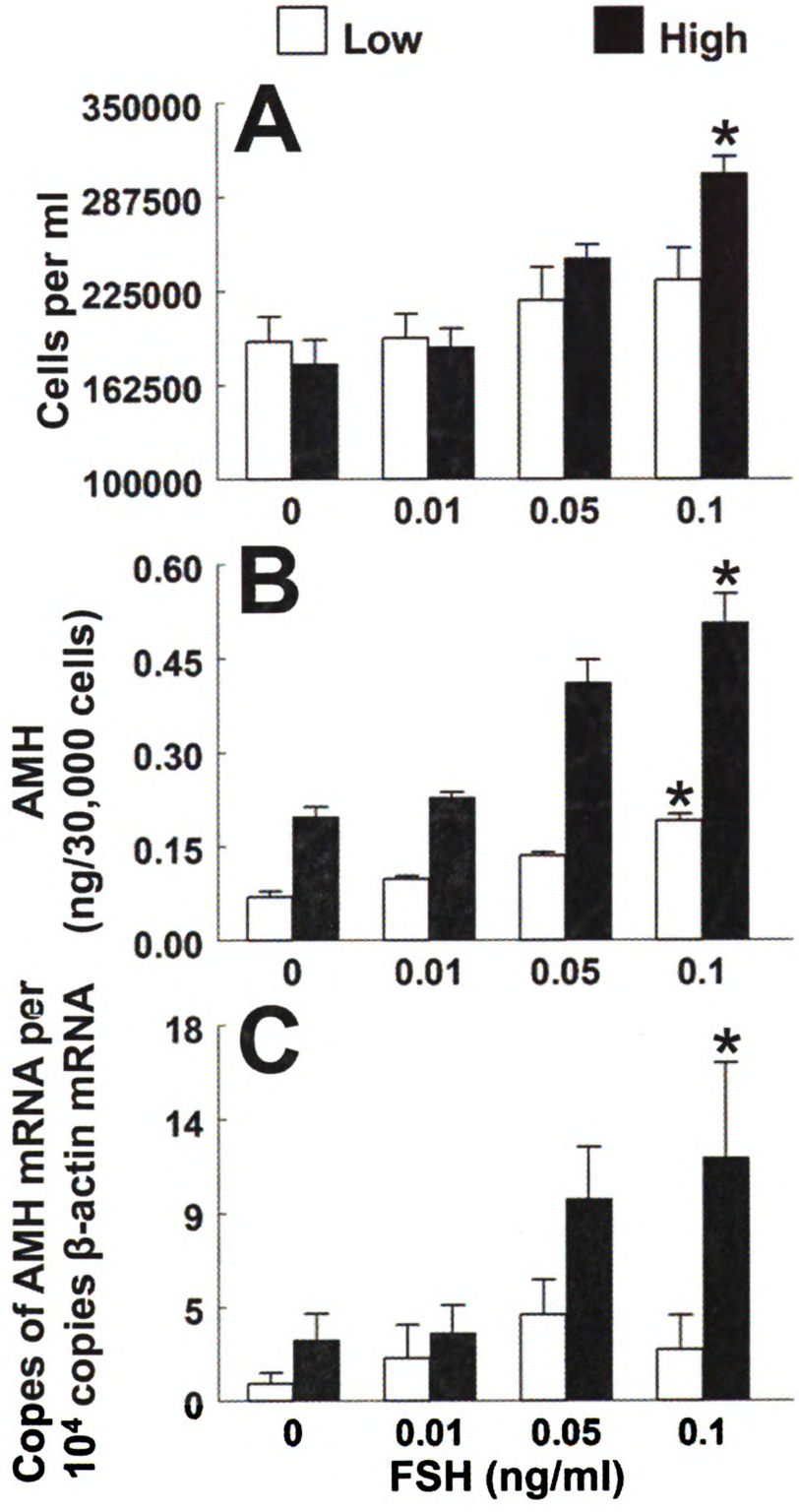
Study 1: Effect of FSH on cell numbers, AMH production, and abundance of AMH mRNA in granulosal cells from cattle with high versus a low number of follicles.

After 6 days of treatment of granulosal cells with different doses of FSH, the High Group had an overall ~ 3-fold higher ($P < 0.01$) AMH concentrations, and ~ 3-fold greater ($P < 0.01$) abundance of AMH mRNA compared to the Low Group (Figure 14) but similar ($P > 0.10$) cell numbers. Doses of FSH ≤ 0.1 ng/ml resulted in a linear increase ($P < 0.01$) in number of granulosal cells in the High Group and a tendency ($P = 0.08$) for cell number to increase in the Low Group. In addition, AMH concentrations were increased ($P < 0.01$) in a dose response fashion in both the High and Low Groups, and abundance of AMH mRNA was increased ($P < 0.01$) in a dose response fashion in the High but not ($P = 0.30$) the Low Group.

Figure 14. Effect of FSH on cell numbers, AMH production, and abundance of AMH mRNA in granulosal cells from cattle with high versus a low number of follicles.

Granulosal cells were treated with various doses of FSH for 6 days. Cell numbers (Panel A), AMH production (Panel B), and abundance of AMH mRNA (Panel C) were measured on Day 6 of culture in the High and Low Group as described in Materials and Methods. AMH production was normalized to 30,000 cells and abundance of AMH mRNA was normalized to copies of AMH per 10,000 copies β-actin mRNA in the same samples. Cell number bars represents the mean $\pm$ SEM of 9 pools of granulosal cells (3 experiments x 3 pools per experiment) and AMH production and AMH mRNA bars represent the mean $\pm$ SEM for 3 pools of granulosal cells (3 experiments x 1 pool per experiment). Results of ANOVA indicated that overall AMH concentrations and abundance of AMH mRNA were lower ($P < 0.01$) for granulosal cells from the Low versus High Group. Asterisk above bar indicates a significant ($P < 0.05$) linear increase.

Figure 14


Low
High
A
350000
287500
225000
162500
100000
Cells per ml
0
0.01
0.05
0.1
B
0.60
0.45
0.30
0.15
0.00
AMH
(ng/30,000 cells)
C
18
14
9
5
0
Copes of AMH mRNA per
10⁴ copies β-actin mRNA
FSH (ng/ml)

Study 2: Effect of FSH on estradiol production and abundance of CYP19A1, LHCGR, and FSHR mRNAs in granulosal cells from cattle with high versus a low follicle number.

After 6 days of treatment of granulosal cells with different doses of FSH, the High Group had an overall ~ 2.6-fold higher ($P < 0.01$) concentration of estradiol, ~ 3-fold greater ($P < 0.05$) abundance of CYP19A1 mRNA, ~ 3-fold greater ($P < 0.01$) abundance of FSHR mRNA, and ~ 3-fold greater ($P < 0.01$) abundance of LHCGR mRNA compared with the Low Group (Figures 15 and 16). Increasing doses of FSH resulted in a linear increase ($P < 0.01$) in estradiol production and abundance of CYP19A1 mRNA in both the High and Low Groups. In contrast, FSH had no effect ($P > 0.15$) on abundance of FSHR or LHCGR mRNA in the High and Low Groups.

Discussion

The present study utilized a long-term bovine granulosal cell culture system to examine FSH action in non-luteinized granulosal cells. The most significant findings of the present study demonstrated that: 1) FSH-induced AMH production and expression of AMH mRNA were lower for granulosal cells from cattle with low versus high follicle numbers, and 2) FSH action was diminished in granulosal cells from cattle with low versus high follicle numbers. These findings imply that chronically heightened FSH concentrations in cattle with a low number of follicles and correspondingly a reduced ovarian reserve may negatively impact FSH regulation of AMH production and follicular function.

Figure 15. Effect of FSH on estradiol production and abundance of CYP19A1 mRNA in granulosal cells from cattle with high versus a low follicle number.

Granulosal cells were treated with various doses of FSH for 6 days. Estradiol production (Panel A) and CYP19A1 (Panel B) expression was measured on Day 6 of culture in the High and Low Group as described in Materials and Methods. Estradiol production was normalized to 30,000 cells and abundance of CYP19A1 mRNA was normalized to copies of CYP19A1 per 10,000 copies β-actin mRNA. Bars represent the mean ± SEM of 9 pools of granulosal cells (3 experiments x 3 pools per experiment) for estradiol or the mean ± SEM for 3 pools of granulosal cells (3 experiments x 1 pool per experiment) for CYP19A1 mRNA. Results of ANOVA indicated that overall concentration of estradiol and abundance of CYP19A1 mRNA were lower ($P < 0.01$) for granulosal cells from the Low versus High Group. Asterisk above bar indicates a significant ($P < 0.05$) linear increase.

