# Influence of the Size and Age of the Ovulatory Follicle on Fertility in Beef Cattle

Dissertation

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ABSTRACT

Artificial insemination (AI) is an underutilized technology in the beef industry. Four experiments were performed to improve understanding of the reproductive system of cattle and how this information can be incorporated into estrous control programs to advance the use of AI in the beef industry. In the first experiment, cows were permitted to either spontaneously exhibit estrus and ovulate from a dominant follicle, or were induced to ovulate from a dominant follicle when it had reached 10 mm in diameter; which is considered smaller than the diameter at which ovulation normally occurs (13-15 mm). This model allowed corpus luteum (CL) function and fertility to be measured in animals that ovulated from dominant follicles differing in diameter and that matured within two different endocrine environments. Conception rate to artificial insemination (AI) was greater in those animals that ovulated from a follicle following spontaneous estrus as compared with animals that ovulated from a smaller dominant follicle in response to treatment with exogenous GnRH. Results from this experiment were the basis for the second experiment designed to examine differences in luteal function and fertility between cows induced with exogenous GnRH to ovulate from either small or large dominant follicles. This approach eliminated the confounding effect that

occurrence of spontaneous estrus may have exerted on fertility. Animals that ovulated from a large follicle in response to exogenous GnRH were more fertile than those animals induced to ovulate from a smaller follicle.

The third experiment was designed to begin to elucidate mechanisms responsible for reduction in fertility in cows induced to ovulate from small follicles. Embryos from untreated donors were implanted into animals that were treated in a similar fashion as in the second experiment, but not inseminated preceding ovulation. This approach allowed separation of effects of the ovum, uterus and luteal function on fertility. Fertility was decreased in cows that had previously ovulated from a small follicle, as compared to those induced to ovulate from larger follicles when embryo transfer was used to impregnate the cows. The final experiment was performed to determine if decreased fertility observed in the previous experiments was due to size of the follicle from which ovulation occurred or duration of proestrus. In previous experiments the interval from luteal regression to induction of ovulation was confounded with diameter of the follicle from which ovulation was induced to occur. Hence, this experiment was essential to identify whether follicle diameter or duration of proestrus was having the greater impact on fertility. Fertility was dependent on length of proestrus when ovulation from follicles of similar diameter was induced.

Taken together, results of the experiments described in this dissertation provide evidence that fertility is significantly influenced by duration of proestrus. Information

garnered from these experiments can readily be incorporated into future estrous cycle regulation programs and superovulation regimens for embryo transfer.

# DEDICATION

To:  Jana, Lane and Phoebe

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## FIELDS OF STUDY

Major Field:  Animal Science

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# CHAPTER 1

# INTRODUCTION

To further genetic progress in the beef cattle industry with artificial insemination (AI), development and implementation of effective methods to regulate the estrous cycle are of primary importance. To effectively incorporate AI in most beef cattle operations, estrous synchronization programs that allow AI at a predetermined time (timed AI) are desirable. The use of timed AI eliminates the need for estrous detection and the increased labor that is inherent when estrous detection is used to determine the appropriate time for AI. Programs that incorporate timed AI have increasingly been accepted by cattle producers as their efficacy has been improved through research. A large data set has been compiled describing the successes and inefficiencies of various programs, however, only a small amount of information is available that identifies how such variables such as follicle size and age of the follicle from which ovulation occurs influence fertility. To continue to make improvements in estrous control programs and in the understanding of the reproductive system of cattle, an evaluation of the fertility of various subclasses of animals following a typical estrous cycle control program would be useful. A series of experiments were designed to understand influence of size of the follicle from which ovulation occurs has on fertility.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

This review pertains to the applied use of estrous control systems in cattle and impacts on the fundamental biology of cattle reproduction. Challenges associated with reproductive performance and implementation of estrous control systems are addressed. Basic principles of reproductive physiology in cattle are reviewed with an emphasis on ovarian follicular development. Current methods of estrous control are described as are the inherent deficiencies associated with these systems. A section is included to address current knowledge of fertility of cattle that ovulate from ovarian follicles at different stages of development. The review concludes with a problem statement and a rationale for conducting the research.

## 2.2 Challenges associated with implementation of estrous control systems

Reproductive performance has been considered to be the single largest contributing factor to economic efficiency of a cattle operation for many years (Dickerson et al., 1974). While many producers strive for enhanced growth rate on an individual cow and calf basis, the most important factor contributing to economic profit is

considered to be number of calves born compared with number of cows exposed or submitted to the breeding program (Dearborn et al., 1973). Artificial insemination (AI) is one method to improve reproductive efficiency of a cow-calf production system. Artificial insemination and many of the estrous control programs associated with AI promote the concept of 1) cows conceiving early in the breeding season, 2) providing a more uniform calf crop, 3) induction of ovulation in otherwise anestrous cows and 4) providing the opportunity to utilize superior sires not attainable for natural mating. Many of the current methods of estrous control used to support incorporation of AI require a substantial labor and time investment with results that are often unsatisfactory to the producer. To take advantage of AI and to further knowledge of the cattle estrous cycle, an enhanced understanding of factors affecting fertility in cattle is needed. The following is a review of the current knowledge in the field of cattle reproduction as well as a description of the current methods of estrous control.

## 2.3 The Estrous Cycle of Cattle

### 2.3.1 Hypothalamic-Pituitary-Ovarian Axis

Regulation of ovarian function in mammals occurs primarily through the stimulatory actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are synthesized by the gonadotropes within the anterior pituitary gland. The actual function of the pituitary gland was discovered in the 20$^{th}$ century, and this gland is now known to produce at least six hormones responsible for coordinating several important body functions (Thorner et al., 1998). The gonadotropins FSH and LH are

heterodimeric glycoproteins sharing a common α-subunit of about 96 amino acids with two asparagine-linked carbohydrate chains (Bhasin and Swerdoff, 1995). The β-subunits of LH and FSH which are made up of approximately 115 amino acids are specific to their individual receptors; LHR and FSHR located in ovarian follicles (Bhasin and Swerdoff, 1995).

Regulation of FSH and LH synthesis and release is controlled by gonadotropin-releasing hormone (GnRH; Asa et al., 1995), secreted by the hypothalamus. The hypothalamus is a poorly defined anatomic region in the base of the brain (Sealfon et al., 1997). Hypothalamic hormones travel from the median eminence of the hypothalamus into a portal plexus capillary system which then supplies the anterior pituitary (Asa et al., 1995). GnRH is a decapeptide synthesized in the medial basal hypothalamus and secreted in a pulsatile manner into the hypothalamo-hypophyseal portal system (Sealfon et al., 1997) to act on the anterior pituitary. The hypothalamo-hypophyseal portal system provides a direct route to the anterior pituitary, thus preventing GnRH from entering general circulation before reaching the cells of the anterior pituitary. Within the anterior pituitary, GnRH binds to its G-protein-coupled, 7-transmembrane domain receptor (Sealfon et al., 1997; Nett, 2005) located on the gonadotropes and induces synthesis and secretion of LH and FSH (Brinkley, 1981).

The episodic release of GnRH from the hypothalamus is required for synthesis and secretion of the gonadotropins (Anderson et al., 1981). LH and FSH are both released from the anterior pituitary in a pulsatile fashion and frequency and amplitude of pulse episodes vary with stage of the estrous cycle. However, the episodic release of LH

and FSH are not directly linked. Observations in cows (Schallenberger et al., 1984; 1985) indicated that all LH pulses were secreted concomitantly with FSH but there were often separate FSH pulses between LH pulses, suggesting a possible non-GnRH induced release of FSH. In ewes, it was observed every GnRH pulse induced a pulse of FSH and LH, however, non-GnRH associated episodes of FSH release occurred in the absence of GnRH release (Padmanabahn et al., 1997). In addition, administering GnRH antagonist to ewes effectively inhibited LH pulses but was unable to completely block FSH pulse release, suggesting existence of an episodic component of FSH secretion independent of GnRH I stimulation (Padmanabhan et al., 2003). These authors suggested this non-GnRH episodic release of FSH may be mediated via an alternative isoform of GnRH (GnRH II) and an alternative receptor (GnRH II receptor).

Although GnRH induces the release of both FSH and LH, frequency of GnRH release from the hypothalamus appears to determine the ratio of FSH and LH secreted from the anterior pituitary (Vizcarra et al., 1999; Ferris and Shupnik, 2006). A lesser pulse frequency of GnRH preferentially induces release of FSH while a greater frequency of GnRH release induces LH secretion (Molter-Ferard et al., 1999; Ferris and Shupnik, 2006). Pulses of LH occur at approximately hourly intervals during the follicular or proestrous stage, while during the luteal phase; pulses are detected only once every 3 or 4 hours (Rahe et al., 1980; Schallenberger et al., 1985). During the luteal phase when pulses of LH are less frequent pulses are of greater amplitude as compared with those which occur during proestrus (Rahe et al., 1980). Studies suggest that 82 to 85% of GnRH pulses result in a corresponding pulse of LH during proestrus and 60% of pulses

of GnRH result in a LH pulse during the luteal phase (Yoshioka et al., 2001). In both periods, pulses of LH were not observed unless thereis a prior pulse of GnRH.

In addition to GnRH the ovarian steroids progesterone and estradiol regulate release of LH and FSH from the pituitary (Walters et al., 1984; Cupp et al., 1995). Through a negative feedback mechanism, elevated progesterone concentrations reduce LH pulse frequency (Ireland and Roche, 1982; Stumpf et al., 1993; Bergfeld et al., 1996). During the luteal phase, GnRH receptor gene expression in the pituitary is reduced (Turzillo et al., 1998). Progesterone does not directly down regulate GnRH receptors in the pituitary (Nett et al., 2002), rather GnRH upregulates it's own receptor at the gonadotropes (Nett et al., 2002). Elevated progesterone concentrations prevented this positive feedback mechanism and subsequently caused decreased GnRH receptors in the gonadotropes. Removing the inhibitory effect of progesterone, results in increased LH pulse frequency and increased estradiol concentrations during the preovulatory period (Schallenberger et al., 1985). Ovariectomized cows were treated with progesterone, estradiol, or the combination of both progesterone and estradiol (Stumpf et al., 1993). Estradiol increased mean LH and LH pulse frequency compared to treatment with only progesterone. The combination of estradiol and progesterone together resulted in a decrease in mean LH and LH pulse frequency when compared to cows treated with only progesterone. Estradiol increases pituitary responsiveness to GnRH (Reeves et al., 1971), by increasing GnRH receptors in the pituitary (Vizcarra et al., 1997). Other studies in which greater concentrations of estradiol in circulation were achieved and where gonadotropin responses were examined within a shorter time frame, found concentrations

of LH and amplitude of the LH pulses were reduced (Wolfe et al., 1992; Price and Webb, 1988). These same studies provice evidence that estradiol also reduces concentrations of FSH in circulation. Estradiol inhibits synthesis of FSH in cultured ovine pituitary cells (Nett et al., 2002), but the more potent inhibitor of FSH secretion is ovarian follicular inhibin (Turzillo and Fortune, 1993; Kaneko et al., 2002).

There are negative feed back actions of follicular estradiol and inhibin on gonadotropin secretion, in particular FSH. (Price and Webb, 1988; Turzillo and Fortune, 1993). Kaneko et al., (2002) found a close inverse relationship between circulating FSH and inhibin A during the follicular growth phase in cattle in various reproductive states, but no relationship between plasma FSH and estradiol in postpartum anovulatory cows or during the mid-luteal phase of the estrous cycle. These data indicate that inhibin A is the more potent negative regulator of FSH as compared with estradiol. Inhibin molecules are disulphide-linked heterodimeric glycoproteins and members of the TGF-β superfamily (Knight and Glister, 2001). Various isoforms exist, but the mature bioactive forms are inhibin A and inhibin B. Each comprises a common α subunit, but differs with the β subunit being activin A or activin B. Intrafollicular inhibin B declines throughout the follicular selection process, while inhibin A increases after selection and is the predominant form in ovulatory follicles (Beg et al., 2002).

### 2.3.2 Luteal Phase of the Estrous Cycle

The luteal phase of the estrous cycle is characterized by the presence of a functional CL in the ovary and is defined as the time period following ovulation of the dominant follicle to the onset of luteal regression. The luteal phase can further be divided into early, mid and late stages. The early luteal phase is characterized by heightened LH pulse frequency due to lesser concentrations of progesterone and rapid growth of the luteal cells in the ovary (Rahe et al., 1980). During the early and mid luteal phases of the estrous cycle, diameter and weight of the CL rapidly increases from approximately day 4 and reaches maximum between day 7 and 12 (Donaldson and Hansel, 1995). This increase in luteal tissue results in increased concentrations of progesterone in circulation and a decrease in LH pulse frequency; while LH pulse amplitude increases (Cupp et al., 1995). Progesterone remains elevated in circulation until day 17 to 19 of the estrous cycle at which time luteal regression occurs if maternal recognition of pregnancy does not occur (Rahe et al., 1980; Cupp et al., 1995).

### 2.3.3 Luteal Regression

During the luteal phase of the estrous cycle, circulating ovarian steroids interact to promote an intrauterine environment conducive for embryonic development and establishment of pregnancy. However, if mating does not occur, or mating does occur and there is a failure in fertilization or early embryonic development, the luteal phase is terminated and the female again becomes sexually receptive. The role of the uterus during luteal regression was detected when it was learned that complete hysterectomy can

extend the lifespan of CL (Wiltbank and Casida, 1956; Malven and Hansel, 1964). Further studies revealed that complete hysterectomy was not required and that removal of the uterine horn ipsilateral to the ovary containing the functional CL also resulted in a prolonged luteal phase (Inskeep and Butcher 1966). Removal of the uterine horn contralateral to the functional CL resulted in normal luteal regression (Inskeep and Butcher, 1966; McCracken and Caldwell, 1969). This previous research indicates that during luteal regression, communication between the uterus and the CL is local and not through systemic circulation.

Prostaglandin $F_{2\alpha}$ of uterine origin is the agent responsible for normal regression of the CL (Hixon and Hansel, 1974, Goding, 1974; Horton and Poyser, 1976; Niswender et al., 1985). Transport of prostaglandin $F_{2\alpha}$ to the ovary occurs via local counter current mechanisms and is not mediated through systemic circulation (Hixon and Hansel, 1974). During luteal regression, release of uterine prostaglandin $F_{2\alpha}$ results in an increase in circulating concentrations of oxytocin (Flint and Sheldrick, 1982). Increased circulating concentrations of oxytocin are believed to be of luteal origin and function at the uterus to increase the release of prostaglandin $F_{2\alpha}$ (Mitchell et al., 1975). The complex interaction between oxytocin and prostaglandin $F_{2\alpha}$ establishes a positive feedback loop that results in a prolonged surge like release of prostaglandin $F_{2\alpha}$ and ensures rapid and complete luteal regression (Mitchell et al., 1975). If pregnancy occurs, oxytocin receptors are suppressed at the time of expected luteolysis and the surge-like release of prostaglandin $F_{2\alpha}$ from the uterus does not occur (Jenner et al., 1991). Luteal regression marks the end of the luteal phase of the estrous cycle and there is a rapid decline in the concentration of

progesterone followed by a decline in size of the CL. With the decline in the concentration of progesterone in circulation, there is a decrease in the inhibitory effect of progesterone on the pulse frequency of GnRH from the hypothalamus and increased frequency of LH pulses. This is supported experimentally in studies where administration of progesterone to cattle reduced LH pulse frequency (Ireland and Roche, 1982; Price and Webb, 1988), and in studies where pulse frequency increased after luteolysis was induced (Ireland et al., 1984). The effect of exogenous progesterone in reducing the frequency of LH pulses in cattle is rapid and dose-dependent (Bergfeld et al., 1996). During luteal regression there is a gradual transition into the follicular phase of the estrous cycle.