Figure 15

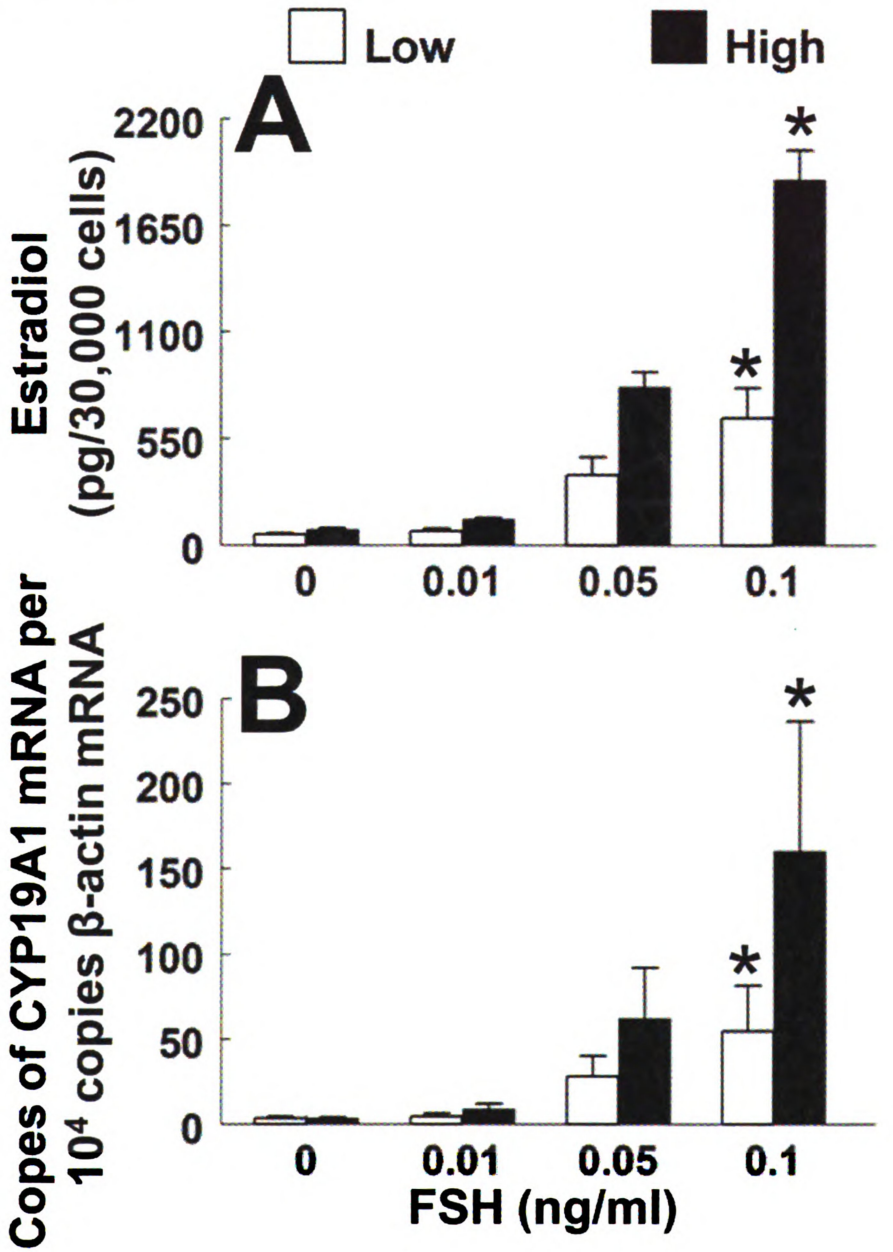
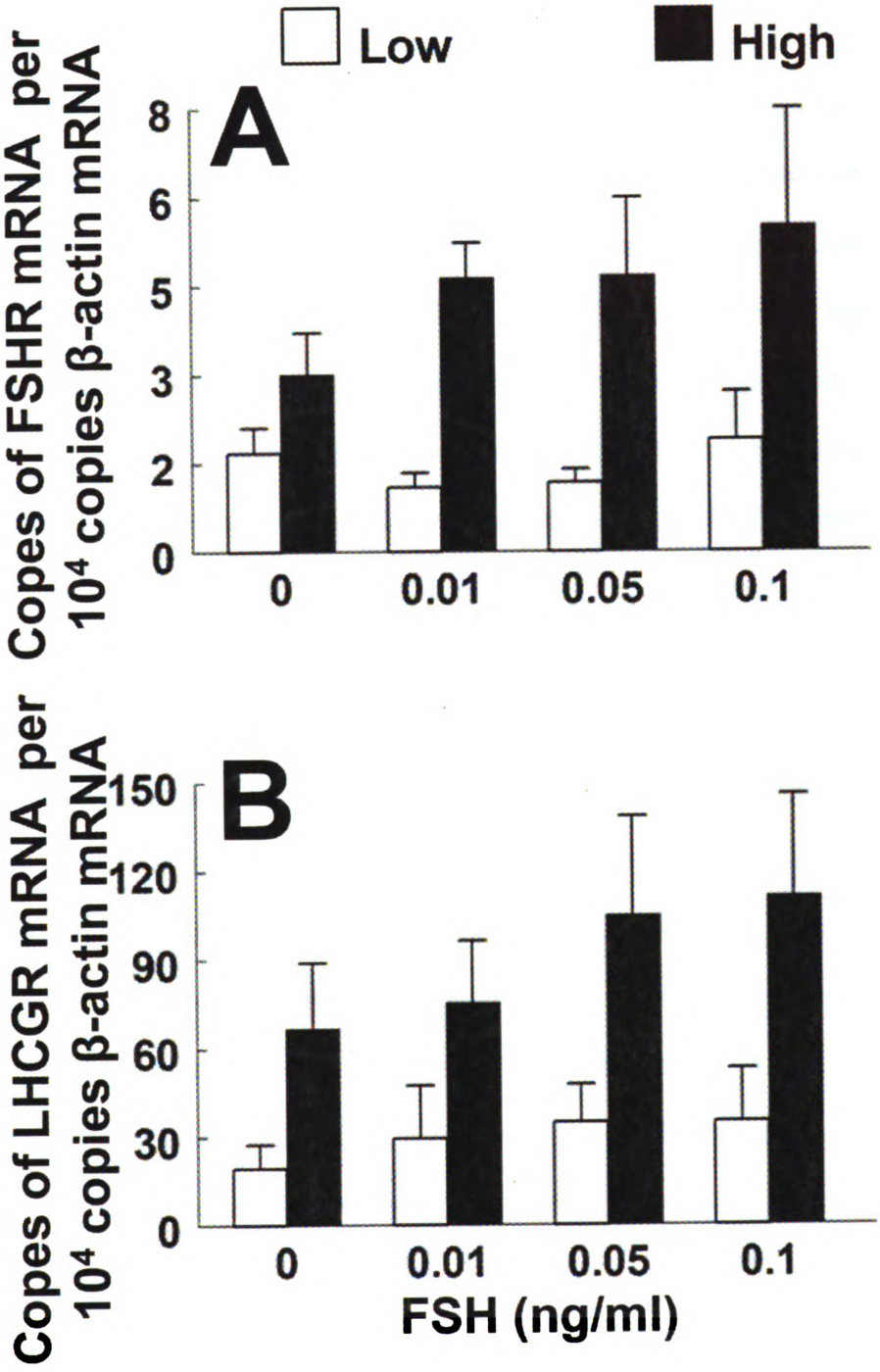

Low
High
A
Estradiol (pg/30,000 cells)
2200
1650
1100
550
0
0
0.01
0.05
0.1
*
*
B
Copes of CYP19A1 mRNA per 10⁴ copies β-actin mRNA
250
200
150
100
50
0
0
0.01
0.05
0.1
FSH (ng/ml)
*
*

Figure 16. Effect of FSH on abundance of FSHR and LHCGR mRNA in granulosal cells from cattle with high versus a low follicle number.

Granulosal cells were treated with various doses of FSH for 6 days. Abundance of FSHR (Panel A) and LHCGR (Panel B) mRNAs was measured on Day 6 of culture in the High and Low Group as described in Materials and Methods. Abundance of FSHR and LHCGR mRNAs were expressed as copies of target gene per 10,000 copies β-actin mRNA. Each bar represents the mean $\pm$ SEM for 3 pools of granulosal cells (3 experiments x 1 pool per experiment). Results of ANOVA indicated that overall abundance of FSHR and LHCGR mRNA were lower ($P < 0.01$) for granulosal cells from the Low versus High Group.

Figure 16


Low
High
A
Copes of FSHR mRNA per 10⁴ copies β-actin mRNA
0
2
3
5
6
8
0
0.01
0.05
0.1
B
Copes of LHCGR mRNA per 10⁴ copies β-actin mRNA
0
30
60
90
120
150
0
0.01
0.05
0.1
FSH (ng/ml)

Our laboratory has recently established that young adult cattle with a low number of follicles growing during follicular waves have 15 to 50% higher circulating FSH concentrations, but 70 to 80 % lower serum AMH concentrations during estrous cycles compared with cattle with a higher number of follicles [11]. Moreover, in vitro studies using bovine granulosal cells show that non-luteinizing doses of FSH stimulates AMH production (Chapter 5). Results of the present study extended these observations by showing that FSH-induced capacity of granulosal cells to produce AMH and express AMH mRNA was markedly reduced in cattle with low versus high follicle numbers. Taken together, these in vivo and in vitro findings imply that the low circulating AMH concentrations reflect not only a diminished ovarian reserve in young adult cattle but also a diminished capacity of granulosal cells to produce AMH in response to FSH.

Studies in AMH "knockout" mice indicate that AMH has an inhibitory role in regulation of recruitment of primordial follicles into the pool of growing follicles [83, 157]. Moreover, number of follicles in ovaries is inversely associated with rate of recruitment [158-162]. Consequently, from a physiologically viewpoint, low circulating AMH concentrations and a diminished ovarian reserve, coupled with a potentially reduced capacity of granulosal cells to produce AMH in response to FSH, as observed in the present study, would likely result in enhanced recruitment of primordial follicles into the growing pool of follicles. A greater rate of follicular recruitment in individuals with low circulating AMH concentrations would also result in a more rapid depletion of the ovarian reserve and further explain why young adult cattle with a reduced ovarian reserve would also be expected to potentially have a much shorter reproductive lifespan

compared with their age-matched counterparts with a larger ovarian reserve. This hypothesis, however, has never been examined.

The reason granulosal cells from cattle with low follicle numbers had a reduced capacity to produce AMH in response to FSH in the present study is unknown. Nevertheless, it is well established that women and cattle with a reduced ovarian reserve, low circulating AMH concentrations, and high FSH concentrations respond poorly to gonadotropin treatments [9-11, 63, 163-166]. In addition, increased serum FSH concentrations are associated with reduced success of in vitro fertilization [21] and decreased fertility during aging of women [21, 52-62, 167] and cattle [1-8, 25]. These well established observations imply that capacity of FSH to stimulate follicular development is reduced in individuals with chronically low circulating AMH but high FSH concentrations. In support of the diminished FSH action in individuals with a reduced ovarian reserve, results of the present in vitro studies clearly demonstrated that granulosal cells from ovaries of cattle with low follicle numbers (and presumably high circulating FSH concentrations and reduced ovarian reserve) had a markedly reduced capacity to respond to FSH, as measured by alterations in a variety of well established FSH targets including estradiol production and abundance of mRNAs for aromatase and FSH and LH receptors [168-172]. This finding strongly supports the possibility that chronically high FSH concentrations may not only diminish capacity of granulosal cells to produce AMH, but also have a general negative impact on granulosal cell differentiation and function.

Several studies report that AMH also has a negative impact on FSH action. For example, AMH inhibits FSH-induced aromatase activity, number of luteinizing hormone

(LH) receptors, and estradiol production in granulosal cells isolated from rats, porcine, and humans [173, 174]. In support of the potential inhibitory effects of AMH on FSH action, FSH-stimulated follicle growth is indeed greater in AMH knockout compared with wild type mice [157]. Nevertheless, results of the present study using bovine granulosal cells show that, despite much lower AMH production, FSH action is markedly diminished in granulosal cells from cattle with low versus high follicle numbers. Although not directly tested, the reason why AMH has a potential negative or positive effect on FSH action among different species is unknown, but may be due to differences in experimental models (e.g., rat, human, porcine versus bovine) or use of different follicle types as a source of granulosal cells [e.g. 1-3 mm follicles, 173, preovulatory follicles, 174 versus 3-5 mm follicles].

In summary, capacity of granulosal cells to respond to FSH in vitro is markedly reduced in granulosal cells isolated from cattle with low versus a high number of follicles. Based on these results, it is concluded that granulosal cells isolated from cattle with low versus high follicle numbers are refractory to FSH. This finding may explain why cattle with low follicle numbers and correspondingly diminished ovarian reserve and low circulating AMH concentrations, but high circulating FSH concentrations respond poorly to superovulation [10].

CHAPTER 7

G. Does AMH alter FSH action in granulosal cells from animals with high versus a low number of antral follicles?

Introduction

Anti-Müllerian hormone (AMH) is a homodimeric glycoprotein [178] responsible for the regression of the Müllerian ducts in male fetuses [179]. In females, AMH is produced exclusively by granulosal cells from healthy growing follicles [19]. However, little is known about the role of AMH in female reproduction.

Previous studies in AMH "knockout" mice imply that AMH controls rate of recruitment of primordial follicles into the pool of growing follicles [83, 180]. In the absence of AMH, number of growing preantral and small antral follicles increase and primordial follicles are depleted earlier than controls [180]. In addition, AMH reduces capacity of follicles in mice to respond to follicle-stimulating hormone (FSH), which is a key regulator of granulosal cell differentiation and function [122]. Moreover, addition of AMH to cultures inhibits FSH-induced preantral follicle growth in mice [157], decreases aromatase activity in rat [173] and porcine granulosal cells [173], and decreases estradiol production by human granulosal cells [174].

While the aforementioned studies imply that AMH has an inhibitory impact on FSH action, previous studies from our laboratory conflict with these findings. For

example, cattle with a low number of follicles growing during follicular waves have chronically high circulating FSH concentrations, but much lower AMH concentrations during estrous cycles [11] compared with cattle with a high number of follicles growing during follicular waves. Based on the aforementioned studies in mice and in porcine and human granulosal cells [157, 173, 174, 180, 181], FSH action in cattle with a high AFC (and correspondingly high follicle numbers during follicular waves, high circulating AMH concentrations, but low FSH concentrations) would be expected to be much lower compared with cattle with a low AFC (and correspondingly low follicle numbers during waves, low circulating AMH concentrations but high FSH concentrations). However, as observed in our previous study (Chapter 6), FSH action, as measured by a variety of FSH targets such as estradiol production and aromatase mRNA, was much greater in granulosal cells from cattle with high versus low follicle numbers, despite a much greater production of AMH and a greater abundance of AMH mRNA. Therefore, these results using the bovine model imply that AMH may enhance rather than inhibit FSH action. To test this hypothesis, this study was designed to examine the effects of AMH on FSH-induced estradiol and progesterone production in granulosal cells isolated from cattle with low or high follicle numbers.

Methods and Materials

Long-term bovine granulosal cell culture

Pairs of ovaries from cattle (unknown ages and breeds) at random stages of the estrous cycle were obtained from JBS Packing Company Inc. (Plainwell, MI) and placed into one of two groups (High, Low) based on the number of antral follicles: High = ≥ 25

antral follicles ≥ 3 mm per pair of ovaries; Low = ≤ 15 antral follicles ≥ 3 mm per pair of ovaries. Ovaries were placed in ice-cold supplemented Dulbecco's phosphate buffered saline solution (DPBS) and transported to the laboratory. Granulosal cells were then cultured in serum-free media as described previously [131] with some modifications. Briefly, granulosal cells from 3 to 5 mm follicles from High and Low Groups were collected and separately pooled in a 15-ml centrifuge tube containing MEM-α culture media supplemented with sodium bicarbonate (10 mM), HEPES (20 mM), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), Fungizone-amphotericin B (0.625 µl/ml), nonessential amino acids (1.1 mM), bovine insulin (1 ng/ml), long R3-IGF-I (2 ng/ml), sodium selenite (4 ng/ml), apo-transferrin (5 µg/ml), and androstenedione (10^{-6} M). The cells were washed with culture media three times and resuspended in 2 ml media. Cell number was estimated by a Coulter Counter Particle Z1 (Beckman Coulter, Inc., Fullerton, CA) while cell viability was estimated using Trypan Blue dye exclusion [133]. Cells (50,000 live cells per well) were plated in 96-well Falcon Primaria plates and cultured at 37°C in a humidified atmosphere (5% CO_2 and 95% air). During culture, 75% of media was removed and replaced with fresh media on days 2 and 4 of culture, and cultures were terminated after 6 days of culture.

Estradiol and progesterone assays

Commercially available RIA kits (Diagnostic Products Corp., Los Angeles, CA) previously validated by our laboratory [13, 134, 135] were used to measure concentrations of estradiol and progesterone in spent media. Estradiol assay sensitivity was 0.5 pg/ml and progesterone assay sensitivity is 0.05 ng/ml [13, 131]. Intra- and

interassay coefficients of variation for both RIA assays were < 9%. Results were expressed as ng or pg per 30,000 cells.

Effect of AMH on FSH-induced increases in granulosal cell numbers and estradiol and progesterone production in granulosal cells from cattle with high versus a low number of follicles.

Granulosal cells from cattle with high versus a low follicle number were treated with 0 or 0.5 ng/ml ovine FSH (oFSH; AFP7558C; National Hormone and Pituitary Program, Baltimore, MD) and 0, 0.1, 0.25, 0.5, 0.75, 1, 5, 10, or 100 ng/ml recombinant human AMH (R & D Systems, Inc., Minneapolis, MN) for 6 days. The present study treated granulosal cells with 0.5 ng/ml FSH because previous studies demonstrate that 0.5 ng/ml FSH stimulates peak estradiol production (Chapter 5). AMH doses were selected to span AMH concentrations observed in serum of heifers [11], in media during culture of bovine granulosal cells (Chapter 6), and in follicular fluid (unpublished Scheetz and Ireland 2010). Each combination of FSH/AMH dose was replicated 6 times. On day 2 and 4 of culture, 75% media was removed as explained earlier. To terminate cultures (on day 6 of culture), 75% of the media was removed from 6 wells and stored at -20°C until subsequent measurement of AMH. Wells were then washed with 150 µl DPBS twice, incubated with trypsin-EDTA (0.125mg/well), and cells were removed from each well by trituration. Cell numbers were determined using 3 pools of granulosal cells (2 wells per pool x 3 pools) per combination of FSH/AMH dose. Estradiol and progesterone were expressed as pg or ng per 30,000 cells. Each experiment was replicated three times.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.1 Institute, Cary, NC). Data are presented as means ± SEM for all experiments. Results were analyzed statistically using a linear regression or a multivariate ANOVA followed by Tukey-Kramer test to determine if means differed. Data were log transformed when necessary to meet the assumptions of normality [77]. A value of $P \leq 0.05$ was considered significant [77].

Results

Effect of AMH on FSH-induced increases in number of granulosal cells and estradiol and progesterone production in granulosal cells from cattle with high versus a low number of follicles.

Treatment of granulosal cells with 0.5 ng/ml versus 0 ng/ml of FSH for 6 days resulted in a increase ($P < 0.01$) in estradiol production in both the High and Low Group (Figure 17). After 6 days of treatment of granulosal cells with 0.5 ng/ml FSH and different doses of AMH, the High Group had an overall ~1.4-fold greater ($P < 0.01$) number of granulosal cells, ~3-fold higher ($P< 0.01$) estradiol concentrations, but 40% lower ($P <0.01$) progesterone concentrations compared with the Low Group (Figure 18). However, treatment with AMH resulted in a dose dependent linear decrease ($P < 0.01$) in FSH-induced estradiol and progesterone production by granulosal cells in the High but not the Low Group ($P > 0.14$). Higher doses of AMH up to 100 ng/ml did not further suppress FSH-induced estradiol production. AMH had no effect ($P >0.10$) on basal estradiol or progesterone production (data not shown).

Figure 17. Effect of FSH on estradiol production in granulosal cells from cattle with high versus a low number of follicles.

Granulosal cells were treated with 0 or 0.5 ng/ml FSH for 6 days. Estradiol production was determined on Day 6 of culture, as described in Materials and Methods. Estradiol production was normalized to 30,000 cells. Bars represent the mean $\pm$ SEM of 9 pools of granulosal cells (3 experiments x 3 pools per experiment). Asterisk above bar indicates a significant ($P < 0.01$) increase compared with untreated controls.

Figure 17

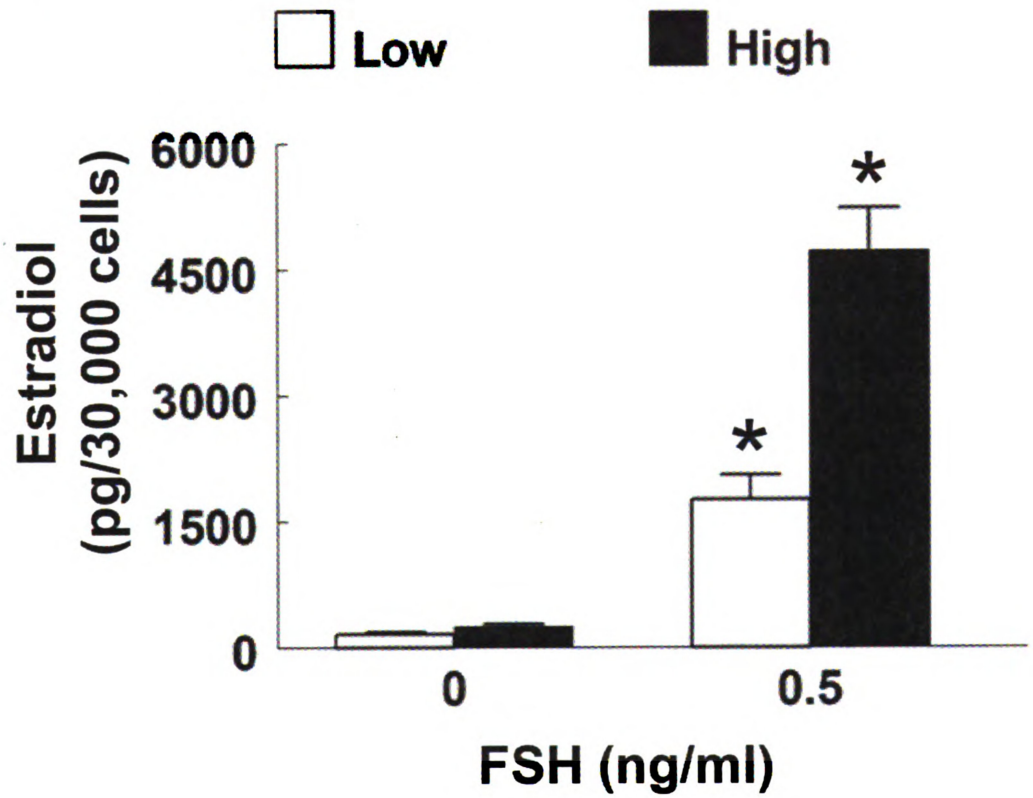
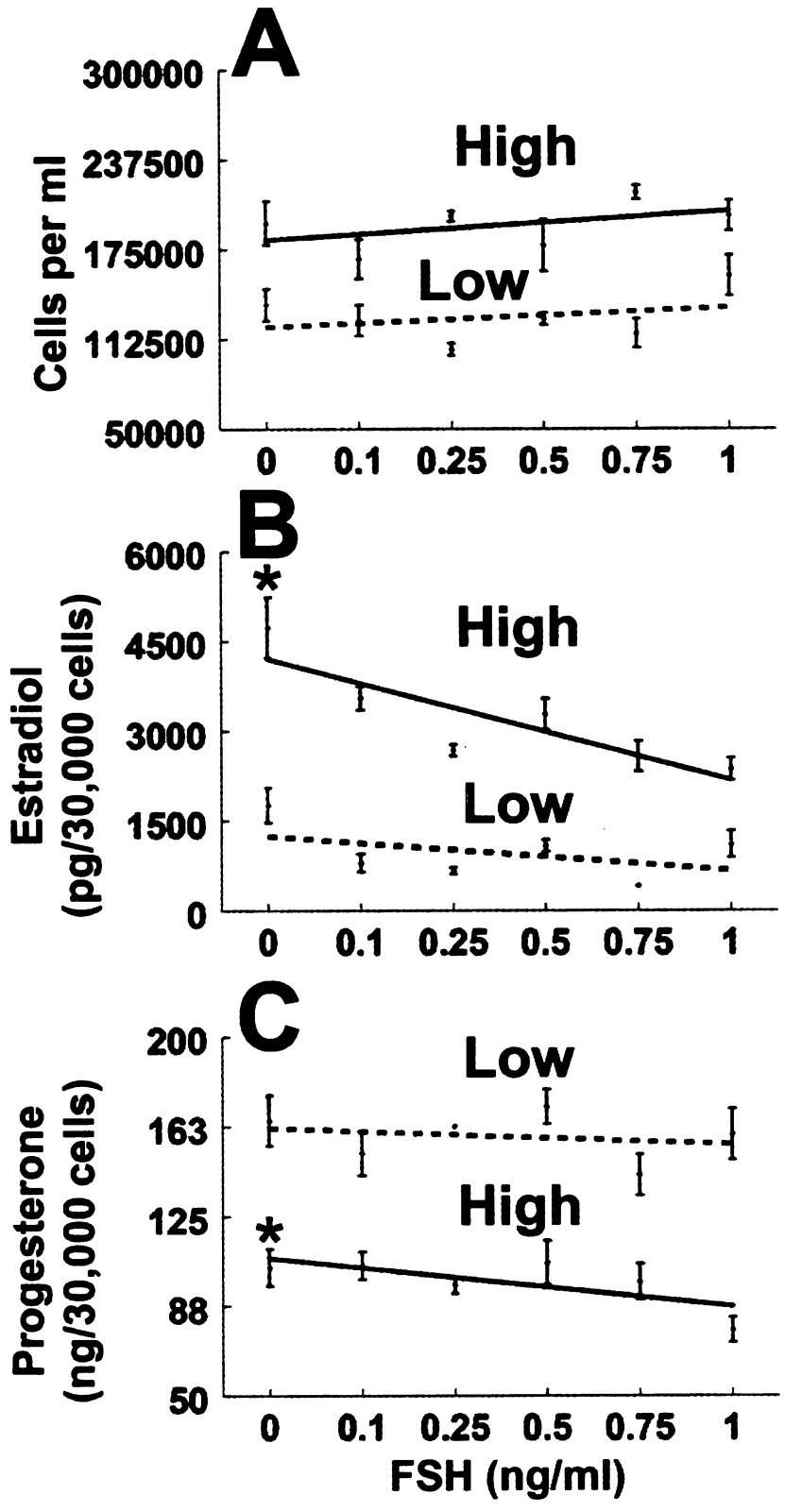