### 2.3.4 Follicular Phase of the Estrous Cycle

The follicular phase of the estrous cycle can be characterized as a period without progesterone influence. The start of the follicular phase occurs during luteal regression and is noted by increased concentrations of 17β-estradiol. The 17β-estradiol functions in a synergistic fashion to increase LH pulse frequency and amplitude which further stimulates 17β-estradiol synthesis. The increase in LH pulse frequency that occurs during proestrus, in response to declining progesterone concentrations and increased 17β-estradiol, results in a linear increase in mean concentration of LH (Chenault et al., 1975, Walters et al., 1984). LH pulse frequency and amplitude and 17β-estradiol concentrations continue to increase until the preovulatory LH surge occurs (Walters et al., 1984; Stumpf et al., 1991). During or just prior to the LH surge 17β-estradiol reaches

adequate concentrations to induce behavioral estrus (Swanson and Hafs, 1971). During the preovulatory surge of LH, final ovarian follicular maturation occurs with activation of the oocyte and the first meiotic division is completed before ovulation. Ovulation occurs approximately 30 hours after the onset of behavioral estrus and the preovulatory surge of LH (Chenault et al., 1975; Bernard et al., 1983, Pinheiro et al., 1998).

The follicular phase culminates with the preovulatory LH surge and behavioral estrus. The oocyte is released from the follicle approximately 30 hours after this LH surge which also signifies the end of the follicular phase.

## 2.4 Ovarian Follicular Development

### 2.4.1 Embryonic Origin of Oocytes and Formation of Primary Follicles

Primordial follicles are derived from oocytes (female germ cells) and pre-granulosa cells (somatic cells surrounding the oocytes). The mechanisms involved in this process called gonadogenesis are not well understood. Studies performed in laboratory animals provide evidence that the involvement of cell-to-cell communications via gap junctions and stimulatory actions from members of the transforming growth factor family, specifically bone morphogenetic proteins (Richards, 2001). Once in the region of the embryonic gonad, primordial germ cells undergo a period of mitotic divisions to increase total number of gametes; which occurs in cattle between 80 to 100 days of gestation (Ohno and Smith, 1964). After repeated mitoses, oogonia differentiate into oocytes and begin meiotic division (Ohno and Smith, 1964). Oocytes are arrested after metaphase I and the oocytes enter the interphase-like dictyotene stage. During this time

period a dense single layer of cumulus cells surround the oocyte to form a primordial follicle (Ohno and Smith, 1964). Development of primordial follicles through the primary (single layer of granulosa cells) and secondary stages (greater than a single layer of granulosa cells) of preantral development takes several weeks or months and is referred to as initial recruitment (McGee and Hsueh, 2000).

### 2.4.2 Ovarian Follicle Recruitment

As assessed by transrectal ovarian ultrasonography (Ginther et al., 2001), recruitment of ovarian follicles from the resting pool is characterized by emergence of several follicles about 4 mm in diameter that continue growing 1 to 2 mm per day during a 'parallel' growing phase for 2 to 3 days. An average of seven follicles develops beyond 5 mm in diameter during a wave of follicular development (Ginther et al., 2001). A transient increase in FSH that peaks about one day before or at emergence of the wave of follicular development is responsible for follicular recruitment (Adams et al., 1992; Ginther et al., 1996). The increase in FSH that promotes new follicular development occurs as a consequence of the previous dominant follicle becoming atretic or ovulation occurring from this follicle, and no longer suppressing circulating FSH through production of estradiol and inhibin (Kaneko et al., 1997; Tohei et al., 2001). Follicles recruited in the growing phase have FSH receptors on granulosa cells and LH receptors

on theca cells (Richards, 1994). At this stage of development, follicles have not yet attained LH receptors on the granulosa cells (Ireland and Roche, 1982, 1983; Xu et al., 1995).

### 2.4.3 Ovarian Follicle Selection

Following recruitment of a cohort of follicles, selection of several of the larger follicles out of this cohort occurs. The exact mechanism by which selection occurs is not fully identified, but has been identified as a two way coupling system between declining FSH concentrations and follicular selection (Ginther et al., 2000). FSH is considered to be the primary stimulator of all ovarian follicles that reach the recruitment phase. When FSH is administered exogenously during the selection phase, multiple follicles attain dominant follicle status (Adams et al., 1993; Mihm et al., 1997). As each ovarian follicle is stimulated to grow, it in turn contributes to the FSH decrease through production of estradiol and inhibin. This FSH decrease ultimately leads to demise of all the ovarian follicles that reached the recruitment phase with the exception of the dominant ovarian follicle. Only the largest follicle of the group is able to continue to grow following the selection phase. Subordinate follicles are atretic at 5 days following emergence of the ovarian follicular wave (Adams et al., 1993; Ko et al., 1991). The most commonly accepted theory for why only the largest follicle is able to continue its growth phase is that it is the first follicle of the cohort to attain functional LH receptors (Ginther et al., 2001). The time at which the future dominant follicle continues growing while the other subordinate follicles cease growth is referred to as deviation (Ginther et al., 2001). At the

beginning of deviation, only the more developed largest follicle is able to utilize the FSH in basal amounts that exist in circulation and becomes the only follicle involved in FSH/coupling (Kulick et al., 2001). On average, a small transient elevation in LH begins before deviation and decreases after deviation (Bergfelt et al., 2000). In some cases where two large follicles continue growing beyond 10 mm, co-dominance occurs and the nature of deviation is similar except that it occurs between the second and third largest follicle of the follicular wave (Kulick et al., 2001). Thecal cells which contain mRNA for LH receptors are present in all ovarian follicle size classes however, granulosa cells appear to have mRNA only after reaching 9 mm in diameter (Xu et al., 1995, Bao et al., 1998). Interestingly, one report concluded that mRNA for the LH receptor did not appear until after deviation was determined (Ginther et al., 2001). This implies that while LH may be important for maintenance and growth of the dominant follicle it is not necessarily the deciding factor as to why a follicle is selected to become the dominant follicle.

The final phase in selection is marked by a 2 to 3 mm deviation in the diameters of the largest compared to second largest follicle of the follicular wave. Mean time to deviation occurs about 3 days after follicular emergence when the largest growing follicle is about 8.5 mm in diameter (Ginther et al., 1996). Studies performed (Ko et al., 1991; Ginther et a., 2001) found that if the dominant follicle is physically removed through ovarian follicle aspiration, a new follicle develops from the same wave of follicular development and achieves dominance. This procedure was repeated and each time a new

follicle became dominant from the same original cohort of follicles or if the subordinate follicles had begun the process of atresia, an entirely new wave of follicles started to grow. These studies and others provide evidence that the dominant follicle is not necessarily predetermined, rather, it merely has a size and developmental advantage at the time of deviation.

### 2.4.4 Ovarian Follicle Dominance

The dominance phase of ovarian follicular development is characterized morphologically by the presence of a single large follicle approximately 12 to 17 mm in diameter, while subordinate follicles regress and there is an absence of new follicular wave development. Dominant follicles exist in this "dominance" phase for typically 5 to 7 days (Sirois and Fortune, 1988; Savio et al., 1988). Concentrations of estradiol in ovarian venous blood and in follicular fluid of the dominant follicle increase during the growth phase of the dominant follicle, and declines throughout the plateau phase (Badinga et al., 1992; Price et al., 1995; Rhodes et al., 1995). The dominant follicle is responsible for preventing development of other gonadotropin dependent follicles through secretion of estradiol and inhibin (Rhodes et al., 1995). Cessation in growth appears to be due to the dominant follicle obtaining a developmental status whereby concurrent LH support is sufficient for maintenance, but not further growth. This has been elucidated through studies where concentrations of LH were artificially increased by providing doses of progesterone that result in lesser concentrations of progesterone in circulation than those typically occurring during the mid-luteal phase of the cattle estrous

cycle resulting in increased concentrations of LH (Sirois and Fortune, 1990), or by direct administration of LH (Taft et al., 1996). In both studies, the dominant follicle was observed to increase in diameter and persist beyond its normal lifespan.

### 2.4.5 Ovarian Follicle Atresia

The wavelike pattern of ovarian follicular development is essentially an uninterrupted process throughout the lifetime of cattle beginning within the first four weeks after birth (Hopper et al., 1993). Given the limited number of follicles from which ovulation ever occurs, the majority of ovarian follicles are destined for death and regression, a physiological process referred to as atresia. This happens relatively quickly for subordinate follicles in each follicular wave as described earlier. In dominant follicles from which ovulation never occurs, the early stages of atresia include a reduction in aromatase activity and estradiol synthesis (Price et al., 1995) without substantial changes to gonadotropin receptor numbers in granulosa cells (Bodensteiner et al., 1996). Estradiol content in follicular fluid is basal, while concentrations of progesterone are increased (Price et al., 1995).

Atresia in ovarian follicles is facilitated by a "programmed-cell death" process called apoptosis. Apoptosis was initially detected to occur in the ovaries of rats (Hughes and Gorospe, 1991), chickens and pigs (Tilly et al., 1991), and subsequently considered to be an evolutionarily conserved process for ovarian follicular atresia in all animals (Kaipia and Hsueh, 1997). Cellular demise by apoptosis is distinguished from the other

two forms of death, necrosis and terminal differentiation, in that it involves a pre-programmed and orderly dismemberment of the cell.

### 2.4.6 Ovulation

A surge of FSH and LH from the anterior pituitary induces ovulation. The process of ovulation is actually quite rare in a random population of beef cattle. On average an individual cow may only ovulate from an ovarian follicle 2 or 3 times per year. In fact less than 1% of all ovarian follicles go through the process of ovulation. The LH surge which induces ovulation is preceded by a surge of GnRH from the hypothalamus (Karsch et al., 1997). Estradiol from the preovulatory follicle in the absence of progesterone is responsible for the release of GnRH as well as behavioral estrus (Evans et al., 1997). Prior to ovulation from the preovulatory follicle, it becomes increasingly sensitive to LH primarily through the increased binding of LH receptors in the thecal and granulosa cells. The surge of FSH which occurs simultaneously as the LH surge is referred to as the primary FSH surge. A secondary FSH surge occurs 4 to 24 hours after the LH surge and it is this increase in FSH which induces a new wave of ovarian follicular development to occur in the subsequent estrous cycle (Dobson, 1978; Kaneko et al., 1991).

## 2.5 Regulation of the Estrous Cycle

### 2.5.1 Synchronization of the Stage of the Estrous Cycle

The objective of estrous synchronization systems is to group animals into a similar stage of the estrous cycle to decrease labor and time requirements. The systems

used today are based off of three primary principles to synchronize estrus in cattle. The first principle is the use of a luteolytic agent to induce luteal regression which, in turn, initiates the follicular phase and ultimately an LH surge and ovulation. The second component of estrous synchronization involves utilizeing an exogenous progestin to either artificially extend the normal luteal phase in animals or to group animals into similar stages of the estrous cycle. The third principle involves resetting the stage of follicular development to ensure all animals are in a similar stage of follicle growth at the initiation of the follicular phase. Often these principles are used in combination for enhanced capability to regulate the estrous cycle in large groups of cattle.

### 2.5.2 Synchronization of Stage of the Estrous Cycle Utilizing Luteolytic Agents

Prostaglandin $F_{2\alpha}$ was demonstrated to be the luteolytic compound in cows (Rowson et al., 1972; Auletta and Flint, 1988). Natural luteal regression requires a complex interaction between ovarian steroids (progesterone and 17β-estradiol) and oxytocin in the uterine endometrium to regulate timing and magnitude of prostaglandin $F_{2\alpha}$ release (Knickerbocker et al., 1988). The limiting factor in using prostaglandin $F_{2\alpha}$ as an agent for synchronization of estrus is that cattle must have a functional CL that is responsive to prostaglandin $F_{2\alpha}$ action at the time of treatment. During the early luteal phase, from the day of ovulation until approximately 5 days or CL development, it is unresponsive to prostaglandin $F_{2\alpha}$ treatment (Rowson et al., 1972). Therefore, in a group of animals in random stages of the estrous cycle, a synchronization system which utilizes only one dose of prostaglandin $F_{2\alpha}$ will theoretically induce luteal regression and result in

behavioral estrus in approximately 75% of female cattle that are having normal estrous cycles (cyclic). A typical response would be approximately 60% of cyclic females in estrus (Lauderdale; 1975). Furthermore, in most groups of cattle in which time of estrus is synchronized, some are anestrous (either prepubertal or postpartum anestrus) and do not have a corpus luteum in their ovaries. In these animals, prostaglandin $F_{2\alpha}$ has no effect to synchronize estrus. Therefore, the total percentage that exhibit estrus in response to a single prostaglandin $F_{2\alpha}$ treatment is often less than 60%.

Several systems to synchronize time of estrus using prostaglandin $F_{2\alpha}$ have been developed to avoid this non-responsive period of luteal regression in cyclic females. It is important to note that none of these systems corrects for the losses associated with anestrous females. In one system, estrous detection and AI is performed for a period of 5 days prior to the prostaglandin $F_{2\alpha}$ treatment to eliminate the cows in early stages of corpus luteum development which would not respond to the injection of prostaglandin $F_{2\alpha}$. Following the injection of prostaglandin $F_{2\alpha}$ another period of estrous detection and AI occurs for 3 to 6 days. The major problem with this approach is the extended period (10+ days) of estrous detection.

An improvement of this system was the addition of a second injection of prostaglandin $F_{2\alpha}$ 10 to 12 days following the first injection. This eliminated the need for nearly half the time of estrous detection, but still required animals be cycling to respond to treatment (Lamb et al., 2009). The fertility of animals artificially inseminated following this treatment regimen resulted in conception rates similar to those achieved

with animals in which AI occurs following spontaneous estrus (Liehr et al., 1972; Inskeep, 1973; Lauderdale, 1973, 1975).

### 2.5.3 Synchronization of the Estrous Cycle Utilizing Exogenous Progestins

Progestins function in a similar fashion to progesterone and suppress behavioral estrus in cattle (Odde, 1990). MGA (melengestrol acetate) is a progestin that is usually fed on a daily basis for a period of time to have its desired effect. The CIDR (controlled intravaginal drug release) contains a natural analog of progesterone and is inserted into the cow's vagina for a period of 5 to 8 days and functions to suppress behavioral estrus. Regardless of the progestin source, the purpose is to suppress spontaneous behavioral estrus and the associated pre-ovulatory LH surge until withdrawal of this source of progestin. Used in this manner, the progestin eliminates the randomness of estrous activity in a group of animals. After the progestin source is removed a large percentage of the herd is allowed to exhibit normal estrous activity. In many animals the corpus luteum regresses during the progestin treatment and this exogenous progestin is solely responsible for prevention an LH surge and behavioral estrus. In those animals which did not go through natural corpus luteum regression, a luteolytic injection of prostaglandin$F_{2\alpha}$ is administered at the end of the progestin treatment to induce luteal regression and allow those animals with a functional corpus luteum at the time of progestin removal to also exhibit signs of normal estrous activity.

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### 2.5.4 Development of Persistent Ovarian Follicles

Administration of doses of progestin that are approved for commercial use in cattle can result in development of dominant ovarian follicles that grow to larger sizes than typically occur and persist in the ovary for extended periods of time. During the estrous cycle of cattle, concentrations of progesterone that are typical of those during the midluteal phase are necessary for maintenance of ovarian follicular dynamics (Lucy et al., 1992). Development of persistent ovarian follicles occurs with commercially recommended doses of progestin and when the CL is absent for most of the treatment period (Savio et al., 1993; Sanchez et al., 1995). When the CL is present and progesterone concentrations are typical of the midluteal phase of the estrous cycle, LH pulses are less frequent and persistent follicles do not normally develop.

The secretory pattern of LH during treatment with the progestins in the absence of a functional corpus luteum is similar to that during the follicular phase of the estrous cycle of cattle (Imakawa et al., 1986) and is likely the stimulus for development of persistent follicles (Cupp et al., 1992). Greater populations of LH receptors are detected on the granulosa and thecal cells of persistent ovarian follicles than on the same cell types of typical dominant follicles (Cupp et al., 1992). The greater population of LH receptors in the persistent ovarian follicles indicates greater cell differentiation and likely results from greater frequency in episodic releases of LH in these cows with lesser progesterone concentrations. Pulstatile LH episodes increase when progesterone or progestins are used in the absence of a functional CL at doses similar to what is used in commercial estrus synchronization programs (Roberson et al., 1989, Sanchez et al., 1993, 1995). This

increase in frequency of LH pulses results in an increase in circulating concentrations of 17β estradiol to concentrations greater than occurs during the normal midluteal phase of the estrous cycle (Roberson et al., 1989, Savio et al., 1993, Sanchez et al., 1993, 1995). If AI or natural breeding occurs following the ovulation from a follicle that has persisted in the ovary for an extended period of time, fertility is reduced (Savio et al., 1993). Fertilization rates of oocytes that originate from follicles that have persisted in the ovary for an extended period of time are similar to those of oocytes from "normal" follicles, however early embryonic mortality occurs to a greater extent in the oocytes from the aged follicles (Ahmad et al., 1995). This decrease in fertility is believed to be a consequence of the ovulation of "aged" oocytes (Mihm 1994, Revah and Butler, 1995). Completion of the first meiotic division of the oocyte is typically induced by the LH surge near the time of the onset of estrus, however an oocyte in a persistent follicle is exposed to frequent pulses of LH for an extended time, which may prematurely induce resumption of meiosis in the oocyte (Mihm et al., 1994, Revah and Butler 1995).