Low
High
Estradiol (pg/30,000 cells)
6000
4500
3000
1500
0
*
*
0
0.5
FSH (ng/ml)

Figure 18. Effect of AMH on FSH-induced cell numbers and estradiol and progesterone production by granulosal cells from cattle with high versus a low number of follicles.

Granulosal cells were treated with 0.5 ng/ml FSH and various doses of AMH for 6 days. Cell numbers (Panel A) and estradiol (Panel B) and progesterone concentrations (Panel C) were determined on Day 6 of culture, as described in Materials and Methods. Estradiol and progesterone were normalized to 30,000 cells. Bars represent the mean $\pm$ SEM of 9 pools of granulosal cells (3 experiments x 3 pools per experiment) for cell number, estradiol and progesterone. Results of ANOVA indicated that overall cell numbers and estradiol production were higher ($P < 0.05$) while progesterone was lower ($P < 0.05$) for granulosal cells from the High versus Low Group. Asterisk above bar indicates a significant ($P < 0.05$) linear decrease.

Figure 18


A
300000
237500
175000
112500
50000
Cells per ml
High
Low
0
0.1
0.25
0.5
0.75
1
B
6000
4500
3000
1500
0
Estradiol (pg/30,000 cells)
*
High
Low
C
200
163
125
88
50
Progesterone (ng/30,000 cells)
Low
High
*
FSH (ng/ml)

Discussion

Several studies [157, 173, 174] including results from the present study, show that AMH inhibits FSH action. Determination of the precise mechanism whereby AMH inhibits FSH action was beyond the scope of this study. Nevertheless, other members of the TGF-β superfamily that signal through the same pathway (Smad 1, 5, and 8) as AMH also inhibit FSH action. For example, in rats, bone morphogenetic protein-15 (BMP-15) inhibits expression of FSH receptor mRNA in granulosal cells [182] and BMP-6 inhibits FSH-induced adenylate cyclase activity [183]. These findings imply that AMH may inhibit FSH action perhaps by decreasing FSH receptors and/or adenylate cyclase activity similar to BMP-15 and BMP-6.

Our present study extends previous results from other animal models [157, 173] by showing that AMH inhibits FSH-induced estradiol and progesterone production, but in the present study results were specific to granulosal cells isolated from cattle with high but not low follicle numbers. The reason granulosal cells from cattle with low follicle numbers were unresponsive to the inhibitory effects of AMH in the present study is unknown, but several explanations are possible. Firstly, results of the present study show that AMH does not directly inhibit basal estradiol or progesterone production by granulosal cells from cattle with high or low follicle numbers. However, granulosal cells from cattle with low versus high follicle numbers have a markedly reduced capacity to respond to FSH, as measured by diminished estradiol production and abundance of mRNAs for aromatase and FSH and LH receptors (Chapter 6). Consequently, the absence of an inhibitory effect of AMH on FSH-induced steroid production by granulosal

cells from cattle with low follicle numbers observed in the present study may be the result of a general refractoriness of granulosal cells to FSH action.

In summary, AMH inhibits FSH action in granulosal cells from cattle with high but not low follicle numbers. Based on these results, it is concluded that AMH may have an important inhibitory role in regulation of FSH action in granulosal cells during follicular waves in cattle with high, but not low follicle numbers.

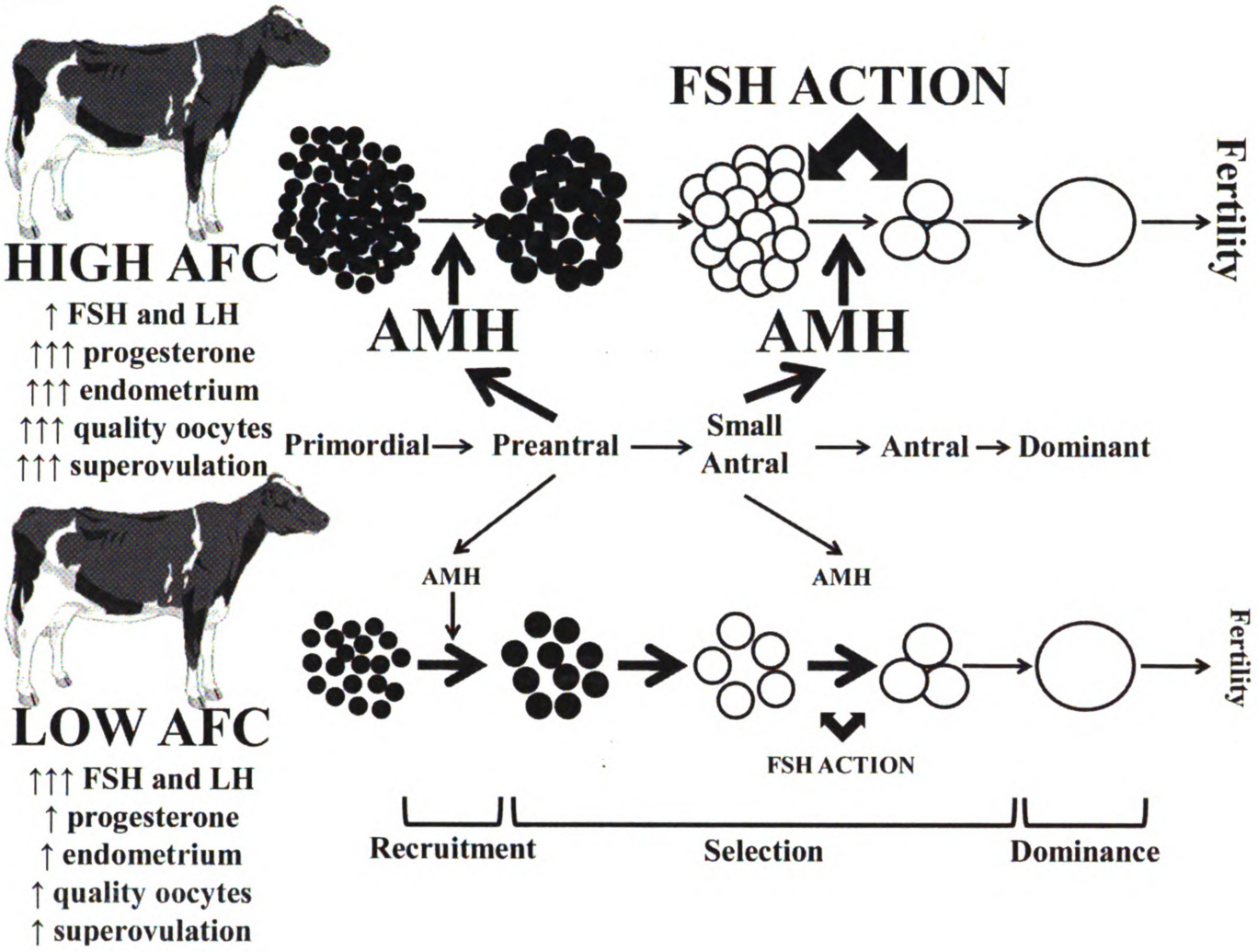
OVERALL SUMMARY, CONCLUSIONS, PHYSIOLOGICAL SIGNIFICANCE AND PRACTICAL APPLICATION

Our laboratory has examined the causes, extent and mechanisms whereby the inherently high variation in number of antral follicles growing during follicular waves in cattle may alter ovarian function and potentially fertility in cattle. Our previous results show that young age-matched cattle with consistently a relatively low ($\leq$ 15 follicles $\geq$ 3 mm in diameter) versus high ($\geq$ 25 follicles) number of antral follicles growing during ovarian follicular waves have many phenotypic characteristics of older less fertile animals [1-8], including a markedly reduced total number of morphologically healthy follicles and oocytes in ovaries (ovarian reserve), poor oocyte quality, low progesterone concentrations during estrous cycles, poor endometrial development, diminished responsiveness to superovulation, decreased number of transferable embryos, but chronically higher FSH and LH secretion, [see Figure 20; 9, 10-13, 189]. These findings clearly demonstrated that the inherently high variation in number of follicles growing during follicular waves has a negative impact on ovarian function in cattle. However, whether the variation in follicle numbers growing during waves and corresponding alterations in ovarian function also impact fertility has never been directly tested, primarily because distinguishing differences between cattle with low or high follicle numbers or low or high secretion patterns of hormones is too time-consuming to routinely use to accurately phenotype the large number of cattle required to complete a

Figure 19. Model depicting role of AMH in cattle with consistently low versus a high antral follicle count (AFC).

The model depicts the phenotypic differences and stages of follicular growth in cattle with a consistently low versus a high AFC. The model shows that cattle with a low versus high AFC possess phenotypic characteristics usually associated with aging and reduced fertility [1-8] such as a smaller ovarian reserve, higher circulating FSH and LH concentrations, much lower AMH and progesterone concentrations, thinner endometrium, reduced oocyte quality, and reduced responsiveness to superovulation [9-13]. Stages of follicular growth are shown as follows: smallest solid circles (●) = primordial follicles, larger solid circles = preantral follicles, and open circles (○) = different sized antral follicles and the dominant follicle. The model also shows that AMH, which is primarily produced by healthy growing preantral and small antral follicles [19], may differentially inhibit both the rate of recruitment of primordial follicles into the growing pool of preantral follicles and FSH-induced development of preantral into antral follicles during follicular waves in cattle with a high or low AFC. Note. AMH inhibits FSH action (indicated by ⮟) in granulosal cells from cattle with high but not low follicle numbers, which may explain why cattle with low follicle numbers and low circulating AMH concentrations have a significantly larger proportion of their ovarian reserve comprised of growing antral follicles [11]. Also note that despite having chronically higher circulating FSH concentrations, granulosal cells from cattle with low follicle numbers are refractory to FSH, which may explain why cattle with a low AFC respond poorly to superovulation.

Figure 19


FSH ACTION
Fertility
HIGH AFC
↑ FSH and LH
↑↑↑ progesterone
↑↑↑ endometrium
↑↑↑ quality oocytes
↑↑↑ superovulation
AMH
AMH
Primordial
Preantral
Small Antral
Antral
Dominant
AMH
AMH
Fertility
LOW AFC
↑↑↑ FSH and LH
↑ progesterone
↑ endometrium
↑ quality oocytes
↑ superovulation
FSH ACTION
Recruitment
Selection
Dominance

statistically valid fertility trial [64]. Consequently, part of my thesis research examined whether AMH, a hormone produced by granulosal cells of healthy growing follicles [19], could be used as a reliable biomarker for the ovarian reserve in cattle.

Results demonstrated the following:

1) Serum AMH concentrations are highly variable among nulliparous young adult cattle, but remain relatively static in individuals [11].
2) Circulating AMH concentrations are highly positively correlated with number of antral follicles growing during ovarian follicular waves and much greater in cattle with high versus a low number of antral follicles during follicular waves [11].
3) A single AMH measurement is highly correlated with multiple daily AMH measurements on different days of an estrous cycle and positively correlated with follicle numbers and ovary size in cattle.

These findings led to the conclusions that AMH is a reliable biomarker to use in future fertility trials to test the hypothesis that variation in follicle numbers is positively associated with fertility, and to use to monitor the impact of the environment (*e.g.*, nutrition, toxins, disease) on the ovarian reserve and potentially fertility of cattle.

Although maternal environment (e.g., nutrition, toxins, disease) has an important role on health of offspring [190], little is known about its impact on ovarian function and fertility of offspring. Therefore, my thesis research also examined whether a persistent mammary gland infection and corresponding number of somatic cell count (SCC) measurements in milk $\geq$ 200,000 cells/ml from pregnant dairy cows had a negative

impact on the ovarian reserve in their daughters. A SCC in milk ≥ 200,000 is an index for potential previous or current udder infections or inflammation [98-103].

Results demonstrated that dairy cows with 4 or 5 SCC measurements ≥ 200,000 beginning 2 months before and during pregnancy not only were ~ 1.3 years older (6.1 versus 4.6 years old) and tended to produce less milk, but also had daughters with much lower AMH concentrations as nulliparous young adults compared with the dairy cows with 0 to 3 SCC measurements ≥ 200,000.

This finding implied that a chronic mammary infection or inflammation during pregnancy (as predicted by a high number of SCC measurements ≥ 200,000) may reduce size of the ovarian reserve and correspondingly potential fertility of female offspring.

Although AMH is produced exclusively in females by granulosal cells of healthy growing follicles [19], the factors that regulate AMH production by granulosal cells and the role of AMH in ovarian function are poorly understood. FSH is a key hormone that regulates granulosal cell differentiation and function, follicle growth and survival, and estradiol production [122]. Therefore, the final part of my thesis used granulosal cells isolated from 3 to 5 mm bovine antral follicles and a 6-day serum-free culture system to examine whether FSH also regulated AMH production and whether AMH altered FSH action. Results demonstrated the following:

1) Granulosal cells respond to FSH in a biphasic fashion. Relatively low doses of FSH increase AMH and estradiol production. In contrast, higher FSH doses decrease AMH and estradiol production, but increase progesterone production and abundance of oxytocin mRNA thus inducing luteinization of granulosal cells.

2) Granulosal cells from cattle with low versus high follicle numbers have a much lower capacity to produce AMH and estradiol and a reduced expression of AMH, CPY19A1, FSHR and LHCGR mRNAs in response to FSH.
3) AMH inhibits FSH-induced estradiol and progesterone production in granulosal cells from cattle with high but not low follicle numbers.

These findings led to the following conclusions:

1) FSH modulates AMH production in granulosal cells.
2) FSH-induced AMH production by granulosal cells is much lower for cattle with low versus high follicle numbers.
3) High doses of FSH cause granulosal cells to undergo luteinization;
4) Granulosal cells from cattle with low follicle numbers are refractory to FSH.
5) AMH may inhibit FSH action in granulosal cells from cattle with high but not low numbers of follicles growing during follicular waves.

From a physiological viewpoint, AMH may have a key role in regulating rate of depletion of the ovarian reserve and thus reproductive performance. For example, previous studies show that AMH slows the rate of primordial follicle recruitment in mice [83, 180] and our studies show that AMH inhibits FSH action on granulosal cells from cattle with high but not low follicle numbers. If these findings also apply to cattle with low versus high follicle numbers growing during follicular waves, as depicted in Figure 20, then the high circulating AMH concentrations in cattle with a high number of follicles growing during follicular waves may slow both rate of recruitment of primordial into

growing preantral follicles and development of preantral into antral follicles. In contrast, cattle with a low number of follicles growing during waves and correspondingly much lower AMH concentrations may have an enhanced rate of recruitment (Figure 20). This finding could also explain why cattle with a low number of follicles and correspondingly high circulating FSH concentrations have a significantly *larger proportion* of their ovarian reserve comprised of growing antral follicles compared to cattle with a high number of follicles growing during waves [Figure 20; 11]. Taken together, these observations imply that cattle with a consistently low number of follicles during waves, low circulating AMH concentrations and a diminished ovarian reserve may also have a much shorter reproductive lifespan compared with their age-matched cohorts with a higher number of follicles. In addition, despite chronically higher circulating FSH concentrations [9, 10], granulosal cells from cattle with low follicle numbers are refractory to FSH. This finding could explain why cattle with a low AFC and correspondingly low ovarian reserve respond poorly to superovulation [10].

From a practical viewpoint, discovery of a linkage between AMH concentration, number of follicles growing during follicular waves, and size of the ovarian reserve with fertility in healthy young adult cattle would provide new insights into factors that may alter ovarian function and ultimately cause or contribute to suboptimal fertility independent of aging. Once the aforementioned linkage is firmly established, this new information would: **a**) provide the foundation and compelling rationale for new basic experiments to unravel the mechanisms whereby low circulating AMH concentrations may alter follicular development and ovarian function, which may lead to new methods to regulate depletion of the ovarian reserve and thus reproductive longevity, **b**) provide

reproductive biologists for the first time with a reliable method to monitor the impact of various environmental factors (*e.g.*, nutrition, toxins, disease) on the ovarian reserve and fertility, **c**) improve the likelihood that better diagnostic, selection, therapeutic and perhaps genetic methods be developed to enhance fertility in cattle, and **d**) confirm the utility of the bovine as a novel translational model to develop more precise procedures to improve assisted reproductive technologies and family planning.

LITERATURE CITED

1. Bryner RW, Garcia-Winder M, Lewis PE, Inskeep EK, Butcher RL. Changes in hormonal profiles during the estrous cycle in old lactating beef cows. Domestic Animal Endocrinology 1990; 7: 181-189.

2. Cushman RA, DeSouza JC, Hedgpeth VS, Britt JH. Superovulatory Response of One Ovary Is Related to the Micro- and Macroscopic Population of Follicles in the Contralateral Ovary of the Cow. Biol Reprod 1999; 60: 349-354.

3. Erickson BH. Development and Senescence of the Postnatal Bovine Ovary. J. Anim Sci. 1966; 25: 800-805.

4. Kawamata M. Relationships between the number of small follicles prior to superovulatory treatment and superovulatory response in Holstein cows. J Vet Med Sci 1994; 65: 965-967.

5. Malhi PS, Adams GP, Singh J. Bovine Model for the Study of Reproductive Aging in Women: Follicular, Luteal, and Endocrine Characteristics. Biology of Reproduction 2005; 73: 45-53.

6. Singh J, Domínguez M, Jaiswal R, Adams GP. A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 2004; 62: 227-243.

7. Taneja M, Bols PEJ, de Velde AV, Ju J-C, Schreiber D, Tripp MW, Levine H, Echelard Y, Riesen J, Yang X. Developmental Competence of Juvenile Calf Oocytes In Vitro and In Vivo: Influence of Donor Animal Variation and Repeated Gonadotropin Stimulation. Biol Reprod 2000; 62: 206-213.

8. Wolfenson D, Inbar G, Roth Z, Kaim M, Bloch A, Braw-Tal R. Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. Theriogenology 2004; 62: 1042-1055.

9. Burns DS, Jimenez-Krassel F, Ireland JLH, Knight PG, Ireland JJ. Numbers of Antral Follicles During Follicular Waves in Cattle: Evidence for High Variation Among Animals, Very High Repeatability in Individuals, and an Inverse Association with Serum Follicle-Stimulating Hormone Concentrations. Biol Reprod 2005; 73: 54-62.

10. Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-

quality embryos after ovarian stimulation in cattle. Hum. Reprod. 2007; 22: 1687-1695.

11. Ireland JLH, Scheetz D, Jimenez-Krassel F, Themmen APN, Ward F, Lonergan P, Smith GW, Perez GI, Evans ACO, Ireland JJ. Antral Follicle Count Reliably Predicts Number of Morphologically Healthy Oocytes and Follicles in Ovaries of Young Adult Cattle. Biol Reprod 2008; 79: 1219-1225.

12. Ireland JJ, Zielak-Steciwko AE, Jimenez-Krassel F, Folger J, Bettegowda A, Scheetz D, Walsh S, Mossa F, Knight PG, Smith GW, Lonergan P, Evans ACO. Variation in the Ovarian Reserve Is Linked to Alterations in Intrafollicular Estradiol Production and Ovarian Biomarkers of Follicular Differentiation and Oocyte Quality in Cattle. Biol Reprod 2009; 80: 954-964.

13. Jimenez-Krassel F, Folger JK, Ireland JLH, Smith GW, Hou X, Davis JS, Lonergan P, Evans ACO, Ireland JJ. Evidence That High Variation in Ovarian Reserves of Healthy Young Adults Has a Negative Impact on the Corpus Luteum and Endometrium During Estrous Cycles in Cattle. Biology of Reproduction 2009; 80: 1272-1281.

14. Adams GP, Matteri RL, Kastelic JP, Ko JCH, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J Reprod Fertil 1992; 94: 177-188.

15. Kinder JE, Kojima FN, Bergfeld EG, Wehrman ME, Fike KE. Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. J. Anim Sci. 1996; 74: 1424-1440.

16. Rahe CH, Owens RE, Fleeger JL, Newton HJ, Harms PG. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology 1980; 107: 498-503.

17. Sunderland SJ, Crowe MA, Boland MP, Roche JF, Ireland JJ. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. J Reprod Fertil 1994; 101: 547-555.

18. Walters DL, Schallenberger E. Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow. J Reprod Fertil 1984; 71: 503-512.

19. La Marca A, Volpe A. Anti-Mullerian hormone (AMH) in female reproduction: is measurement of circulating AMH a useful tool? Clinical Endocrinology 2006; 64: 603-610.

20. Hunter RHF. Physiology and Technology of Reproduction in Female Domestic Animals. London: Academic Press; 1980.

21. Beckers NGM, Macklon NS, Eijkemans MJC, Fauser BCJM. Women with regular menstrual cycles and a poor response to ovarian hyperstimulation for in vitro fertilization exhibit follicular phase characteristics suggestive of ovarian aging. Fertility and Sterility 2002; 78: 291-297.

22. Chuang C-C, Chen C-D, Chao K-H, Chen S-U, Ho H-N, Yang Yu-S. Age is a better predictor of pregnancy potential than basal follicle-stimulating hormone levels in women undergoing in vitro fertilization. Fertility and Sterility 2003; 79: 63-68.

23. Cohen MA, Lindheim SR, Sauer MV. Donor age is paramount to success in oocyte donation. Hum. Reprod. 1999; 14: 2755-2758.