### 2.5.5 Synchronizing Ovarian Follicle Growth

The advancement of ovarian ultrasonography to characterize the wave like patterns of follicular growth in cattle (Savio et al., 1988; Sirois and Fortune et al., 1988; Ginther et al., 1989) led to the concept of managing ovarian follicular wave patterns in such a way that all or most animals have a healthy dominant follicle present at the cessation of the treatment. Timed AI became a viable possibility after this idea was established. The benefits of timed AI as opposed to estrous detection and AI are many;

inseminating all animals at a predetermined time, reduced labor requirements because estrous detection is no longer required and that there is not a need to manage animals individually as occurs with estroust detection and AI. Many of the estrous synchronization programs developed have taken into account importance of having a dominant follicle from which ovulation is present at the time of AI. Most estrous synchronization systems in the USA utilize an injection of GnRH at the onset of the program. This injection has multiple effects. If a follicle is present that can respond to a LH surge, ovulation occurs. This resets the ovarian follicle wave pattern due to the secondary FSH increase following the LH surge in addition to forming a new CL (Lamb et al., 2009). If a spontaneous CL was already present at time of GnRH then an accessory CL is formed which coexists with the existing CL. If no CL is present due to post partum anestrus or stage of the estrous cycle, a new CL is formed. This CL in anestrous animals will help to prevent short estrous cycles following breeding and also prevents a premature LH surge before the desired time of AI (Lamb et al., 2009). Typically an injection of prostaglandin $F_{2\alpha}$ is administered 5 to7 days following the GnRH injection. The CO-Synch program involves administering an injection of GnRH at the onset of the program to reset the ovarian follicular wave. Seven days following the first GnRH injection, prostaglandin $F_{2\alpha}$ is administered to induce luteal regression (Geary et al., 1998). This should result in luteal regression occurring at the same time as the dominant follicle of the ensuing follicular wave is achieving dominance. Because progesterone is no longer present in circulation, the dominant follicle becomes a from which ovulation occurs due to the increase in circulating concentrations of LH. This stimulus induces changes in the

follicle described earlier in this section of the dissertation regarding the follicular phase. The GnRH is administered 48 hours after the injection of prostaglandin $F_{2\alpha}$ which coincides with the time of AI in the CO-Synch program or if spontaneous estrus occurs, there will be ovulation from this follicle and release of an ovum as a consequence (Pursley et al., 1998). This program has some limitations, however, because in lactating beef cattle in random stages of the estrous cycle seldom do more than 60% of the animals respond to an injection of GnRH (Geary et al., 2006, Colazo et al., 2007). Vasconcelos (et al., 1999) and Navanukraw (et al., 2004) reported that 10% to 30 % of cows treated in a normal Ovsynch program failed to respond to the final GnRH treatment. Ovsynch is similar to CO-synch with the only difference being that AI occurs 12 hours after the second GnRH injection. In a dairy setting this extra time for animal handling for the Ovsynch program is of little consequence because cows are milked 2 or 3 times per day, however, in beef cattle the extra animal handling required with the Ovsynch program is cumbersome and results in most beef producers choosing the CO-Synch program. Ovulatory response to the first GnRH injection in either program is reported to be the key determinant for a successful estrous control program of this nature (Vasconcelos et al., 1999). Bello (et al., 2005) reported that in cows having estrous cycles at random times only 54% responded to an injection of GnRH at the onset of an Ovsynch program. The CL in cows that had ovulations after the first GnRH of Ovsynch, regardless of treatment , were more likely to undergo luteolysis in response to prostaglandin $F_{2\alpha}$ with the Ovsynch regimen of treatment, and have a greater estrous synchrony compared with cows not responding to first GnRH treatment (Colazo et al., 2007). Moreover, if cows had

ovulations after the first GnRH treatment, these cows were more likely to have a functional dominant follicle from which ovulation was capable at the final GnRH injection of Ovsynch (Vasconcelos et al., 1999). On average, a future dominant follicle takes 7 to 10 d to proceed through emergence, deviation, and dominance, before ovulation from this follicle occurs or becoming atretic (Ginther et al, 1989). Induction of ovulation is possible before atresia of the dominant follicle (Silcox et al., 1993; Moreira et al., 2000). A dominant follicle that emerges after ovulation as a result of the first GnRH treatment with the Ovsynch treatment regimen is likely to still be functional 9 d later, at the time of the last GnRH treatment regimen of Ovsych. In contrast, a follicle that emerges within 3 days before the first GnRH treatment of the Ovsynch regimen will probably not have an ovulation to first GnRH and may be atretic before the last GnRH treatment with the Ovsynch regimen. Thus, it is not surprising that synchronization of ovulation with the Ovsynch regimen increased if cows had ovulations as a result of the first GnRH treatment (Bello et al., 2005). This implies that at the time of the injection of prostaglandin $F_{2\alpha}$ there is a large variability in both the size and age of the dominant follicle. Some of the dominant follicles that are present at the timed of desired AI and ovulation could have originated from a follicular wave that began nearly 13 days prior to ovulation while others could have originated from follicular wave that began only 7 days prior to ovulation. This would result in dominant follicles that are several mm different in diameter and 5 to 6 days different in maturity at the time of ovulation. Very little is known about the resulting fertility of ova from follicles of this wide range in size and maturity.

## 2.6 Statement of the Problem

Current methods to regulate estrus and facilitate use of timed AI in beef and dairy cattle results in wide variation in size of the dominant follicle at ovulation. While some studies have suggested follicular size at ovulation, both lesser and greater diameters than typical influence fertility, these studies are retrospective and confounded by comparison of follicles of unknown origin and functionality. A primary question is; Is there an optimum diameter at ovulation from the dominant follicle that maximizes fertility? The current methods of estrous control utilized in the beef and dairy industries control follicular diameter in only a portion of the animals that are treated. Some of the animals treated respond to some of the steps in the estrous synchronization program, but seldom do all animals respond to all treatments. This provides great variability among individual animals at AI when it is desirable to have all animals in a similar phase of their reproductive cycle. To address this overarching question, a novel animal model was developed in which diameter of the ovulatory follicle at the time of ovulation could be adjusted to meet specific experimental objectives. The schematic diagram (Figure 2.1) depicts the animal model which was developed to control for size of the follicle at time of ovulation. The structure of this model allows for aspiration of the ovarian follicle to be utilized as a means to regulate ovarian follicle growth in all animals. The primary advantage of this model as compared with other models is that it allows the assignment of

animals to treatments prior to the time of ovulation. Most of the other models developed to study fertility and size of the ovulatory follicle are retrospective in design. This means that in the other animal models there are not specific treatments applied to specific groups rather treatments are determined retrospectively. Another advantage of this model is that it allows for greater control of treatment groups. There is an inherent randomness and variation among a group of animals. The animal model developed for the studies reported in this dissertation reduce the opportunity for variation among the treatment groups allowing for results that are more readily interpreted. Four experiments were performed with the following objectives.

Objective 1: Does premature induction of ovulation as compared with spontaneous estrus and ovulation influence fertility?

This first experiment was designed to identify specifically if any differences exist in fertility of cattle that are either induced to ovulate from ovarian follicles that are smaller than what normally occurs or that have a spontaneous estrus and ovulation of a "normal" size ovarian follicle. To maximize the potential to detect differences related to follicle diameter, animals were treated in a manner that resulted in either GnRH-induced ovulation of follicles that were of a minimal diameter expected to respond to the induced LH surge or ovulation from follicles that typically responded to an endogenous and spontaneous LH surge. The working hypothesis was that fertility would be increased in cows ovulating from large follicles that result from a spontaneous estrus and ovulation as compared with animals ovulating from small follicles as a result of a GnRH-induced ovulation.

Objective 2: When GnRH is utilized to induce ovulation, does size of the follicle at the time of ovulation affect fertility?

In experiment 1, treatments differed in both diameter of the ovulatory follicle and the fact that small follicles were induced to ovulate with GnRH whereas the follicles of greater diameter ovulated in response to a spontaneous LH surge. In the second experiment, the animal model was adjusted such that all ovulations were induced in all cows with GnRH, but diameters of follicles differed substantially. The animals that made up these two distinct treatment groups would be representative of follicle diameter existing within the variation among cattle receiving a standard estrous synchronization treatment. By design, size of the ovulatory follicle was closely regulated and allowed for observations of ovulatory follicle size, luteal function and fertility that resulted from ovulation from either small or large follicles following GnRH injection. The working hypothesis was that cows that were induced to ovulate from large follicles would have increased fertility as compared with cows that ovulated from small follicles.

A second experiment of this objective was designed to isolate which mechanisms may contribute to the decrease in pregnancy observed in the previous experiments. The animal model that was used in the first experiment of this objective was again utilized, however there was no AI of animals at time of GnRH, rather a wild type embryo was implanted 7 days following GnRH. This experiment was designed to provide insight as

to the lesion in the reproductive axis that influenced pregnancy rates in the previous studies. If pregnancy rates are similar between treatments in this experiment, it would suggest that the oocyte that originates from small follicles is compromised in some fashion. If pregnancy rates are reduced in the animals induced to ovulate from small follicle in this experiment, reduced fertility would be attributed to something more than the function of the oocyte because wild type embryos were utilized. For example the uterine environment or the function of the corpus luteum may be altered when small follicles are induced to ovulate which could have a negative impact on fertility.

Objective 3: Does duration of proestrus effect fertility if follicular diameter is held constant?

Variation in diameter of the ovulatory follicle for the previous experiments was achieved by changing the interval from the decline in progesterone to the LH surge. Initial results from the first three experiments suggested diameter of ovulatory follicle influenced fertility, however interval from progesterone decline to the LH surge, which we have defined as proestrus for this animal model, was a variable that remained unaccounted for in our previous experiments. The larger follicle at ovulation also had longer proestrus phase representing a confounding effect in our previous experiments which needed to be clarified. Results from the previous experiments provided evidence that ovulatory follicle size at ovulation did influence fertility. However, as size of the ovulatory follicle increases there are subsequent changes throughout the reproductive system which also may influence fertility. It is likely that the uterus is exposed to greater

concentrations of 17β estradiol for a longer duration as the dominant follicle is growing. In addition the ovulatory follicle and the ovum would be exposed to 17β estradiol for a longer period of time as compared with when a small follicle ovulates.

The fourth and final experiment of this dissertation was designed to separate the effects of follicle size and duration of proestrus on fertility. The same animal model as was utilized previously was used in this experiment, however subtle changes were made to identify the variables of follicle size and duration of proestrus. The animal model was adjusted in such a way that ovarian follicle size at time of ovulation would be similar among treatment groups; however duration of proestrus would be different. Results from this experiment would provide evidence as to the relative importance of ovulatory follicle size and duration of proestrus on fertility.

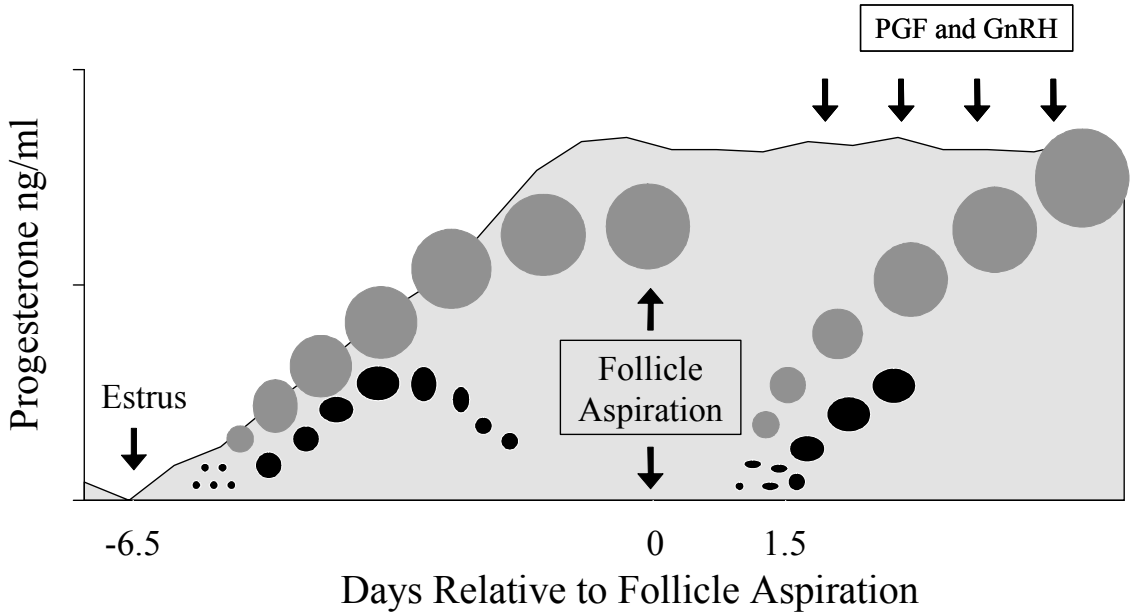


Figure 2.1 General animal model that was utilized in all of the experiments in this dissertation. Animals were synchronized to a common day of estrus prior to ovarian follicular aspiration. Ovarian follicular aspiration was used as a means to precisely regulate ovarian follicular growth. Prostaglandin $F_{2\alpha}$ and GnRH were administered at varying times to control for ovulatory follicle size and duration of proestrus.

# CHAPTER 3

# IINFLUENCE OF A PREMATURE INDUCTION OF AN LH SURGE WITH GNRH ON OVULATION, LUTEAL FUNCTION AND FERTILITY IN CATTLE


## Abstract

We tested the hypothesis that luteal function and fertility would be reduced in cattle induced to ovulate prematurely compared with spontaneous ovulation. Estrus was synchronized in 56 beef cows (24 nonlactating and 32 nursing calves). At 6.4 ± 0.1 d after estrus all follicles ≥ 5 mm were aspirated (day of aspiration = d 0) with a 17 G needle using the ultrasound-guided, transvaginal approach. On D 1.5 and 2, cows were administered two luteolytic doses of prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$ ). Ovarian structures were monitored by transrectal ultrasonography from D –2 to D 12, or ovulation. Emergence of a new follicular wave occurred on D 1.7 ± 0.1. When the largest follicle (F1) of the newly emerged wave was 10 mm in diameter (D 4.8 ± 0.1), animals were assigned on an alternating basis to receive 100 μg GnRH (GnRH-10; n = 29) to induce ovulation, or upon detection of spontaneous estrus, to the spontaneous (SPON) treatment (n = 24). Cows were bred by AI at 12 h after GnRH (GnRH-10) or 12 h after the onset of estrus (SPON) as detected using an electronic surveillance system (HeatWatch, CowChips LLC, Denver, CO). Blood samples were collected every other day beginning 2 d after

ovulation until pregnancy diagnosis 30 d after AI. Ovulation and AI occurred in 29/29 cows in the GnRH-10 and 24/24 cows in the SPON treatment. Ovulation occurred later ($P < 0.05$) in the SPON (D 7.7 ± 0.1) than GnRH-10 (D 6.8 ± 0.1) treatment. Occurrence of double ovulations was similar between treatments and were detected in 47% of cows resulting in 1.5 ± 0.1 ovulations/cow. Diameter of the ovulatory F1 and the second ovulatory follicle (F2; in cows with 2 ovulations) were greater ($P < 0.05$) in the SPON (12.0 ± 0.3 mm and 10.5 ± 0.4 mm, respectively) than GnRH-10 (10.7 ± 0.1 mm and 9.2 ± 0.3 mm) treatment. Cross sectional area of luteal tissue and plasma concentrations of progesterone during the mid-luteal phase were greater ($P < 0.05$) in the SPON (3.62 ± 0.2 $cm^2$ and 6.4 ± 0.3 ng/ml) than GnRH-10 (3.0 ± 0.2 $cm^2$ and 5.4 ± 0.2 ng/ml) treatment. Conception rate to AI in the SPON (100%) was greater ($P < 0.05$) than in the GnRH-10 (76%) treatment. The animal model used in this study resulted in unusually high conception rates and double ovulations. In conclusion, premature induction of the LH surge reduced diameter of the ovulatory follicle(s), luteal function and conception rate to AI.