24. Erickson BH, Reynolds RA, Murphree RL. Ovarian Characteristics and Reproductive Performance of the Aged Cow. Biol Reprod 1976; 15: 555-560.

25. Maurer RR, Echternkamp SE. Repeat-Breeder Females in Beef Cattle: Influences and Causes. J. Anim Sci. 1985; 61: 624-636.

26. Oliveira JFC, Neves JP, Moraes JCF, Gonçalves PBD, Bahr JM, Hernandez AG, Costa LFS. Follicular development and steroid concentrations in cows with different levels of fertility raised under nutritional stress. Animal Reproduction Science 2002; 73: 1-10.

27. Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. Biol Reprod 1996; 55: 1187-1194.

28. Ireland JJ, Mihm M, Austin E, Diskin MG, Roche JF. Historical Perspective of Turnover of Dominant Follicles During the Bovine Estrous Cycle: Key Concepts, Studies, Advancements, and Terms. J. Dairy Sci. 2000; 83: 1648-1658.

29. van Wezel IL, Rodgers RJ. Morphological characterization of bovine primordial follicles and their environment in vivo. Biol Reprod 1996; 55: 1003-1011.

30. Greenwald GS, Terranova PF. Follicular selection and its control. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 1, 1st ed. New York: Raven Press; 1988: 387-445.

31. Lussier JG, Matton P, Dufour JJ. Growth rates of follicles in the ovary of the cow. J Reprod Fert 1987; 81: 1-7.

32. Maurasse C, Matton P, Dufour JJ. Ovarian follicular populations at two stages of an estrous cycle in heifers given high energy diets. J Anim Sci 1985; 61: 1194-1210.

33. Skinner MK, Keski-Oja J, Ostern KG, Moses HL. Ovarian thecal cells produce transforming growth factor-beta which can regulate granulosa cell growth. Endocrinology 1987; 121: 786-792.

34. Spicer LJ, Alpizar E, Echternkamp SE. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. J Anim Sci 1993; 71: 1232-1241.

35. Wandji SA, Pelletier G, Sirard MA. Ontogeny and cellular localization of ^{125}I-labeled insulin-like growth factor-1, ^{125}I-labeled follicle-stimulating hormone, and ^{125}I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. Biol Reprod 1992; 47: 814-822.

36. Kumar TR, Low MJ, Matzuk MM. Genetic Rescue of Follicle-Stimulating Hormone beta-Deficient Mice. Endocrinology 1998; 139: 3289-3295.

37. Fortune J. Follicular dynamics during the bovine estrous cycle: a limiting factor in improvement of fertility? . Anim Repro 1993; 33: 111-125.

38. Ireland JJ, Roche JF. Hypothesis regarding developement of dominant follicles during a bovine estrous cycle. In: Roche JF, O'Callaghan D (eds.), Follicular Growth and Ovulation Rate in Farm Animals. The Hague: Martinus Nijhoff Publishers; 1987: 1-18.

39. Matton P, Adelakoun V, Couture Y, Dufour JJ. Growth and Replacement of the Bovine Ovarian Follicles during the Estrous Cycle. J. Anim Sci. 1981; 52: 813-820.

40. Rajakoski E. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. Ada Endocrinology 1960; 34: 7-68.

41. Roche JF. Control and regulation of folliculogenesis--a symposium in perspective. Rev Reprod 1996; 1: 19-27.

42. Jaiswal RS, Singh J, Adams GP. Developmental pattern of small antral follicles in the bovine ovary. Biol Reprod 2004; 71: 1244-1251.

43. Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. J Reprod Fertil 1988; 83: 663-671.

44. Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 1988; 39: 308-317.

45. Fortune JE, Sirois J, Quirk SM. The growth and differentiation of ovarian follicles during the bovine estrous cycle. Theriogenology 1988; 29: 95-109.

46. Hodgen GD. The dominant ovarian follicle. Fertility and Sterility 1982; 38: 281-300.

47. Knopf L, Kastelic JP, Schallenberger E, Ginther OJ. Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. Domest Anim Endocrinol 1989; 6: 111-119.

48. Goodman AL, Hodgen GD. The ovarian triad of the primate menstrual cycle. Recent Prog Horm Res 1983; 39: 1-73.

49. Ireland JJ, Roche JF. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. Endocrinology 1982; 111: 2077-2086.

50. Ireland JJ, Roche JF. Growth and Differentiation of Large Antral Follicles after Spontaneous Luteolysis in Heifers: Changes in Concentration of Hormones in Follicular Fluid and Specific Binding of Gonadotropins to Follicles. J. Anim Sci. 1983; 57: 157-167.

51. Ireland JJ, Roche JF. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology 1983; 112: 150-156.

52. Scheffer GJ, Broekmans FJM, Looman CWN, Blankenstein M, Fauser BCJM, de Jong FH, te Velde ER. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. Hum. Reprod. 2003; 18: 700-706.

53. Block E. A quantitative morphological investigation of the follicular system in newborn female infants. Acta Anat 1953; 17: 201-206.

54. Chang M-Y, Chiang C-H, Hsieh Ts-Ta, Soong Y-K, Hsu K-H. Use of the Antral Follicle Count to Predict the Outcome of Assisted Reproductive Technologies. Fertility and Sterility 1998; 69: 505-510.

55. Forabosco A, Sforza C, Pol A, Vizzotto L, Marzona L, Ferrario V. Morphometric study of the human neonatal ovary. The Anatomical Record 1991; 231: 201-208.

56. Gougeon A, Echochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. Biol Reprod 1994; 50: 653-663.

57. Hsieh Y-Y, Chang C-C, Tsai H-D. Clinical Assisted Reproduction: Antral Follicle Counting in Predicting the Retrieved Oocyte Number After Ovarian Hyperstimulation. Journal of Assisted Reproduction and Genetics 2001; 18: 320-324.

58. Huang F-J, Chang S-Y, Tsai M-Y, Kung F-T, Wu J-F, Chang H-W. Determination of the Efficiency of Controlled Ovarian Hyperstimulation in the Gonadotropin-Releasing Hormone Agonist-Suppression Cycle Using the Initial

Follicle Count During Gonadotropin Stimulation. Journal of Assisted Reproduction and Genetics 2001; 18: 91-96.

59. Kupesic S, Kurjak A, Bjelos D, Vujisic S. Three-dimensional ultrasonographic ovarian measurements and in vitro fertilization outcome are related to age. Fertility and Sterility 2003; 79: 190-197.

60. Prior JC. Perimenopause: The Complex Endocrinology of the Menopausal Transition. Endocr Rev 1998; 19: 397-428.

61. Richardson SJ, Senikas V, Nelson JF. Follicular Depletion During the Menopausal Transition: Evidence for Accelerated Loss and Ultimate Exhaustion. J Clin Endocrinol Metab 1987; 65: 1231-1237.

62. Tomas C, Nuojua-Huttunen S, Martikainen H. Pretreatment transvaginal ultrasound examination predicts ovarian responsiveness to gonadotrophins in in-vitro fertilization. Hum. Reprod. 1997; 12: 220-223.

63. van Rooij IAJ, Broekmans FJM, te Velde ER, Fauser BCJM, Bancsi LFJMM, Jong FHd, Themmen APN. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum. Reprod. 2002; 17: 3065-3071.

64. Pezzullo JC. Proportion Difference Power / Sample Size Calculation. In, vol. 2008; 2009.

65. Ouellette Y, Price CA, Carriere PD. Follicular fluid concentration of transforming growth factor-beta1 is negatively correlated with estradiol and follicle size at the early stage of development of the first-wave cohort of bovine ovarian follicles. Domest Anim Endocrinol 2005; 29: 623 - 633.

66. Fanchin R, Taieb J, Lozano DHM, Ducot B, Frydman R, Bouyer J. High reproducibility of serum anti-Mullerian hormone measurements suggests a multi-staged follicular secretion and strengthens its role in the assessment of ovarian follicular status. Hum. Reprod. 2005; 20: 923-927.

67. Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BMN, de Jong FH, Groome NP, Themmen APN, Visser JA. Serum Anti-Mullerian Hormone Levels Reflect the Size of the Primordial Follicle Pool in Mice. Endocrinology 2006; 147: 3228-3234.

68. Hehenkamp WJK, Looman CWN, Themmen APN, de Jong FH, te Velde ER, Broekmans FJM. Anti-Mullerian Hormone Levels in the Spontaneous Menstrual Cycle Do Not Show Substantial Fluctuation. J Clin Endocrinol Metab 2006; 91: 4057-4063.

69. La Marca A, Stabile G, Artenisio AC, Volpe A. Serum anti-Mullerian hormone throughout the human menstrual cycle. Hum. Reprod. 2006; 21: 3103-3107.

70. Piltonen T, Morin-Papunen L, Koivunen R, Perheentupa A, Ruokonen A, Tapanainen JS. Serum anti-Mullerian hormone levels remain high until late reproductive age and decrease during metformin therapy in women with polycystic ovary syndrome. Hum. Reprod. 2005; 20: 1820-1826.

71. de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM. Antimüllerian hormone serum levels: a putative marker for ovarian aging. Fertility and Sterility 2002; 77: 357-362.

72. Baerwald AR, Adams GP, Pierson RA. Characterization of ovarian follicular wave dynamics in women. Biol Reprod 2003; 69: 1023 - 1031.

73. Baerwald AR, Adams GP, Pierson RA. A new model for ovarian follicular development during the human menstrual cycle. Fertil Steril 2003; 80: 116-122.

74. Katska L, Smorag Z. Number and quality of oocytes in relation to age in cattle. Anim Reprod Sci 1984; 7: 451-460.

75. Tufan E, Elter K, Durmusoglu F. Assessment of reproductive ageing patterns by hormonal and ultrasonographic ovarian reserve tests. Hum. Reprod. 2004; 19: 2484-2489.

76. Scheffer GJ, Broekmans FJM, Dorland M, Habbema JDF, Looman CWN, te Velde ER. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertility and Sterility 1999; 72: 845-851.

77. SAS. SAS User's Guide: Statistics. Cary, NC: SAS Institute Inc; 2004.

78. Al-Qahtani A, Muttukrishna S, Appasamy M, Johns J, M C, Visser J, Themmen A, Groome N. Development of a sensitive enzyme immunoassay for anti-Müllerian hormone and the evaluation of potential clinical applications in males and females. . Clinical Endocrinology 2005; 63: 267-273.

79. Long W-Q, Ranchin V, Pautier P, Belville C, Denizot P, Cailla H, Lhomme C, Picard J-Y, Bidart J-M, Rey R. Detection of Minimal Levels of Serum Anti-Mullerian Hormone during Follow-Up of Patients with Ovarian Granulosa Cell Tumor by Means of a Highly Sensitive Enzyme-Linked Immunosorbent Assay. J Clin Endocrinol Metab 2000; 85: 540-544.

80. Rico C, Fabre S, Medigue C, Clemente Nd, Clement F, Bontoux M, Touze J-L, Dupont M, Briant E, Remy B, Beckers J-F, Monniaux D. Anti-Mullerian Hormone Is an Endocrine Marker of Ovarian Gonadotropin-Responsive Follicles and Can Help to Predict Superovulatory Responses in the Cow. Biology of Reproduction 2009; 80: 50-59.

81. Wunder DM, Bersinger NA, Yared M, Kretschmer R, Birkhäuser MH. Statistically significant changes of antimüllerian hormone and inhibin levels

during the physiologic menstrual cycle in reproductive age women. Fertility and Sterility 2008; 89: 927-933.