## Introduction

Estrous synchronization regimens designed such that all female cattle are bred by AI at a fixed-time (timed-AI), regardless of spontaneous estrus, are used in beef and dairy cattle production to circumvent the challenges associated with detection of estrus and/or to permit handling of animals in groups rather than as individuals. Commonly used timed-AI protocols include the Ovsynch (Pursley et al., 1998) and CO-Synch (Geary et al., 1998) programs. These synchronization programs use GnRH and $PGF_{2\alpha}$ to sequentially control ovarian follicular dynamics, luteolysis, and ovulation. The intent of the initial treatment with GnRH, given 7 d before an injection of $PGF_{2\alpha}$ is to induce ovulation and reset follicular growth, leading to the synchronized development of mature dominant follicles that are induced to ovulate by a second GnRH injection given 2 to 3 d after $PGF_{2\alpha}$. However, the initial GnRH injection has been reported to reset follicular growth in only 66% of beef (Geary et al., 2000) and 64% of dairy cows (Vasconcelos et al., 1999), and variation in follicle size when the second GnRH treatment is given has been characterized (Perry et al., 2002).

It is inevitable that the lack of precision in resetting follicle waves with GnRH will alter age and diameter of the pre-ovulatory follicle at the time of synchronized ovulation and timed-AI; resulting in ovulatory follicles of varying maturity. Determination of the impact of variation in maturity of follicles at the time of synchronized ovulation on fertility is critical to modify timed-AI programs to optimize conception rate. The mechanisms by which follicular maturity at ovulation influences fertility are of primary interest. Our working hypothesis was that luteal function and

conception rate would be reduced when follicles that have not reached full maturity are induced to ovulate compared with cows that spontaneously exhibit estrus and ovulate.

## Materials and Methods

*Animals and Treatments*

Multiparous Angus and Angus x Simmental cows either nursing calves (n = 32) or non-lactating (n = 24) were enrolled in the study. Cows were 4.6 $\pm$ 0.2 yr of age, with all animals having a least one prior calf. Animals that were lactating were 74.3 $\pm$ 4.3 d post partum. Animals were fed mixed grass hay *ad libitum* throughout the experiment. All animals were handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. Estrus was synchronized in all animals with two injections of $PGF_{2\alpha}$ (Lutalyse, 25 mg dinoprost tromethamine per injection, Pfizer Animal Health, New York, NY) separated by 11 d. At 6.4 ± 0.1 d following the synchronized estrus, all ovarian follicles $\geq$ 5 mm in diameter were aspirated (D 0 of the experiment) with a 17-gauge needle by the ultrasonography-guided transvaginal approach (Bergfelt et al., 1994) using a 5 MHz convex array transducer (Aloka 500V, Corometrics Inc., Wallingford, CT). Subsequent to follicular aspiration, injections of $PGF_{2\alpha}$ (Lutalyse, 25 mg each) were administered on D 1.5 and 2 to induce luteolysis (Figure 3.1). Location and diameter of all ovarian follicles ≥ 5 mm in diameter were monitored daily from D -2 until detection of ovulation by transrectal ultrasonography using a 7.5 mHz linear array transducer attached to the Aloka 500V. At the time the largest follicle of the new cohort of follicles that emerged after aspiration

was determined to be ≥ 10 mm in diameter, animals were alternately assigned, within lactation status, to receive GnRH (100 μg, Cystorelin, Merial, Inselin, NJ) at that time (GnRH-10, n = 29) or no treatment (n = 27). All untreated cows that exhibited a spontaneous estrus (n = 24) were assigned to the spontaneous (SPON) treatment. Day of ovulation was defined as the first day that the preovulatory follicle was no longer visible. Diameter of the corpus luteum (CL) was determined 12 d after ovulation and cross-sectional area was also determined at this time using the ellipse function of the Aloka 500V instrument. In cows with two CL, cross-sectional area was calculated as the sum of the area for the two CL.

*Estrous detection, AI and pregnancy diagnosis*

Estrus was monitored with the Heatwatch estrous detection system (CowChips LLC, Denver, CO) in both treatments. Onset of estrus was defined as the first time that an animal was recorded to have been mounted by herd mates at least three times within a 4-h period. Artificial insemination was performed 12 h after onset of estrus in the SPON treatment and 12 h after the GnRH injection in the GnRH-10 treatment. A single experienced technician performed AI with semen from three bulls, which were used equally across treatments. Pregnancy diagnosis was performed at 30 and 60 d after AI using a 7.5 mHz linear array transducer (Aloka). Additionally, at 60 d after AI the number of fetuses present was determined for cows with double ovulations.

*Determination of Progesterone Concentrations*

Blood samples were collected every other day from D 2 to d 30 after ovulation to quantify progesterone concentrations. Blood was collected into tubes containing an anticoagulant (EDTA) from a coccygeal vessel and centrifuged at 1500 x *g* for 15 min within 1 h after collection. Plasma was stored at -20º C until concentrations were determined using a commercially available RIA kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA) as described previously for our laboratory (Burke et al., 2003). All samples from an individual animal were included in a single assay and each assay had equal numbers of animals from both treatments. The average intra-assay CV was 2.6%, and inter-assay CV (2 assays) for pooled plasma samples containing 1.5 and 7.5 ng/ml were 18.2 and 14.9% respectively. The average sensitivity of the assays was 0.2 ng/ml.

*Statistical Analyses*

The effects of treatment, time and the treatment by time interaction on concentrations of progesterone were analyzed by ANOVA using the MIXED procedure in SAS (SAS, 1996) accounting for repeated measures. The model was $Y_{ijk} = \mu + T_i + c_{j:i} + H_k + (TH)_{ik} + e_{ijk}$; where $Y_{ijk}$ is the observation of the *j*th cow in the *i*th treatment at the *k*th time, $\mu$ is the overall mean, $T_i$ is the *i*th treatment, $c_{j:i}$ is the random effect of the jth cow within the *i*th treatment ($c_{j:i} \sim N[0, \sigma^2_c]$), $H_k$ is the *k*th time, $(TH)_{ik}$ is the treatment by time interaction term, and $e_{ijk}$ is the random residual effect ($e_{ijk} \sim N[0, \Sigma]$); where $\Sigma$ is the variance-covariance of the residual errors with a first-order autoregressive structure for

repeated measures within cows. Lactation status of cows and the interaction with treatment were tested in the initial analyses of all data. This interaction was not significant in any analyses therefore the interaction term for lactation status by treatment was removed from final analyses. The effects of treatment on the interval from follicle emergence to ovulation and follicle diameter at ovulation were analyzed using the MIXED procedure in SAS ($Y_{ij} = \mu + T_i + e_{ij}$; with notations defined above). The effect of treatment on pregnancy rate and incidence of double ovulation were tested using the Fishers Exact Test (PROC FREQ, SAS). Data are expressed as the mean ± SEM.

## Results

Aspiration of ovarian follicles was performed 6.4 ± 0.1 d after the synchronized estrus (D 0). Emergence of a new wave of follicular development was detected on D 1.7 ± 0.1 and the largest follicle of this cohort attained a diameter of 10 mm on D 4.8 ± 0.1. Three cows that were eligible to be assigned to the SPON treatment were not detected in estrus and were not used in the experiment**;** resulting in 29 animals in the GnRH-10 treatment and 24 females in the SPON treatment. No cows in the GnRH-10 treatment were detected in estrus before, or at the time of the GnRH injection.

The interval from follicle aspiration to the injection of GnRH in the GnRH- 10 treatment was 4.8 ± 0.1 d and the interval to the onset of estrus in the SPON treatment was 6.2 ± 0.2 d. Thus, the intervals from $PGF_{2\alpha}$ to the preovulatory LH surge were approximated as 3.3 and 4.7 d in the GnRH-10 and SPON treatments, respectively. The day of ovulation and the diameter of the largest follicle (F1) that ovulated were less ($P <$

0.05) in the GnRH-10 than SPON treatment (Table 3.1). The incidence of double ovulations was 41% in the GnRH-10 and 54% in the SPON treatment and did not differ between treatments. Diameter of the second ovulatory follicle (F2) in animals with double ovulations was also less ($P < 0.05$) in the GnRH-10 than SPON treatment (Table 3.1).

A treatment x day interaction was detected ($P < 0.05$) for concentrations of progesterone from D 2 to 16 after ovulation when all animals (pregnant and not pregnant) were included in the analyses. The interaction was largely due to greater concentrations of progesterone in the SPON than GnRH-10 treatment from D 8 to 16 after ovulation (Figure3. 2). Consistent with the observation of greater concentrations of progesterone during the mid-luteal phase, cross-sectional area of the CL was greater ($P < 0.05$) in the SPON ($3.6 \pm 0.2$ cm$^2$) than GnRH-10 ($3.0 \pm 0.2$ cm$^2$) treatment on D 12 after ovulation. A second analysis of progesterone concentrations that included only pregnant animals and observations through D 30 after ovulation (Figure 3.3) indicated greater ($P < 0.05$) concentrations of progesterone in the SPON treatment were maintained through D 24 after ovulation (treatment x day, $P < 0.05$). Cross-sectional area of CL size did not differ when nonpregnant animals were excluded from this comparison (SPON $3.6 \pm 0.2$ cm$^2$; GnRH-10; $3.3 \pm 0.2$ cm$^2$). Across treatments, animals with two CL had greater ($P < 0.05$) concentrations of circulating progesterone (Figure 3.4; group x day, $P < 0.05$) and total cross-sectional area of the CL 12 d after ovulation than cows with a single CL (2 CL, $4.2 \pm 0.2$ cm$^2$; 1 CL; $2.7 \pm 0.1$ cm$^2$; $P< 0.05$).

The proportion of animals that were pregnant 30 d after AI was less ($P < 0.05$) in the GnRH-10 compared with SPON treatment by 24 percentage points (Table 3.1). All cows in the GnRH-10 treatment that were pregnant on D 30 were pregnant on D 60 whereas pregnancies were lost in 3 cows in the SPON treatment between the D 30 and D 60 pregnancy diagnosis.

In animals with double ovulations, 11 of 12 in the GnRH-10 treatment and 13 of 13 in the SPON treatment were pregnant on D 30. In animals with a single ovulation, conception rate on D 30 was 65% (11/17) in the GnRH-10 and 100% (11/11) in the SPON treatment. Accordingly, conception rate was greater ($P < 0.05$) for animals ovulating two follicles (96%) than cows that ovulated a single follicle (79%). Two fetuses were detected at 60 d after AI in 50% (6/12) of cows with double ovulations in the GnRH-10 treatment and 62% (8/13) of females in the SPON treatment; this proportion did not differ between treatments.

## Discussion

The present study was designed to compare luteal function and subsequent fertility between cows induced to ovulate immature follicles and those that spontaneously ovulate. Animals that were induced to ovulate immature follicles had lesser concentrations of progesterone during the ensuing luteal phase and early stages of pregnancy. Furthermore, it was demonstrated that fertility is reduced when animals are induced to ovulate a less mature dominant follicle compared with spontaneously ovulating animals.

The differences in "follicle maturity" were achieved by inducing ovulation in one treatment when follicles were of smaller diameter than typical ovulatory follicles versus permitting follicles to spontaneously mature and ovulate in the second treatment. While diameter was used as a benchmark for maturity, the animal model used, provided precise control of other aspects of follicular development. First, the origin of all ovulatory follicles was known. The ovulatory follicle was the largest growing follicle(s) of a new follicular wave that emerged in a synchronized endocrine environment after aspiration of follicles at a similar stage of the estrous cycle. Second, age (days from emergence) of ovulatory follicles, in addition to diameter was known. Third, the interval from $PGF_{2\alpha}$ to the GnRH-induced LH surge or the onset of estrus (referred to hereafter as "duration of proestrus"), and thus, the approximate interval from luteal regression to the LH surge was known.

The immature ovulatory follicles in the GnRH-10 treatment in the present study were smaller by approximately 1.3 mm and younger by 1.1 d than in the SPON treatment, and had experienced a proestrus period that was shorter by approximately 1.5 d. Follicle diameter at ovulation and the duration of proestrus have been identified in other reports as sources of variation in conception rate in cattle. Decreased conception rates have been observed in female beef cattle induced to ovulate follicles of lesser diameter within a CO-Synch synchronization program (Lamb et al., 2001; Perry et al., 2005). The influence of duration of proestrus on fertility was evaluated in dairy cows through modification of the interval from $PGF_{2\alpha}$ to the second GnRH treatment in an Ovsynch program (Peters and Pursley, 2003). These authors demonstrated that conception rate to timed-AI was greater

in animals with a 48 h interval from $PGF_{2\alpha}$ to GnRH than when GnRH was given at the same time as $PGF_{2\alpha}$, and that follicle size and conception rate increased when GnRH was given either 0, 12, 24 or 36 h after $PGF_{2\alpha}$. In these reports, the origin, functional status and age of the follicles that were induced to ovulate, and the endocrine status of the animals during the interval from follicle emergence to the onset of proestrus were not standardized among animals.

In the present study, variables of follicular development and endocrine environment were closely controlled, however, it is important to reiterate that age, diameter and duration of proestrus were varied between treatments. While follicle maturity has traditionally been defined by size of the follicle, results of the present study, taken together with the reports cited above, suggest that follicle maturity is more complex and may be the result of an interaction of several inputs. For example, it has been reported (Perry et al., 2005) that GnRH-induced ovulation of follicles that are ≤ 11 mm in size resulted in low pregnancy rates (18 to 29%) and a high rate of embryonic mortality (39%) between approximately 27 and 68 d after AI, but animals that spontaneously ovulated follicles ≤ 11 mm had similar fertility and embryonic mortality as animals that ovulated larger follicles after AI. In the present study, cows that were induced to ovulate with GnRH when follicles were 10 to 11 mm in diameter had conception rates of 76% (65% in single ovulating animals) and 0% embryonic mortality between 30 and 60 d postpartum. The question remains as to the relative importance of age/diameter, endocrine

environment during development, duration of proestrus preceding ovulation, and the interactions of these events, to influence maturity of ovulatory follicles and subsequent fertility.

Differences in follicle maturity likely result in differences in fertility through influencing one or more of a variety of physiological processes. Potential points of influence could include preovulatory estradiol concentrations, competence of the oocyte, oviductal function and sperm transport, uterine environment and luteal phase progesterone concentrations. Lower concentrations of estradiol were detected in dairy cows induced to ovulate follicles of lesser diameter (Vasconcelos et al., 2001) whereas in beef cows, this response was inconsistent among experiments (Perry et al., 2005). Estradiol concentrations were not measured in the present experiment; however, the GnRH-induced LH surge, which induces a rapid decrease in estradiol concentrations, occurred approximately 1.5 d before detection of the onset of estrus. Presumably this would coincide with the attainment of the preovulatory peak in estradiol concentrations. The influences of GnRH-induced ovulation of immature follicles on competence of the oocyte, oviductal function and sperm transport, and the uterine environment have not been investigated in depth.

In the present experiment, concentrations of progesterone did not differ during the early luteal phase, but were less during the mid-luteal phase and early pregnancy in cows that were induced to ovulate immature follicles. We (Burke et al., 2001) and others (Vasconcelos et al., 2001) have demonstrated that progesterone concentrations and cross-sectional area of the CL were reduced in animals that were induced to ovulate

prematurely. Others have noted decreased progesterone concentrations with ovulation of smaller follicles (Perry et al., 2005); however, this finding has not been consistent across reports (Perry et al., 2002, Taponen et al., 2002; Peters and Pursley, 2003). Maintenance of a functional CL is paramount to maintenance of early pregnancy. Decreased mid-luteal phase progesterone concentrations have been detected in nonpregnant versus pregnant cows (Lukaszewska and Hansel, 1980; Mann et al., 1995) and others have observed decreased pregnancy rates in cows that experienced a delayed rise in progesterone concentrations during the early luteal phase following insemination (Shelton et al., 1990; Mann and Lamming, 2001). A physiological minimum for the concentration of progesterone needed to support pregnancy has not been determined (Mann and Lamming, 1999). Because the signal in cattle for maternal recognition of pregnancy is provided via interferon-tau (IFN-τ) secretion from the embryo (Anthony et al., 1988) and progesterone concentrations and IFN-τ secretion by the embryo are highly correlated (Kerbler et al., 1997), it is possible that insufficient progesterone concentrations were responsible for the reduced conception rate in cows induced to ovulate immature follicles in the present study. However, further research is necessary to determine if subtle reductions in progesterone concentrations (approximately 1 ng/ml beginning on d 8 after ovulation in the present study) will decrease conception rates in cows.