82. Hirobe S, He WW, Lee MM, Donahoe PK. Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 1992; 131: 854-862.

83. Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Anti-Mullerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary. Endocrinology 2002; 143: 1076-1084.

84. Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA. Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 1995; 136: 4951-4962.

85. Bridges PJ, Brusie MA, Fortune JE. Elevated temperature (heat stress) in vitro reduces androstenedione and estradiol and increases progesterone secretion by follicular cells from bovine dominant follicles. Domestic Animal Endocrinology 2005; 29: 508-522.

86. Rensis FD, Scaramuzzi RJ. Heat stress and seasonal effects on reproduction in the dairy cow--a review. Theriogenology 2003; 60: 1139-1151.

87. Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, Braw-Tal R, Berman A. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. Biol Reprod 1995; 52: 1106-1113.

88. Wolfenson D, Roth Z, Meidan R. Impaired reproduction in heat-stressed cattle: basic and applied aspects. Animal Reproduction Science 2000; 60-61: 535-547.

89. Jurisicova A, Taniuchi A, Li H, Shang Y, Antenos M, Detmar J, Xu J, Matikainen T, Benito HernÃ¡ndez A, Nunez G, Casper RF. Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of Harakiri. The Journal of Clinical Investigation 2007; 117: 3971-3978.

90. Steckler T, Wang J, Bartol FF, Roy SK, Padmanabhan V. Fetal Programming: Prenatal Testosterone Treatment Causes Intrauterine Growth Retardation, Reduces Ovarian Reserve and Increases Ovarian Follicular Recruitment. Endocrinology 2005; 146: 3185-3193.

91. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic Syndrome in Childhood: Association With Birth Weight, Maternal Obesity, and Gestational Diabetes Mellitus. Pediatrics 2005; 115: e290-296.

92. Nelson SM, Fleming RF. The preconceptual contraception paradigm: obesity and infertility. Hum. Reprod. 2007; 22: 912-915.

93. Hillier TA, Pedula KL, Schmidt MM, Mullen JA, Charles M-A, Pettitt DJ. Childhood Obesity and Metabolic Imprinting. Diabetes Care 2007; 30: 2287-2292.

94. Mossa F, Kenny D, Jimenez-Krassel F, Smith GW, Berry DP, Butler ST, Fair T, Lonergan P, Ireland JJ, Evans ACO, . Undernutrition of heifers during the first trimester of pregnancy diminishes size of the ovarian reserve in female offspring. In 42nd Annual Meeting of the Society for Study of Reproduction; Pittsburg, PA. 2009; Abstract 135: 135.

95. Lumey LH. Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944–1945. Paediatric and Perinatal Epidemiology 1992; 6: 240-253.

96. Ibanez L, Potau N, Enriquez G, De Zegher F. Reduced Uterine and Ovarian Size in Adolescent Girls Born Small for Gestational Age. Pediatric Research 2000; 47: 575-577.

97. Ibanez L, Potau N, Enriquez G, Marcos MV, Zegher Fd. Hypergonadotrophinaemia with reduced uterine and ovarian size in women born small-for-gestational-age. Hum. Reprod. 2003; 18: 1565-1569.

98. Caraviello DZ, Weigel KA, Shook GE, Ruegg PL. Assessment of the Impact of Somatic Cell Count on Functional Longevity in Holstein and Jersey Cattle Using Survival Analysis Methodology. J. Dairy Sci. 2005; 88: 804-811.

99. Dohoo IR, Leslie KE. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Preventive Veterinary Medicine 1991; 10: 225-237.

100. Cook NB, Bennett TB, Emery KM, Nordlund KV. Monitoring Nonlactating Cow Intramammary Infection Dynamics Using DHI Somatic Cell Count Data. Journal of Dairy Science 2002; 85: 1119-1126.

101. Laevens H, Deluyker H, Schukken YH, De Meulemeester L, Vandermeersch R, De Muecaronlenaere E, De Kruif A. Influence of Parity and Stage of Lactation on the Somatic Cell Count in Bacteriologically Negative Dairy Cows. J. Dairy Sci. 1997; 80: 3219-3226.

102. Schepers AJ, Lam TJGM, Schukken YH, Wilmink JBM, Hanekamp WJA. Estimation of Variance Components for Somatic Cell Counts to Determine Thresholds for Uninfected Quarters. J. Dairy Sci. 1997; 80: 1833-1840.

103. Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez RN. Monitoring udder health and milk quality using somatic cell counts. Vet. Res. 2003; 34: 579-596.

104. Valley Agricultural Software. DairyComp 305. In, vol. 2010. Tulare, CA: Valley Agricultural Software; 2007.

105. Grohn YT, Wilson DJ, Gonzalez RN, Hertl JA, Schulte H, Bennett G, Schukken YH. Effect of Pathogen-Specific Clinical Mastitis on Milk Yield in Dairy Cows. J. Dairy Sci. 2004; 87: 3358-3374.

106. Reneau JK. Effective Use of Dairy Herd Improvement Somatic Cell Counts in Mastitis Control. J. Dairy Sci. 1986; 69: 1708-1720.

107. Dohoo IR, Martin SW, Meek AH. Disease, production and culling in Holstein-Friesian cows VI. Effects of management on disease rates. Preventive Veterinary Medicine 1984; 3: 15-28.

108. Wilson DJ, Grohn YT, Bennett GJ, Gonzalez RN, Schukken YH, Spatz J. Milk Production Change Following Clinical Mastitis and Reproductive Performance Compared Among J5 Vaccinated and Control Dairy Cattle. J. Dairy Sci. 2008; 91: 3869-3879.

109. Lush JL, Shrode RR. Changes in Milk Production with Age and Milking Frequency. J. Dairy Sci. 1950; 33: 338-357.

110. Robison OW, Yusuff MKM, Dillard EU. Milk Production in Hereford Cows I. Means and Correlations. J. Anim Sci. 1978; 47: 131-136.

111. Riollet C, Rainard P, Poutrel B. Cell Subpopulations and Cytokine Expression in Cow Milk in Response to Chronic Staphylococcus aureus Infection. J. Dairy Sci. 2001; 84: 1077-1084.

112. Blum JW, Dosogne H, Hoeben D, Vangroenweghe F, Hammon HM, Bruckmaier RM, Burvenich C. Tumor necrosis factor-[alpha] and nitrite/nitrate responses during acute mastitis induced by Escherichia coli infection and endotoxin in dairy cows. Domestic Animal Endocrinology 2000; 19: 223-235.

113. Waller K, Colditz I, Lun S, Östensson K. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. Research in Veterinary Science 2003; 74: 31-36.

114. Nakajima Y, Mikami O, Yoshioka M, Motoi Y, Ito T, Ishikawa Y, Fuse M, Nakano K, Yasukawa K. Elevated levels of tumor necrosis factor-alpha, TNF-alpha and interleukin-6 (IL-6) activities in the sera and milk of cows with naturally occurring coliform mastitis. Research in Veterinary Science 1997; 62: 297-298.

115. Davidson J, Tiemann U, Betts J, Hansen P. DNA synthesis and prostaglandin secretion by bovine endometrial cells as regulated by interleukin-1. Reproduction, Fertility and Development 1995; 7: 1037-1043.

116. Barros CM, Betts JG, Thatcher WW, Hansen PJ. Possible mechanisms for reduction of circulating concentrations of progesterone by interferon-alpha in cows: effects on hyperthermia, luteal cells, metabolism of progesterone and secretion of LH. J Endocrinol 1992; 133: 175-182.

117. Newton GR, Martinod S, Hansen PJ, Thatcher WW, Siegenthaler B, Gerber C, Voirol MJ. Effect of Bovine Interferon on Acute Changes in Body Temperature and Serum Progesterone Concentration in Heifers. J. Dairy Sci. 1990; 73: 3439-3448.

118. Geisert R, Morgan G, Short E, Zavy M. Endocrine events associated with endometrial function and conceptus development in cattle. J Reproduction, Fertility and Development 1992; 4: 301-305.

119. Rae MT, Palassio S, Kyle CE, Brooks AN, Lea RG, Miller DW, Rhind SM. Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. Reproduction 2001; 122: 915-922.

120. Ibanez L, Potau N, Ferrer A, Rodriguez-Hierro F, Marcos MV, de Zegher F. Reduced Ovulation Rate in Adolescent Girls Born Small for Gestational Age. J Clin Endocrinol Metab 2002; 87: 3391-3393.

121. Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J. Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3. Hum. Reprod. 2003; 18: 323-327.

122. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 1997; 15(2): 201-204.

123. La Marca A, Malmusi S, Giulini S, Tamaro LF, Orvieto R, Levratti P, Volpe A. Anti-Mullerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation. Hum. Reprod. 2004; 19: 2738-2741.

124. Eldar-Geva T, Margalioth EJ, Gal M, Ben-Chetrit A, Algur N, Zylber-Haran E, Brooks B, Huerta M, Spitz IM. Serum anti-Mullerian hormone levels during controlled ovarian hyperstimulation in women with polycystic ovaries with and without hyperandrogenism. Hum. Reprod. 2005; 20: 1814-1819.

125. Dumesic DA, Lesnick TG, Stassart JP, Ball GD, Wong A, Abbott DH. Intrafollicular antimüllerian hormone levels predict follicle responsiveness to follicle-stimulating hormone (FSH) in normoandrogenic ovulatory women

undergoing gonadotropin releasing-hormone analog/recombinant human FSH therapy for in vitro fertilization and embryo transfer. Fertility and Sterility 2009; 92: 217-221.

126. Seifer DB, MacLaughlin DT, Penzias AS, Behrman HR, Asmundson L, Donahoe PK, Haning RV, Jr., Flynn SD. Gonadotropin-releasing hormone agonist-induced differences in granulosa cell cycle kinetics are associated with alterations in follicular fluid mullerian-inhibiting substance and androgen content. J Clin Endocrinol Metab 1993; 76: 711-714.

127. Klein NA, Battaglia DE, Clifron DK, Bremner WJ, Soules MR. The gonadotropin secretion pattern in normal women of advanced reproductive age in relation to the monotropic FSH rise. J Soc Gynecol Invest 1996; 3: 27-32.

128. te Velde ER, Dorland M, Broekmans FJ. Age at menopause as a marker of reproductive ageing. Maturitas 1998; 30: 119-125.

129. te Velde ER, Pearson PL. The variability of female reproductive ageing. Hum Reprod Update 2002; 8: 141-154.

130. Ottolenghi C, Uda M, Hamatani T, Crisponi L, Garcia J-E, Ko M, Pilia G, Sforza C, Schlessinger D, Forabosco A. Aging of oocyte, ovary, and human reproduction. Ann NY Acad Sci 2004; 1034: 117-131.

131. Sen A, Bettegowda A, Jimenez-Krassel F, Ireland JJ, Smith GW. Cocaine- and Amphetamine-Regulated Transcript Regulation of Follicle-Stimulating Hormone Signal Transduction in Bovine Granulosa Cells. Endocrinology 2007; 148: 4400-4410.

132. Sen A, Lv L, Bello N, Ireland JJ, Smith GW. Cocaine- and Amphetamine-Regulated Transcript Accelerates Termination of Follicle-Stimulating Hormone-Induced Extracellularly Regulated Kinase 1/2 and Akt Activation by Regulating the Expression and Degradation of Specific Mitogen-Activated Protein Kinase Phosphatases in Bovine Granulosa Cells. Mol Endocrinol 2008; 22: 2655-2676.