The high incidence of double ovulations (47% of all animals) observed in the present study was unexpected. One characteristic of the model used was that the time of luteal regression (d 1.5) coincided with emergence of a new follicle wave (d 1.7 ± 0.1). Thus,

the follicle wave progressed from emergence in an environment that would be characterized by low progesterone concentrations and increased frequency of LH pulses characteristic of the follicular phase. Since the FSH increase in response to follicle aspiration peaks at approximately 30 h after aspiration (Burke et al., 2003), it is presumed that FSH was beginning to decline on d 1.5. The development of co-dominant follicles occurred more often in the first-follicular wave (35%) than in the second-follicular wave (4%; Kulick et al., 2001), and Bleach et al. (1998) reported that cattle with three follicle waves during the estrous cycle exhibited more (30%) double ovulations than those with two waves (0%). Both studies are consistent with the idea that a high rate of co-dominance is associated with low peripheral concentrations of progesterone around the time of emergence of a follicle wave. Accordingly, Lane et al. (2005) reported that progesterone suppressed the increase in FSH that initiates the first follicular wave in cattle. In other experiments, we have used the same model except that luteal regression was initiated 4 d after aspiration (~2.5 d after emergence of a new follicular wave; Mussard et al., 2003) or 5 d after aspiration (~3.5 d after emergence; Bailey et al., 2004) resulting in 15% and 0% incidence of double ovulation respectively. Gibbons et al. (1997), using a follicle aspiration model similar to the present study, detected double ovulations in 3/6 heifers when luteal regression was induced approximately 1.5 d after emergence of a follicle wave. Taken together, these reports suggest that as the interval of luteal regression relative to emergence of a follicle wave after aspiration increases, the incidence of double ovulations decreases. The changes in circulating hormone patterns that are associated with luteolysis (i.e. reduced progesterone and increased LH) may have

perturbed the normal process of dominant follicle selection. This remains speculative and studies are ongoing to elucidate the mechanism responsible for the high incidence of double ovulations observed in the present study.

It is likely that the high incidence of double ovulations contributed to the greater than normal conception rates in this experiment. All but 1 of the 25 cows with two ovulations conceived to AI. In 56% of cows with two ovulations (14/25), two fetuses were present at 60 d after AI. This is noteworthy as the mean diameter of the second follicle that ovulated was 9.2 ± 0.3 mm in the GnRH-10 treatment and 10.5 ± 0.4 mm in the SPON treatment. The finding that over 50% of these "small" follicles were represented as fetuses at 60 d after AI emphasizes that if follicle maturity is defined as the capacity of a follicle to result in pregnancy, then diameter, in itself, is a questionable indicator of maturity of ovulatory follicles.

In conclusion, premature ovulation of a dominant follicle with GnRH reduced size of the ovulatory follicle(s), reduced fertility and decreased subsequent luteal function. The underlying mechanisms that may be responsible for this reduction in conception rate include reduced preovulatory estradiol concentrations, incompetence of the oocyte, diminished oviductal function and sperm transport, improper uterine environment and/or impaired luteal function. It is possible that one of these represents the key mechanism for reduced conception or alternatively, that deficiencies in several of these physiological systems may have contributed to a cumulative depression in conception rate. Systematic studies that investigate these potential sources of infertility in isolation are required.

| Variable | GnRH-10 | SPON |
|---|---|---|
| Day[a] of GnRH injection or estrus | 4.8 ± 0.1* | 6.2 ± 0.2 |
| Incidence of Ovulation, % | 29/29 (100) | 24/24 (88.9) |
| Day of Experiment at Ovulation | 6.8 ± 0.1* | 7.7 ± 0.1 |
| F1 Diameter at Ovulation, mm | 10.7 ± 0.1* | 12.0 ± 0.3 |
| F2 Diameter at Ovulation, mm | 9.2 ± 0.3* | 10.5 ± 0.4 |
| Pregnant 30 d after AI, % | 22/29 (75.9)* | 24/24 (100) |

Table 3.1. Timing of ovulation, characteristics of ovulatory follicles and conception rate in cows induced to ovulate follicles of approximately 10 mm in diameter with GnRH (GnRH-10) or in cows that ovulated spontaneously (SPON). The day of follicular aspiration = d 0. F1 denotes the largest follicle, which ovulated and F2 denotes the second largest follicle which ovulated due to treatment. *GnRH-10 vs. SPON; $P < 0.05$.

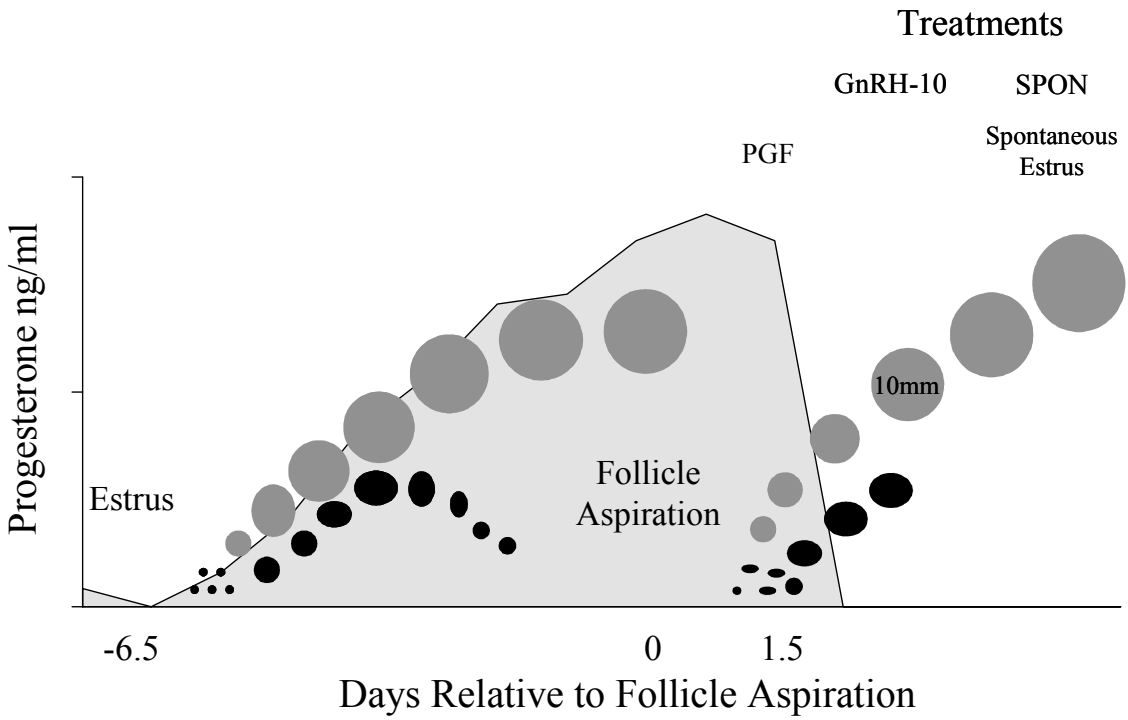


Figure 3.1 The animal model utilized in this experiment. Estrus was synchronized to a common day prior to aspiration. Ovarian follicle aspiration was utilized as a means to closely control ovarian follicular wave development. PGF was administered to all animals at a similar time and animals were randomly allocated to receive either GnRH or allowed to spontaneously exhibit estrus.

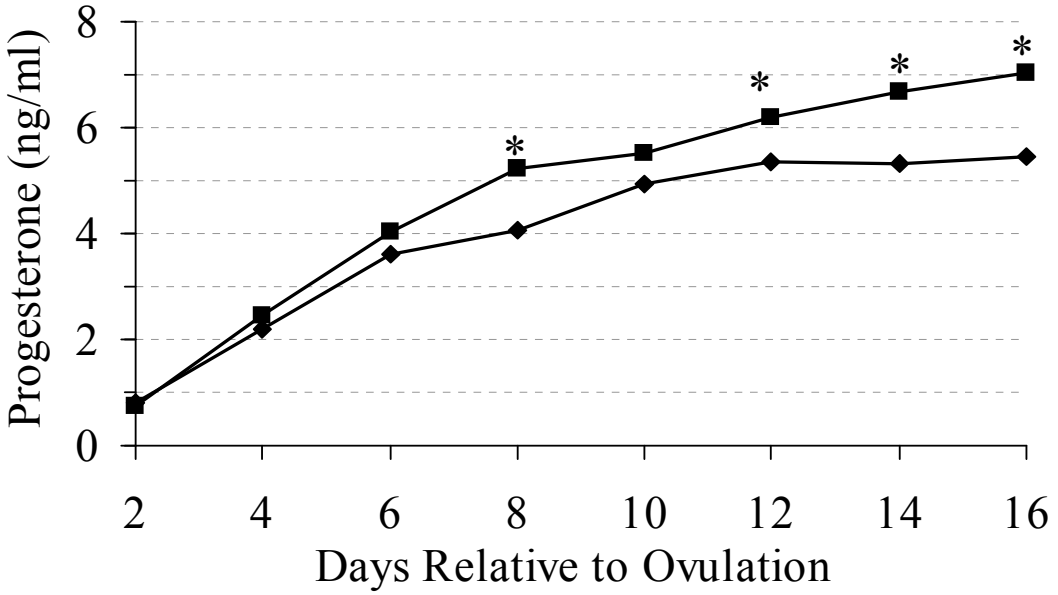


Figure 3.2. Circulating concentrations of progesterone in cows induced to ovulate follicles of approximately 10 mm in diameter with GnRH (GnRH-10 = ♦) or in cows that ovulated spontaneously (SPON = ■) following a $PGF_{2\alpha}$-induced luteal regression that occurred 1.5 d after follicular aspiration. Day 0 represents the day of ovulation (treatment x time, $P < 0.05$; * indicates that means within time differ, $P < 0.05$).

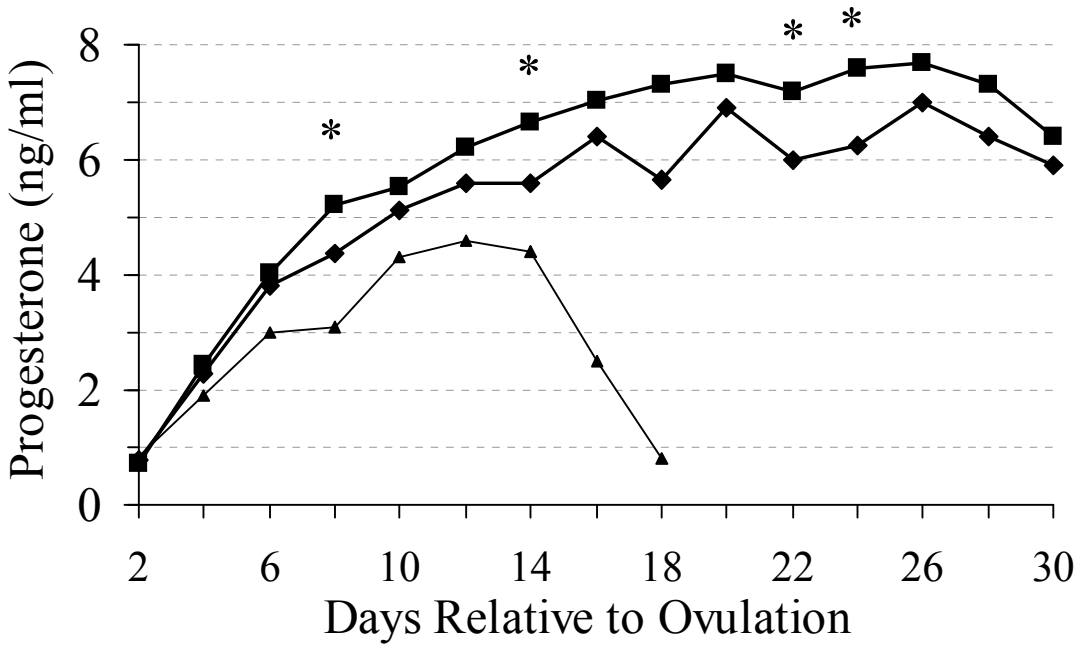


Figure 3.3. Circulating concentrations of progesterone in cows induced to ovulate follicles of approximately 10 mm in diameter with GnRH (GnRH-10 = ♦) or in cows which ovulated spontaneously (SPON= ■) that were pregnant 30 d after AI either 12 h after GnRH (GnRH-10) or 12 h after detection of estrus (SPON). Concentrations of progesterone in cows from the GnRH-10 treatment that were not pregnant 30 d after AI (Not pregnant = ▲) are included for illustrative purposes. Day 0 represents the day of ovulation. A treatment x time interaction ($P < 0.05$; pregnant cows in the GnRH-10 and SPON treatment) was detected (* indicates that means within time differed between treatments, $P < 0.05$).

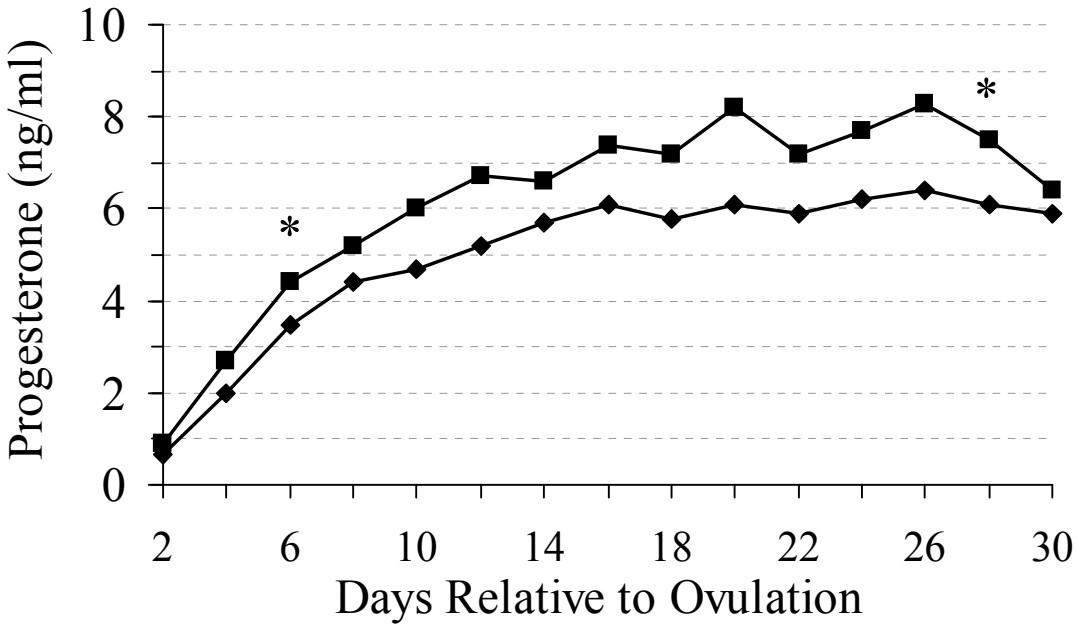


Figure 3.4. Circulating concentrations of progesterone in cows with either one corpus luteum ( 1 CL= ♦) or two CL (2 CL= ■). Cows across the GnRH-10 and SPON treatments were grouped according to number of CL. Day 0 represents the day of ovulation (treatment x group, $P < 0.05$; *indicates that means within time differ, $P < 0.05$).