133. Jimenez Krassel F, Ireland JJ. Development and validation of a short-term, serum-free culture system for bovine granulosa cells: evaluation of the effects of somatotropin and growth hormone-releasing factor on estradiol production. J Dairy Sci 2002; 85: 68 - 78.

134. Kobayashi Y, Jimenez Krassel F, Li Q, Yao J, Huang R, Ireland JJ, Coussens PM, Smith GW. Evidence that cocaine- and amphetamine-regulated transcript is a novel intraovarian regulator of follicular atresia. Endocrinology 2004; 145: 5373 - 5383.

135. Jimenez-Krassel F, Winn ME, Burns D, Ireland JLH, Ireland JJ. Evidence for a Negative Intrafollicular Role for Inhibin in Regulation of Estradiol Production by Granulosa Cells. Endocrinology 2003; 144: 1876-1886.

136. Bettegowda A, Patel OV, Ireland JJ, Smith GW. Quantitative analysis of messenger RNA abundance for ribosomal protein L-15, cyclophilin-A, phosphoglycerokinase, beta-glucuronidase, glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and histone H2A during bovine oocyte maturation and early embryogenesis in vitro. Molecular Reproduction and Development 2006; 73: 267-278.

137. Bettegowda A, Yao J, Sen A, Li Q, Lee K-B, Kobayashi Y, Patel OV, Coussens PM, Ireland JJ, Smith GW. JY-1, an oocyte-specific gene, regulates granulosa cell function and early embryonic development in cattle. Proceedings of the National Academy of Sciences 2007; 104: 17602-17607.

138. Marshall OJ. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 2004; 20: 2471-2472.

139. Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. Journal of Immunological Methods 2003; 278: 261-269.

140. Murphy BD. Models of Luteinization. Biology of Reproduction 2000; 63: 2-11.

141. Berndtson AK, Vincent SE, Fortune JE. Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. Biology of Reproduction 1995; 52: 1334-1342.

142. Saumande J. Culture of bovine granulosa cells in a chemically defined serum-free medium: The effect of insulin and fibronectin on the response to FSH. The Journal of Steroid Biochemistry and Molecular Biology 1991; 38: 189-196.

143. McArdle CA, Kohl C, Rieger K, Gröner I, Wehrenberg U. Effects of gonadotropins, insulin and insulin-like growth factor I on ovarian oxytocin and progesterone production. Molecular and Cellular Endocrinology 1991; 78: 211-220.

144. Wrathall JHM, Knight PG. Production of immunoactive inhibin by bovine granulosa cells in serum-free culture: Effects of exogenous steroids and FSH. Domestic Animal Endocrinology 1993; 10: 289-304.

145. Lucy MC, Savio JD, Badinga L, De La Sota RL, Thatcher WW. Factors that affect ovarian follicular dynamics in cattle. J. Anim Sci. 1992; 70: 3615-3626.

146. Fanchin R, Mendez Lozano DH, Louafi N, Achour-Frydman N, Frydman R, Taieb J. Dynamics of serum anti-Mullerian hormone levels during the luteal phase of controlled ovarian hyperstimulation. Hum. Reprod. 2005; 20: 747-751.

147. Fanchin R, Schonauer LM, Righini C, Frydman N, Frydman R, Taieb J. Serum anti-Mullerian hormone dynamics during controlled ovarian hyperstimulation. Hum. Reprod. 2003; 18: 328-332.

148. Fauser BCJM, van Heusden AM. Manipulation of Human Ovarian Function: Physiological Concepts and Clinical Consequences. Endocr Rev 1997; 18: 71-106.

149. Weenen C, Laven JSE, von Bergh ARM, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BCJM, Themmen APN. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol. Hum. Reprod. 2004; 10: 77-83.

150. Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, Mason H. Granulosa Cell Production of Anti-Mullerian Hormone Is Increased in Polycystic Ovaries. J Clin Endocrinol Metab 2007; 92: 240-245.

151. Ball BA, Conley AJ, MacLaughlin DT, Grundy SA, Sabeur K, Liu IKM. Expression of anti-Müllerian hormone (AMH) in equine granulosa-cell tumors and in normal equine ovaries. Theriogenology 2008; 70: 968-977.

152. Meduri G, Massin N, Guibourdenche J, Bachelot A, Fiori O, Kuttenn F, Misrahi M, Touraine P. Serum anti-Mullerian hormone expression in women with premature ovarian failure. Hum. Reprod. 2007; 22: 117-123.

153. Voutilainen R, Miller WL. Human Mullerian Inhibitory Factor Messenger Ribonucleic Acid is Hormonally Regulated inthe Fetal Testis and in Adult Granulosa Cells. Mol Endocrinol 1987; 1: 604-608.

154. Winkler N, Bukulmez O, Hardy DB, Carr BR. Gonadotropin releasing hormone antagonists suppress aromatase and anti-Müllerian hormone expression in human granulosa cells. Fertility and Sterility 2009; In Press.

155. Knight PG, Glister C. Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 2001; 121: 503-512.

156. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. Methods 2001; 25: 402-408.

157. Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Anti-Mullerian Hormone Attenuates the Effects of FSH on Follicle Development in the Mouse Ovary. Endocrinology 2001; 142: 4891-4899.

158. Krarup T, Pederson T, Faber M. Regulation of oocyte growth in the mouse ovary. Nature 1969; 224: 187-188.

159. Peters H, Byskov AG, Grinsted J. Follicular growth in fetal and prepubertal ovaries of humans and other primates. Clin Endocrinol Metab 1978; 7: 469-485.

160. Driancourt MA. Ovarian features contributing to the variability of PMSG-induced ovulation rate in sheep. J Reprod Fert 1987; 80: 207-212.

161. Hirshfield AN. Relationship between supply of primordial follicles and the onset of follicular growth in rats. Biol Reprod 1994; 50: 421-428.

162. Shirota M, Soda S, Katoh C, Asai S, Sato M, Ohta R, Watanabe G, Taya K, Shirota K. Effects of reduction of the number of primordial follicles on follicular development to achieve puberty in female rats. Reproduction 2003; 125: 85-94.

163. Seifer DB, ScottJr RT, Bergh PA, Abrogast LK, Friedman CI, Mack CK, Danforth DR. Women with declining ovarian reserve may demonstrate a decrease in day 3 serum inhibin B before a rise in day 3 follicle-stimulating hormone. Fertility and Sterility 1999; 72: 63-65.

164. Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM. Early follicular serum müllerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertility and Sterility 2002; 77: 468-471.

165. Penarrubia J, Fabregues F, Manau D, Creus M, Casals G, Casamitjana R, Carmona F, Vanrell JA, Balasch J. Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist-gonadotropin treatment. Hum. Reprod. 2005; 20: 915-922.

166. Visser JA, de Jong FH, Laven JSE, Themmen APN. Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 2006; 131: 1-9.

167. Lass A, Silye R, Abrams DC, Krausz T, Hovatta O, Margara R, Winston RM. Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. Hum Reprod 1997; 12: 1028-1031.

168. Silva JM, Price CA. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells in vitro. Biol Reprod 2000; 62: 186-191.

169. Findlay JK, Drummond AE. Regulation of the FSH Receptor in the Ovary. Trends in Endocrinology and Metabolism 1999; 10: 183-188.

170. LaPolt PS, Tilly JL, Aihara T, Nishimori K, Hsueh AJ. Gonadotropin-induced up- and down-regulation of ovarian follicle- stimulating hormone (FSH) receptor gene expression in immature rats: effects of pregnant mare's serum gonadotropin,

human chorionic gonadotropin, and recombinant FSH. Endocrinology 1992; 130: 1289-1295.

171. Gutierrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle- stimulating hormone, and morphological characteristics. Biol Reprod 1997; 56: 608-616.

172. Ireland JJ, Richards JS. A previously undescribed role for luteinizing hormone (LH:hCG) on follicular cell differentiation. Endocrinology 1978; 102: 1458-1465.

173. di Clemente N, Goxe B, Remy JJ, Cate RL, Josso N, Vigier B, Salesse R. Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 1994; 2: 553-558.

174. Grossman MP, Nakajima ST, Fallat ME, Siow Y. Müllerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. Fertility and Sterility 2008; 89: 1364-1370.

175. Hsueh AJW, Liu Y-X, Cajander S, Peng X-R, Dahlh K, Kristensen P, Ny TOR. Gonadotropin-Releasing Hormone Induces Ovulation in Hypophysectomized Rats: Studies on Ovarian Tissue- Type Plasminogen Activator Activity, Messenger Ribonucleic Acid Content, and Cellular Localization. Endocrinology 1988; 122: 1486-1495.

176. Conti M, Harwood JP, Hsueh AJ, Dufau ML, Catt KJ. Gonadotropin-induced loss of hormone receptors and desensitization of adenylate cyclase in the ovary. J Biol Chem 1976; 251: 7729-7731.

177. Amsterdam A, Hanoch T, Dantes A, Tajima K, Strauss JF, Seger R. Mechanisms of gonadotropin desensitization. Mol Cell Endocrinol 2002; 187: 69-74.

178. Picard J-Y, Tran D, Josso N. Biosynthesis of labelled anti-müllerian hormone by fetal testes: Evidence for the glycoprotein nature of the hormone and for its disulfide-bonded structure. Molecular and Cellular Endocrinology 1978; 12: 17-30.

179. Jost A. Problems of fetal endocrinology: the gonadal and hypophyseal hormones. Recent Prog Horm Res 1953; 8: 379-418.

180. Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Control of Primordial Follicle Recruitment by Anti-Mullerian Hormone in the Mouse Ovary. Endocrinology 1999; 140: 5789-5796.

181. Durlinger AL, Visser JA, Themmen AP. Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 2002; 124: 601-609.

182. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S. Bone Morphogenetic Protein-15 Inhibits Follicle-stimulating Hormone (FSH) Action by Suppressing FSH Receptor Expression. Journal of Biological Chemistry 2001; 276: 11387-11392.

183. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The Bone Morphogenetic Protein System In Mammalian Reproduction. Endocr Rev 2004; 25: 72-101.

184. Erickson G, Shimasaki S. The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reproductive Biology and Endocrinology 2003; 1: 9.

185. Visser JA, Themmen APN. Anti-Müllerian hormone and folliculogenesis. Molecular and Cellular Endocrinology 2005; 234: 81-86.

186. Yi SE, LaPolt PS, Yoon BS, Chen JYC, Lu JKH, Lyons KM. The type I BMP receptor BmprIB is essential for female reproductive function. Proc Nat Acad Sci U S A 2001; 98: 7994 - 7999.

187. Voss AK, Fortune JE. Levels of messenger ribonucleic acid for cytochrome P450 17 alpha- hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after the luteinizing hormone surge. Endocrinology 1993; 132: 2239-2245.

188. Luck MR, Rodgers RJ, Findlay JK. Secretion and gene expression of inhibin, oxytocin and steroid hormones during the in vitro differentiation of bovine granulosa cells. Reprod Fertil Dev 1990; 2: 11-25.

189. Mossa F, Jimenez-Krassel F, Walsh S, Berry DP, Butler ST, Folger J, Smith GW, Ireland JLH, Lonergan P, Ireland JJ, Evans ACO. Inherent capacity of the pituitary gland to produce gonadotropins is not influenced by the number of ovarian follicles ≥3 mm in diameter in cattle. J Reproduction, Fertility and Development 2010; 22: 550-557.

190. Gluckman PD, Hanson MA. Living with the Past: Evolution, Development, and Patterns of Disease. Science 2004; 305: 1733-1736.