# CHAPTER 4

## . OVULATORY RESPONSE, LUTEAL FUNCTION AND FERTILITY IN CATTLE INDUCED TO OVULATE DOMINANT FOLLICLES OF EARLY OR LATE MATURITY


### Abstract

In previous experiments, a reduction in luteal function and fertility occurred when ovulation from a premature dominant follicle was induced with GnRH as compared to when animals had a spontaneous LH surge that caused ovulation. To isolate the cause of this reduction in luteal function and fertility, two experiments were performed to test the hypothesis that luteal function and fertility would be also compromised when animals are induced to ovulate a small premature dominant follicle as compared to animals that are induced to ovulate a dominant follicle of normal size and maturity. In Exp 1, AI was performed following the timed LH surge and in Exp 2 embryos from untreated animals were implanted 7 days following the timed LH surge. In Exp 1, estrus was synchronized to a common day in 101 lactating crossbred cows. At 6.8 $\pm$ 0.1 days (d) after estrus all follicles > 5 mm were aspirated (d of aspiration = D 0) with a 17 G needle using the ultrasound-guided transvaginal approach. When the largest follicle following aspiration reached 8 mm, a luteolytic dose of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was administered. Ovarian

structures were monitored by transrectal ultrasonography from D –2 until D 12, or ovulation. When the largest follicle was 10 mm in diameter, animals were assigned to either receive 100 μg of GnRH at that time (GnRH-10) or when the largest follicle reached 13 mm (GnRH-13). Cows were bred by AI 12 hours after injection of GnRH. Blood samples were collected on D 8, 10, 12 and 14 after ovulation for analysis of circulating concentrations of progesterone during the mid-luteal phase. In Exp 2, the same animal model was utilized (GnRH-10ET n = 12, GnRH-13ET n = 12), however embryos from untreated animals were implanted 7 days following the synchronized ovulation in beef heifers. In both experiments pregnancy was determined with ultrasonography 30 d after AI. In Exp 1, in the GnRH-10 treatment 45/47 of animals ovulated and 54/54 of the animals in the GnRH-13 treatment ovulated following GnRH treatment. Ovulation occurred earlier and the dominant follicle was smaller in the GnRH-10 (7.1 $\pm$ 0.1 d, 11.1 $\pm$ 0.2 mm) than in the GnRH-13 treatment (8.3 $\pm$ 0.1 d, 13.6 $\pm$ 0.2 mm, respectively, $P < .05$). Circulating concentrations of progesterone were greater in the GnRH-13 treatment than the GnRH-10 treatment during the mid-luteal phase ($P < .05$). Conception rate to AI was decreased in the GnRH-10 treatment (2/45) vs. the GnRH-13 treatment (31/54) at pregnancy diagnosis on D 30 ($P < .01$). In Exp 2 conception rates were reduced by 59 percentage points in the GnRH-10ET treatment. Circulating concentrations of progesterone were also reduced in the GnRH-10ET treatment as compared with the GnRH-13ET treatment ($P < .05$). In conclusion, induced

ovulation of a premature dominant follicle resulted in decreased ovulatory size, reduced luteal function and compromised conception rates to both AI and ET as compared to animals that were induced to ovulate a more mature dominant follicle.

## Introduction

Estrous synchronization regimens designed such that all female cattle are bred by AI at a fixed-time (timed-AI), regardless of spontaneous estrus, are used in beef and dairy cattle enterprises to circumvent challenges associated with detection of estrus and/or to permit handling of animals in groups rather than as individuals. Commonly used timed-AI protocols include the Ovsynch (Pursley et al., 1998) and CO-Synch (Geary et al., 1998) programs. These synchronization programs use GnRH and $PGF_{2\alpha}$ to sequentially control ovarian follicular dynamics, luteolysis, and ovulation. The intent of the initial treatment with GnRH, given 7 d before an injection of $PGF_{2\alpha}$ is to induce ovulation and reset follicular growth, leading to the synchronized development of mature dominant follicles that are induced to ovulate by a second GnRH injection given 2 to 3 d after $PGF_{2\alpha}$. However, the initial GnRH injection has been reported to reset follicular growth in only 66% of beef (Geary et al., 2000) and 64% of dairy cows (Vasconcelos et al., 1999).

Determination of the impact of variation in maturity of follicles at the time of synchronized ovulation on fertility is important for to modifying timed-AI programs to optimize conception rate. Failure to control follicular development within a timed AI program can result in the ovulation of follicles that are either immature or aged. Fertility

following ovulation of aged follicles is reduced (Stock and Fortune, 1993; Mihm et al., 1994), thus the majority of estrous control programs are structured to minimize the development and ovulation of persistent dominant follicles. The ovulation of immature follicles with GnRH and its impact on fertility are not as well defined. Decreased conception rates have been observed in female beef cattle induced to ovulate follicles of lesser diameter within a CO-Synch synchronization program (Lamb et al., 2001; Perry et al., 2005). Peters and Pursley (2003) demonstrated that decreasing the interval from $PGF_{2\alpha}$ to GnRH-induced ovulation in an Ovsynch program resulted in decreased conception rates. In these, and other reports regarding this relationship, the origin, functional status, and age of the follicles that were induced to ovulate, and the endocrine status of the animals during the interval from follicular emergence to the onset of proestrus were not standardized among animals. Using an animal model that controlled these variables, we have demonstrated that when premature dominant follicles are induced to ovulate with GnRH, fertility is reduced as compared with dominant follicles that ovulate following a spontaneous LH surge (Mussard et al., 2007). The mechanisms by which follicular maturity at ovulation influences fertility are of primary interest. The conclusions that were derived from this research are somewhat confounded however, because the cows with smaller follicles were induced to ovulate with GnRH, whereas those that ovulated larger follicles experienced a spontaneous LH surge.

Two studies were conducted to more precisely examine the effects of ovulatory follicle size on fertility in females in which the LH surge was induced with GnRH. In the first experiment effects of ovulatory follicle size on AI conception rate were compared

whereas the second experiment was designed to compare pregnancy rate following implantation of embryos on d 7 of the estrous cycle; thereby indirectly assessing the influence of ovulatory follicle diameter on the uterine environment. Our working hypothesis was that luteal function and conception rate would be reduced when follicles that have not reached full maturity are induced to ovulate as compared with follicles that are induced to ovulate after they have reached what is projected to be full maturity, and that this difference in fertility between treatments would be negated when embryo transfer was used to impregnate the cows.

## Materials and Methods

### Experiment 1

*Animals and Treatments*

Multiparous Angus and Angus x Simmental cows nursing calves (n = 101) that were 4.1 $\pm$ 0.2 yr of age were enrolled in the study. Cows were 65.4 $\pm$ 5.6 d post partum at the onset of the experiment. Animals were allowed to graze mixed pasture, *ad libitum*, throughout the experiment. All animals were handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. To synchronize estrus, an intravaginal progesterone-releasing device (CIDR, Pfizer Animal Health, New York, NY) was inserted coincident with administration of GnRH (100 μg, Cystorelin, Merial, Inselin, NJ). Seven days later, the CIDR was removed and $PGF_{2\alpha}$ (Lutalyse; 25 mg dinoprost tromethamine per injection; Pfizer Animal Health, New York, NY) was administered. At 6.8 ± 0.1 d following the synchronized estrus, all

ovarian follicles ≥ 5 mm in diameter were aspirated (D 0 of the experiment) with a 17 G needle by the ultrasonography-guided transvaginal approach (Bergfelt et al., 1994) using a 5 MHz convex array transducer (Aloka 500V, Corometrics Inc., Wallingford, CT). Location and diameter of all ovarian follicles ≥ 5 mm in diameter were monitored daily from D 0 until D 12 or detection of ovulation by transrectal ultrasonography using a 7.5 mHz linear array transducer attached to the Aloka 500V. The day of emergence was defined as the day when the largest ovarian follicle of an ensuing wave of ovarian follicles reached 5 mm in diameter. When the largest follicle of the subsequent follicular wave following aspiration reached 8 mm, a luteolytic dose of $PGF_{2\alpha}$ was administered to all cows. At the time the largest follicle of the new cohort of follicles that emerged after aspiration was determined to be ≥ 10 mm in diameter, animals were alternately assigned to receive GnRH (100 μg, Cystorelin, Merial, Inselin, NJ) at that time (GnRH-10, n = 47) or when the largest follicle reached 13 mm in diameter (GnRH-13, n = 54). Day of ovulation was defined as the day on which the preovulatory follicle was no longer visible.

*Estrous Detection, AI and Pregnancy Diagnosis*

Artificial insemination was performed 10 to 14 h after the GnRH injection in both treatments. A single experienced technician performed AI with semen from four bulls, which were used equally across treatments. Pregnancy diagnosis was performed at 30 and 90 d after AI using a 7.5 mHz linear array transducer (Aloka).

*Determination of Progesterone Concentrations*

Blood samples were collected 8, 10, 12 and 14 days following the induced ovulation to determine mid-luteal phase concentrations of circulating progesterone. Blood was collected into tubes containing an anticoagulant (EDTA) from a coccygeal vessel and centrifuged at 1500 x *g* for 15 min within 1 h after collection. Plasma was stored at -20º C until concentrations were determined using a commercially available RIA kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA) as described previously for our laboratory (Burke et al., 2003). All samples from an individual animal were included in a single assay and each assay had equal numbers of animals from both treatments. The average intra-assay CV was 3.2%, and inter-assay CV (2 assays) for pooled plasma samples containing 1.5 and 7.5 ng/ml were 2.4 and 3.5% respectively. The average sensitivity of the assays were 0.2 ng/ml.

*Statistical Analyses*

The effects of treatment, time and the treatment by time interaction on concentrations of progesterone were analyzed by ANOVA using the MIXED procedure in SAS (SAS, 1996) accounting for repeated measures. The model was $Y_{ijk} = \mu + T_i + c_{j:i} + H_k + (TH)_{ik} + e_{ijk}$; where $Y_{ijk}$ is the observation of the *j*th cow in the *i*th treatment at the *k*th time, $\mu$ is the overall mean, $T_i$ is the *i*th treatment, $c_{j:i}$ is the random effect of the jth cow within the *i*th treatment ($c_{j:i} \sim N[0, \sigma^2_c]$), $H_k$ is the *k*th time, $(TH)_{ik}$ is the treatment by

time interaction term, and $e_{ijk}$ is the random residual effect ($e_{ijk} \sim N[0, \Sigma]$); where $\Sigma$ is the variance-covariance of the residual errors with a first-order autoregressive structure for repeated measures within cows. Effects of treatment on the interval from follicle emergence to ovulation and follicle diameter at ovulation were analyzed using the MIXED procedure in SAS ($Y_{ij} = \mu + T_i + e_{ij}$; with notations defined above). Effect of treatment on pregnancy rate and incidence of double ovulation were tested using the Fishers Exact Test (PROC FREQ, SAS). Data are expressed as the mean ± SEM.

**Experiment 2**

*Animals and Treatments*

Angus and Angus x Simmental heifers (n = 24) were enrolled in the study. Heifers were 1.4 $\pm$ 0.2 yr of age, with all animals exhibiting normal estrous cycles prior to the onset of the experiment. Animals were allowed to graze mixed pasture, *ad libitum*, throughout the experiment. All animals were handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. Estrus was synchronized and treatments assigned and administered as described for Exp 1.

*Embryo Implantation and Pregnancy Diagnosis*

Embryo implantation was performed 7.1 ± 0.2 d following GnRH injection. Embryos that had previously been collected from untreated cows using standard superovulation and embryo collection procedures ("wild type" embryos) were implanted

in the upper one third of the uterine horn ipsilateral to the ovary that had a CL. The embryos utilized were previously frozen using the glycerol method of freezing. Embryo quality was evaluated during the rehydration process. All embryos that were implanted were grade 1 embryos from two matings which were stratified between treatments. A single experienced embryo transfer technician implanted all of the embryos. Pregnancy diagnosis was performed at 30 d after GnRH injection using a 7.5 mHz linear array transducer (Aloka).

*Determination of Progesterone Concentrations*

Blood samples were collected on D 8, 12 and 14 following the induced ovulation to determine mid-luteal phase concentrations of circulating progesterone. Blood was collected and stored as described in Exp 1. All samples from an individual animal were included in a single assay and each assay had equal numbers of animals from both treatments. The average intra-assay CV was 3.3%, and inter-assay CV's for pooled plasma samples containing 1.5 and 7.5 ng/ml were 2.5 and 3.4% respectively. The average sensitivity of the assay was 0.2 ng/ml.

*Statistical Analyses*

The model for the statistical analysis of this experiment was the same as that described in Exp 1.

## Results

### Experiment 1

Aspiration of ovarian follicles (D 0) was performed 6.8 ± 0.1 d after the synchronized estrus. Emergence of a new wave of follicular development was detected on D 1.7 ± 0.1 and the largest follicle of this cohort attained a diameter of 8 mm on D 4.1 at which time $PGF_{2\alpha}$ was administered to induce luteal regression. The day of GnRH injection was 5.1 ± 0.1 and 6.3 ± 0.1 for the GnRH-10 and GnRH-13 treatments respectively**.** Thus, the intervals from $PGF_{2\alpha}$ to the GnRH induced preovulatory LH surge were approximated as 1.0± 0.1 d and 2.2 ± 0.1 d in the GnRH-10 and GnRH-13 treatments, respectively. Ovulation in the GnRH- 10 treatment occurred on D 7.1 ± 0.1 d and on D 8.3 ± 0.2 in the GnRH-13 treatment which was approximately 5.4 and 6.6 days after emergence, respectively. The D of ovulation and the diameter of the largest follicle (F1) that ovulated were less ($P < 0.05$) in the GnRH-10 than GnRH-13 treatment (Table 4.1). Diameter of the second ovulatory follicle (F2) in animals with double ovulations was also less ($P < 0.05$) in the GnRH-10 than GnRH-13 treatment. The incidence of double ovulations was 18% in the GnRH-10 and 12% in the GnRH-13 treatment and did not differ between treatments. In the GnRH-10 treatment 2 animals did not ovulate in response to GnRH, whereas all animals in the GnRH-13 treatment did ovulate; resulting

in 45 animals in the GnRH-10 treatment and 54 animals in the GnRH-13 treatment. No cows in either treatment were detected in estrus before, or at the time of the GnRH injection.

A treatment x day interaction was detected ($P < 0.05$) for concentrations of progesterone from D 8 to D 14 after ovulation when all animals (pregnant and not pregnant) were included in the analyses (Fig 4.1). The proportion of animals that were pregnant 30 d after AI was less ($P < 0.05$) in the GnRH-10 compared with GnRH-13 treatment by 53 percentage points (Table 4.1). All cows in the GnRH-10 treatment that were pregnant 30 d after AI were pregnant on D 90 of gestation, whereas 1 pregnancy was lost in the GnRH-13 treatment between 30 and 90 d of gestation.

**Experiment 2**

Aspiration of ovarian follicles (D 0) was performed 7.2 ± 0.1 d after the synchronized estrus. Emergence of a new wave of follicular development was detected on D 1.5 ± 0.1 and the largest follicle of this cohort attained a diameter of 8 mm on D 4.3 ± 0.1. An injection of $PGF_{2\alpha}$ was administered when the largest follicle of the ensuing wave reached 8 mm in diameter. The D of GnRH injection was 5.3 ± 0.1 and 6.4 ± 0.1 for the GnRH-10ET and GnRH-13ET treatments respectively**.** Thus, the intervals from $PGF_{2\alpha}$ to the preovulatory LH surge were approximated as 1.0± 0.1 d and 2.1 ± 0.2 d in the GnRH-10ET and GnRH-13ET treatments, respectively. Ovulation in the GnRH-10ET treatment was detected on D 6.8 ± 0.1 and occurred on D 7.8 ± 0.2 in the GnRH-

13ET treatment. The D of ovulation and the diameter of the largest follicle that ovulated were less ($P < 0.05$) in the GnRH-10ET than GnRH-13ET treatment (Table 4.2). All animals in both treatments ovulated a dominant follicle as a result of the GnRH injection resulting in 12 animals in both the GnRH-10ET and GnRH-13ET treatment. No animals in either treatment were detected in estrus before, or at the time of the GnRH injection. Concentrations of circulating progesterone were decreased ($P < 0.05$) in nonpregnant animals as compared with pregnant animals from both treatments on D 10 and D 12 after ovulation (Fig 4.2; treatment x day, P >.05). The proportion of animals that were pregnant 30 d after ET was less ($P < 0.05$) in the GnRH-10ET compared with GnRH-13ET treatment by 59 percentage points (Table 4.2).

## Discussion

The present studies were designed to compare fertility and subsequent luteal function between animals induced to ovulate immature follicles to those that were induced to ovulate larger, more mature follicles. It was demonstrated that fertility is reduced when animals are induced to ovulate a less mature dominant follicle as compared with those that ovulate a large more mature dominant follicle. Furthermore, animals that were induced to ovulate immature follicles had lesser concentrations of progesterone during the ensuing luteal phase and early stages of pregnancy. Exp 2 was included to delineate the reasons for the reduction in fertility in those animals induced to ovulate an immature

follicle. By using wild type embryos the effects of oocyte quality, oviductal function and sperm transport were removed and permitted an indirect assessment of the influence of follicle maturity on subsequent uterine function.

The differences in "follicle maturity" were achieved by inducing ovulation in one treatment when follicles were of lesser diameter than typical of ovulatory follicles versus permitting follicles to progress in size and maturity to a stage similar to that which is observed at spontaneous ovulation before induction of ovulation with GnRH. While diameter was used as a benchmark for maturity, the animal model used provided precise control of other aspects of follicular development. First, the origin of all ovulatory follicles was the largest growing follicle(s) of a new follicular wave that emerged in a synchronized endocrine environment after aspiration of follicles at a similar stage of the estrous cycle. Second, age (days from emergence) of ovulatory follicles, in addition to diameter was known. Third, the interval from $PGF_{2\alpha}$ to the GnRH-induced LH surge (referred to as "duration of proestrus"), and thus, the approximate interval from luteal regression to the LH surge was known. Thus, in addition to the 3 mm difference in follicular diameter between the GnRH-10 and GnRH-13 treatments, follicles in the GnRH-10 treatment were younger in age by approximately 1.2 days, and exposed to a shorter proestrus than in the GnRH-13 treatment. Follicle diameter at ovulation (Lamb et al., 2001; Perry et al., 2005; Mussard et al., 2007), duration of proestrus (Taponen et al., 1999, 2002, 2003; Peters and Pursley, 2003) and follicle age as defined by duration of dominance (Santos et al., 2004; Thatcher et al., 2006) have all been identified in other reports as sources of variation in conception rate in cattle. In the present study, variables

of follicular development and endocrine environment were closely controlled; however age and diameter of the ovulatory follicle, as well as, duration of proestrus were intentionally varied between treatments. The relative contributions and interactions of these factors to determine follicular maturity, and hence likelihood of pregnancy following mating or embryo transfer warrants further investigation.

Differences in follicle maturity likely result in differences in fertility by influencing one or more of a variety of physiological processes. We hypothesized at the onset of this experiment that animals that ovulated an immature follicle (GnRH-10) would have reduced fertility as compared with those that ovulated a follicle that was more mature (GnRH-13), however the dramatic decrease (53 percentage points) in fertility in the GnRH-10 treatment was an unexpected result. In an attempt to explain the sources of the large difference in fertility, Exp 2 was performed. Using embryo transfer, influences of follicuar diameter at ovulation on oocyte quality, oviductal function and sperm transport were superceded. The embryos that were implanted on D 7 of the estrous cycle were of excellent quality, as evidence by pregnancy rate in the GnRH-13 treatment. We hypothesized at the onset of this experiment that by using high quality, wild type embryos, the reduced fertility in those animals ovulating an immature follicle, relative to females that ovulated larger follicles, would be negated. We rejected this hypothesis when it was observed that fertility in those animals that received an embryo following induced ovulation of an immature follicle was reduced to a degree similar to those observed with AI in Exp 1.

The results imply that the decreased fertility that we have observed in these and previous experiments is not solely the result of compromised oocyte quality, oviductal function or sperm transport. While it is possible that these aspects have a role in the reduction in fertility when small follicles are induced to ovulate, these findings strongly suggest that uterine function and environment, and perhaps luteal function and the uterine-CL signaling mechanisms may play a greater role than previously hypothesized. In the present experiments, concentrations of progesterone were less during the mid-luteal phase in animals that were induced to ovulate immature follicles. We (Burke et al., 2001, Mussard et al., 2007) and others (Vasconcelos et al., 2001) have demonstrated that progesterone concentrations and cross-sectional area of the CL were reduced in animals that were induced to ovulate prematurely. Others have noted decreased progesterone concentrations with ovulation of smaller follicles, however, this finding has not been consistent across papers (Perry et al., 2002, Taponen et al., 2002; Peters and Pursley, 2003). A physiological minimum for the concentration of progesterone needed to support pregnancy has not been determined (Mann and Lamming, 1999). Because the signal in cattle for maternal recognition of pregnancy (MRP) is provided via interferon-tau (IFN-τ) secretion from the embryo (Anthony et al., 1988) and progesterone concentrations and IFN-τ secretion by the embryo are highly correlated (Kerbler et al., 1997), it is possible that insufficient progesterone concentrations or were responsible for the reduced conception rate in cows induced to ovulate immature follicles in the present study.

In conclusion, premature ovulation of a dominant follicle with GnRH resulted in reduced diameter of the ovulatory follicle(s), compromised fertility and decreased luteal function. Wild type embryos implanted into animals that had CL resulting from ovulation of either a small immature ovulatory follicle or a large more mature follicle did not improve pregnancy rates in those animals that ovulated a smaller ovulatory follicle prior to embryo implantation. Results from these experiments would suggest that decreased fertility in animals that ovulate a small immature follicle is due to factors other than those involved with oocyte quality and oocyte fertilization. Factors related to uterine environment, luteal function and embryo-CL signaling mechanisms are areas that could influence fertility. Further studies to isolate the mechanism(s) by which fertility is reduced following ovulation of a small immature follicle need to be conducted.

| Variable | GnRH-10 | GnRH-13 |
|---|---|---|
| Incidence of Ovulation (%) | 45/47(95.7) | 54/54 (100) |
| D of Experiment at Ovulation | 7.1 $\pm$ 0.1* | 8.3 $\pm$ 0.2 |
| DF1 Diameter at Ovulation (mm) | 11.1 $\pm$ 0.2* | 13.6 $\pm$ 0.2 |
| Pregnant 30 d after AI (%) | 2/45 (4.4)* | 31/54 (57.4) |

Table 4.1. Ovulatory characteristics, luteal progesterone and pregnancy rate of cows induced to ovulate either a small (GnRH-10) or large (GnRH-13) ovarian follicle. Time of ovulation, ovulatory follicle size and pregnancy rates were decreased in the GnRH-10 treatment. [Treatment difference denoted by * ($P < 0.05$)].

| Variable | GnRH-10ET | GnRH-13ET |
|---|---|---|
| Incidence of Ovulation (%) | 12/12 (100) | 12/12 (100) |
| D of Experiment at Ovulation | 6.8 $\pm$ 0.1* | 7.8 $\pm$ 0.2 |
| DF1 Diameter at Ovulation (mm) | 11.1 $\pm$ 0.2* | 13.7 $\pm$ 0.2 |
| Pregnant 30 d after ET (%) | 1/12 (8.3)* | 8/12 (66.7) |

Table 4.2. Ovulatory characteristics, luteal progesterone and pregnancy rate of heifers implanted with wild type embryos on D 7 of the estrous cycle. Time of ovulation, ovulatory follicle size and pregnancy rates were decreased in the GnRH-10ET treatments. [Treatment difference denoted by * ($P < 0.05$)].

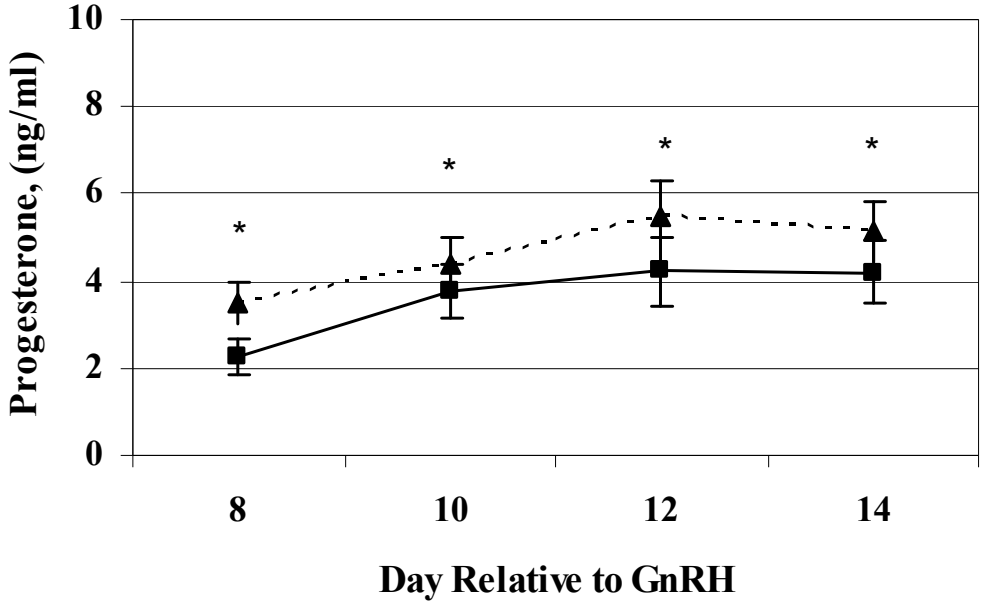


Figure 4.1. Circulating concentrations of progesterone following GnRH induced ovulation in cows manipulated to have either small (GnRH-10; ▲) or large (GnRH-13; ■) ovarian follicles at the time of ovulation. AI was performed at the time of GnRH injection. . [Treatment X time interaction, $P < 0.05$; * indicates treatment means at that time differ ($P < 0.05$)].

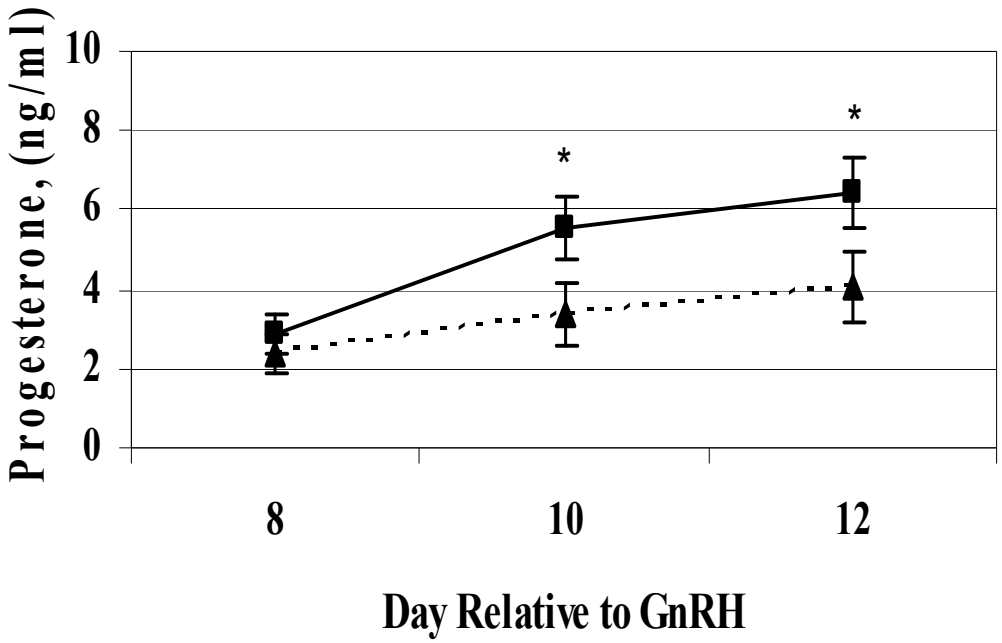


Figure 4.2. Circulating concentrations of progesterone following GnRH induced ovulation in heifers manipulated to have either small (GnRH-10ET; ▲) or large (GnRH-13ET; ■) ovarian follicles at the time of ovulation. AI was not performed and wild type embryos were implanted on Day 7 of the estrus cycle. [Treatment X time interaction, $P < 0.05$; * indicates treatment means at that time differ ($P < 0.05$)].

# CHAPTER 5

# INFLUENCE OF THE LENGTH OF PROESTRUS ON FERTILITY AND ENDOCRINE FUNCTION IN CATTLE.


## Abstract

Previous research suggests that follicle maturity and subsequent fertility is influenced by length of proestrus across a range of follicular sizes. To test this hypothesis we used an animal model in which ovulation of similar sized follicles was induced following either a long (LPE; ~ 2.25 days) or short (SPE; ~1.25 days) proestrus (interval from prostaglandinF $_{2\alpha}$ ($PGF_{2\alpha}$) administration to a GnRH induced LH surge). Specific objectives were to compare pregnancy rates and luteal phase progesterone concentrations between the LPE and SPE treatments. Ovulation of follicles that were previously synchronized using follicular aspiration was induced with GnRH (Day 0) after either 2.25 days (d) (LPE; n = 40) or 1.25 days (SPE; n = 38) of proestrus. Lactating and nonlactating cows were inseminated 12 h following GnRH administration. Ovulatory follicle diameter was similar between treatments. Pregnancy rates to AI were greater ($P < 0.01$) in the LPE (50.0%) compared to the SPE (2.6%) treatment. The proportion of cows having a short luteal phase in the subsequent estrous cycle was greater ($P < 0.01$) in the SPE than LPE treatment. In cows with normal luteal function, timed-AI pregnancy rates and progesterone concentrations in the subsequent luteal phase were greater ($P < 0.05$) in the LPE than SPE treatment. In conclusion, decreasing the length of proestrus

before induction of ovulation of a large follicle resulted in lower pregnancy rates and mid-luteal phase progesterone concentrations and an increased incidence of short luteal phases.

## Introduction

Estrous synchronization programs that permit AI at a predetermined time (timed-AI) rather than AI at a time determined by expression and detection of estrus, circumvent the challenges associated with detection of estrus and may be better suited for many beef cattle management systems. Estrous control programs that facilitate timed-AI, such as Ovsynch (Pursley et al., 1997a) and CO-Synch (Geary et al., 1998), rely on the use of gonadotropin releasing hormone (GnRH) to control follicular dynamics. Ovulation was detected after the initial GnRH injection in only 66% of beef (Geary et al., 2000) and 64% of dairy cattle (Vasconcelos et al., 1999) as the effectiveness of GnRH to induce follicle turnover is dependent, in part, upon the day of the estrous cycle when GnRH treatment is administered (Vasconcelos et al., 1999; Moreira et al., 2000; Cartmill et al., 2001). Therefore, with these approaches to timed-AI, follicles of varying size, maturity, and steroidogenic capacity will be induced to ovulate at the end of the synchronization program.

Fertility following ovulation of aged follicles is reduced (Stock and Fortune, 1993; Mihm et al., 1994) and a majority of estrous control programs are structured to minimize the development and ovulation of persistent dominant follicles. However, the

implications for fertility of GnRH-induced ovulation of immature dominant follicles on fertility are less clear. Decreased conception rates have been observed in beef cattle induced to ovulate follicles of lesser diameter within a CO-Synch synchronization program (Lamb et al., 2001; Perry et al., 2005) and others (Peters and Pursley, 2003) have demonstrated that decreasing the interval from $PGF_{2\alpha}$ to GnRH-induced ovulation (referred to subsequently as "proestrus") in an Ovsynch program resulted in a decreased conception rate. Using an animal model that standardized the functional status and age of the follicles induced to ovulate and the endocrinology during the interval from follicular emergence to the onset of proestrus, we have demonstrated that animals induced to ovulate smaller follicles with GnRH have decreased fertility as compared to cows which either spontaneously ovulate or are induced to ovulate large follicles (Mussard et al., 2003a, 2003b, 2007). Ovarian follicles of lesser diameter that were induced to ovulate in these reports were also younger (shorter interval from emergence to ovulation) and exposed to a shorter proestrus. Consideration of data across these experiments suggested that length of proestrus was a more important determinant of subsequent fertility than follicular diameter or age. From these findings, we hypothesized that a shorter proestrus would lead to lower fertility and luteal phase progesterone concentrations in cattle induced to ovulate follicles of similar diameter and age. The specific objectives were to compare fertility and hormone concentrations between cattle induced to ovulate similar-sized ovarian follicles following two different intervals of proestrus.

## Material and Methods

*Animals and treatments*

Lactating (n=38) and non-lactating (n=44), multiparous Angus and Angus x Simmental cows were used and handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. Estrus was initially synchronized using an intravaginal progesterone insert (CIDR®; Pfizer Animal Health, New York, NY, USA) for 7 d and administration of $PGF_{2\alpha}$ (Lutalyse®, 25 mg dinoprost tromethamine per injection, Pfizer Animal Health, New York, NY, USA) on the day of CIDR withdrawal. Twenty four hours after CIDR withdrawal and $PGF_{2\alpha}$ injection, cows were administered estradiol benzoate (i.m.; EB; 1 mg EB /500 kg BW; β-estradiol 3-benzoate, Sigma, St. Louis, MO, USA; 1 mg EB/ml peanut oil). Estrus detection was performed twice-daily for 2 d after $PGF_{2\alpha}$. All ovarian follicles $\geq$ 5 mm in diameter were aspirated between 5.75 and 7.75 d following the synchronized estrus (D -6.75 of the experiment) with a 17 G needle by the ultrasonography-guided transvaginal approach (Bergfelt et al., 1994; Aloka 500V, 5 MHz convex array transducer, Corometrics Inc., Wallingford, CT, USA). At follicular aspiration, one lactating cow and three non-lactating cow were removed from the experiment due to absence of either a dominant follicle or CL in their ovaries at this time. Within lactation status, cows were stratified by breed and age and then randomly assigned to receive $PGF_{2\alpha}$ (25 mg) on either D -2.25 (19 lactating and 21 non- lactating cows; long proestrus treatment; LPE) or D -1.25 (18 lactating and 20 non- lactating cows; short proestrus treatment; SPE). All cows received GnRH (100 μg, Cystorelin®, Merial, Inselin, NJ, USA) on D 0. Thus, the length of

proestrus was 2.25 d in the LPE and 1.25 d in the SPE treatment. Artificial insemination was performed 12 h after GnRH in all animals by a single technician with semen from one of three sires. Sires were stratified equally across treatment and lactation status and age.

*Ultrasonography and blood sampling*

Location and diameter of all ovarian follicles ≥ 5 mm in diameter were monitored daily from D -3 until GnRH administration (Day 0) by transrectal ultrasonography using a 7.5 mHz linear array transducer (Aloka 500V). On D 2, ultrasonography was performed to verify ovulation of the dominant follicle. Blood samples were collected on D 8, 10, and 12 to determine concentrations of progesterone during the estrous cycle that ensued after GnRH. Blood samples were centrifuged at 1500 x *g* for 15 min within 1 hour after collection and plasma was decanted and stored at -20º C until quantified for concentrations of progesterone. Pregnancy diagnosis was performed on D 30 by transrectal ultrasonography using a 5.0 mHz linear array transducer (Aloka 500V).

*Hormone quantification*

Plasma concentrations of progesterone were determined using a commercially available RIA kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as described previously for our laboratory (Burke et al., 2003). Average intra-

assay coefficient of variation (CV) was 2.8%, and inter-assay CV's (2 assays) for pooled plasma samples containing 1.5 and 7.5 ng/ml were 3.5 and 2.8% respectively. The average sensitivity of the assays was 0.2 ng/ml.

*Data and statistical analysis*

Ovulation was characterized by the disappearance of the dominant ovarian follicle on Day 2. An animal was considered to have a "short luteal phase" if circulatory progesterone concentrations were < 1.0 ng/mL on Day 8, 10, or 12 of the experiment. If progesterone concentrations were >1.0 ng/mL on Day 8, 10, and 12 animals were considered to have a "normal luteal phase". Treatment, lactation status, and the treatment by lactation status interaction were initially included in the model but lactation status, and the lactation by treatment interaction were removed since the interaction was not statistically significant, resulting in the final statistical model including just treatment. The effect of treatment on proportion of animals ovulating one or two ovarian follicles, incidence of short luteal phases, and pregnancy rate to timed-AI were analyzed using the GEN MOD procedures of SAS. The effect of treatment on the diameter of the ovulatory follicle was compared using MIXED procedures of SAS. A second series of analyses were performed to compare pregnancy rate and circulating concentration of progesterone between treatments in only those cows that experienced a normal luteal phase. Only one nonlactating cow in the SPE treatment had a normal luteal phase, therefore lactation status was not included in the model. The effects of treatment, day, and the treatment by day interaction for progesterone concentrations on Day 8, 10, and 12 in cows with normal

luteal function were analyzed using the MIXED procedures of SAS with repeated measure analysis included in the model. Akaike's Information Criteria (AIC) was used to determine the best variance-covariance structure for the models. Data are expressed as the mean ± SEM.

## Results

All cows in the LPE (n = 40) and SPE (n = 38) treatments ovulated within 2 days after GnRH and diameter of the largest ovulatory follicle at the time of GnRH administration did not differ between treatments (13.0 ± 0.2 and 12.6 ± 0.2 mm, respectively). The proportion of cows induced to ovulate 2 follicles following GnRH administration tended ($P = 0.07$) to be greater in the LPE (9/40) than the SPE (3/38) treatment. Pregnancy rate to timed-AI was greater ($P < 0.01$) in the LPE (50.0%) than SPE (2.6%) treatment and more cows ($P < 0.01$) in the LPE treatment had a normal luteal phase (26/40) than in the SPE treatment (7/38). In a second analysis that included only cows that were classified as having normal luteal phases, pregnancy rate to timed-AI was greater ($P < 0.05$) in the LPE (73%; 19/26) than SPE (14.3%; 1/7) treatment and progesterone concentrations were greater ($P < 0.05$) in the LPE than SPE treatment during the mid-luteal phase of the subsequent estrous cycle.

## Discussion

In the present study, cows that had a shorter proestrus were less likely to become pregnant than those that experienced a longer proestrus when follicular diameter was

similar between treatments. These findings emphasize that follicular characteristics beyond diameter are critical in determining follicle maturity, and the likelihood that normal luteal function and pregnancy will result. Manipulation of the length of proestrus altered luteal progesterone concentrations and given the multiple roles of this steroid in processes leading to pregnancy, it is plausible that these changes contribute to infertility that occurs with a shortened proestrus.

Previous studies from our laboratory (Mussard et al., 2003a, 2003b, 2007) have also focused on the hypothesis that fertility following ovulation of immature follicles is reduced. In earlier experiments, follicle maturity was largely defined as the diameter of the preovulatory follicle just before ovulation. Accordingly, we observed that GnRH-induced ovulation of follicles of a smaller diameter than normal resulted in lower pregnancy rates than in cows that spontaneously ovulated (Mussard et al., 2007) or that were induced with GnRH to ovulate follicles that were of similar diameter to those present at spontaneous ovulation (Mussard et al., 2003a; 2003b). Furthermore, this reduction in pregnancy rate occurred whether AI (Mussard et al., 2003a, 2007) or embryo transfer (Mussard et al., 2003b) were used to impregnate the cows. Follicular development and endocrine environment were closely controlled with the animal model used in our earlier experiments (similar to the present experiments), but age of ovulatory follicles and length of proestrus were intentionally varied between treatments to obtain the desired differences in follicle diameter. Further, in order to achieve the objectives of individual experiments, modification of either the time of $PGF_{2\alpha}$ treatment relative to follicle emergence or the interval from $PGF_{2\alpha}$ to GnRH resulted in varying age of

ovulatory follicles and/or length of proestrus between experiments. The relationship of conception rate to follicle diameter, length of proestrus, and follicle age across six treatments in three experiments (Mussard et al., 2003a, 2003b, 2007) are presented in order of increasing conception rate in Table 1. Consideration of these data suggests that ovulatory follicle diameter and age do not appear to be accurate predictors of subsequent fertility; within the confines of this animal model. Secondly, it appears that length of proestrus and conception rate are positively associated. While these observations are speculative since they are based upon comparisons across experiments, they served as the basis for the hypothesis tested in the present study. Results of the present experiment further strengthen this view, emphasizing that the length of proestrus influences fertility independent of follicle size or age. Additionally, modification of the length of proestrus had major impacts on concentrations of circulating progesterone and luteal function in the ensuing estrous cycle.

It was also hypothesized that progesterone concentrations in the subsequent estrous cycle would be greater in animals with a longer proestrus before induction of ovulation. In ewes, shortening proestrus before induced ovulation reduced the numbers of granulosa cells in the follicle and resulted in a CL that were smaller, contained fewer large luteal cells, and produced lesser amounts of progesterone both *in vivo* and *in vitro* (Murdoch and Van Kirk, 1998). Length of proestrus had variable effects on progesterone concentrations during the subsequent estrous cycle in the present experiments. A surprising finding was that, a large proportion of cows experienced short luteal phases, which will be discussed later. For cows that had normal luteal phases, mid-luteal phase

progesterone concentrations were lower in cows with a short proestrus. Differences in progesterone concentrations may have contributed to the very low fertility, since several reports (Shelton et al., 1990; Kerbler et al., 1997; Mann and Lamming, 2001; Hommeida et al., 2004) indicate that pregnancy rates in cows were lower in cows that experienced a delayed rise in progesterone concentrations during the early luteal phase and a strong association between circulating progesterone concentrations, conceptus growth, and conceptus secretion of IFN-τ has been reported (Kerbler et al., 1997; Mann et al., 2006). The relationship of length of proestrus with subsequent progesterone production by the CL requires further investigation.

The high incidence of short luteal phases following induced ovulation in the SPE treatment was unexpected even though others (Taponen et al., 1999, 2002, 2003; Peters and Pursley, 2003) have observed short luteal phases in cows with a shortened proestrus when using differing animal models. Of the studies summarized in Table 5.2 using the same general approach taken in the present experiments, the present experiments were the first in which short luteal phases were observed. Reduced preovulatory 17β estradiol concentrations in cows with a short proestrus are potentially the cause of the increased incidence of short luteal phases observed. Mann and Lamming (2000) demonstrated that administering low concentrations of 17β estradiol were not sufficient to cause down regulation of the oxytocin receptor within the uterus and allowed for increased secretion of $PGF_{2\alpha}$ following an oxytocin challenge compared to cows receiving elevated concentration of 17β estradiol. Additionally, Kieborz-Loos et al., (2003) observed that

elevated 17β estradiol concentrations along with progesterone priming were required to prevent premature secretion of $PGF_{2\alpha}$ from the uterus. Inevitably, pregnancy failure occured in cows that experienced a short luteal phase.

In summary, cows with a shorter proestrus prior to the induced ovulation of a large follicle had lower pregnancy rates to timed-AI and concentrations of 17β estradiol during the preovulatory period than those with a longer proestrus. Additionally, inducing the ovulation of a large follicle following a shortened period of proestrus resulted in decreased luteal progesterone and an increased incidence of short luteal phases. Follicle maturity is not accurately predicted by any single characteristic, but more likely the cumulative effect of many factors such as length of, and 17β estradiol production during proestrus, diameter and age of the follicle, and progesterone production by the resultant CL. The investigation of causative factors of infertility associated with follicular maturity in cattle requires consideration of a multitude of factors.

| Variable | LPE | SPE |
|---|---|---|
| Incidence of Ovulation (%) | 40/40 (100) | 38/38 (100) |
| D of Experiment at Ovulation | 2.0 ± 0 | 2.0 ± 0 |
| DF1 Diameter at Ovulation (mm) | 12.9 ± 0.2 | 12.6 ± 0.2 |
| Interval from PGF to GnRH | 2.25 ± 0* | 1.25 ± 0 |
| Animals with normal luteal function | 28/40 (70%)* | 10/38 (26%) |
| Pregnant, with normal luteal function (%) | 71%* | 10% |
| Pregnant to AI (%) overall | 50%* | 3% |

Table 5.1. Ovulatory characteristics, luteal progesterone, prevalence of short luteal phases and pregnancy rate of cows induced to ovulate a similar sized follicle following either a long period of proestrus (LPE) or a short period of proestrus (SPE). Time and occurrence of ovulation and ovulatory follicle size were similar ($P > 0.1$); Interval from GnRH to PGF (Proestrus), circulating concentrations of progesterone and pregnancy rates were decreased in the SPE treatment. In addition the occurrence of short luteal phases were greater in the SPE treatment than occurred in the LPE treatment. [Treatment difference denoted by * ($P < 0.05$)].

| Conception Rate (%)[a] | Follicle diameter at Ovulation (mm)[b] | Age of follicle (days)[c] | Duration of Proestrus (days)[d] | n | Experiment |
|---|---|---|---|---|---|
| 4 | 11.1 ± 0.2 | 5.4 | 1.0 ± 0.1 | 45 | Mussard et al., 2003a[f] |
| 8 | 11.1 ± 0.2 | 5.1 | 1.0 ± 0.1 | 12 | Mussard et al., 2003b[g] |
| 57 | 13.6 ± 0.2 | 6.6 | 2.2 ± 0.1 | 54 | Mussard et al., 2003a[f] |
| 67 | 13.7 ± 0.2 | 6.1 | 2.0 ± 0.1 | 12 | Mussard et al., 2003b[g] |
| 76 | 10.7 ± 0.1 | 5.1 | 3.3 ± 0.1 | 29 | Mussard et al., 2007[e] |
| 100 | 12.0 ± 0.3 | 6.0 | 4.7 ± 0.2 | 24 | Mussard et al., 2007[e] |

Table 5.2. Conception rate, diameter and age of the ovulatory follicle, duration of proestrus, and number of cows included from a series of experiments investigating the effect of follicle maturity on fertility.
[a] Percentage of animals determined to be pregnant following insemination. Pregnancy determination was conducted via ultrasonography at approximately 30 days post-insemination.
[b] Diameter of the largest ovulatory follicle as determined by ultrasonography conducted either at GnRH administration or estrus.
[c] Approximate age of the ovulatory follicle.
[d] Interval from $PGF_{2\alpha}$ until GnRH administration.
[e] Cows were either induced with GnRH to ovulate a small (~11 mm) follicle or allowed to spontaneously exhibit estrus. Cows were inseminated 12 hours following estrus or GnRH.
[f] Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Animals were inseminated 12 h following GnRH administration.
[g] Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Embryos from non-treated cows were then transferred 7 days after GnRH.

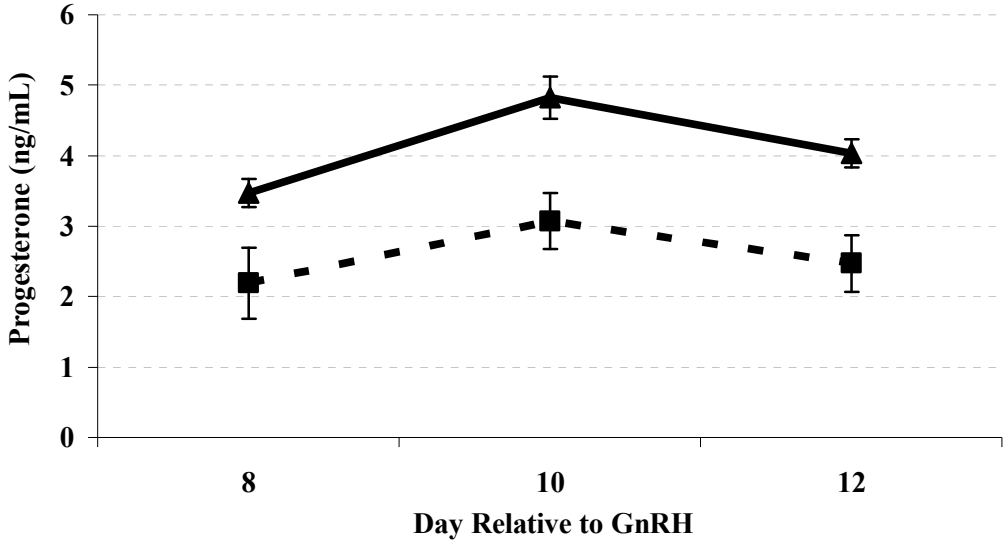


Figure 5.1. Progesterone concentrations of beef cows with normal luteal function that were induced to ovulate a similar sized follicle following either 2.25 days (LongPE; ▲) or 1.25 days (ShortPE; ■) of proestrus. Progesterone concentrations were affected by treatment (treatment; $P < 0.05$).

# CHAPTER 6

## GENERAL DISCUSSION

The experiments reported in this dissertation were performed to answer a relatively straightforward question. Does size of the follicle from which ovulation occurs impact fertility? The first experiment was performed in such a manner to determine if a difference exists in fertility when ovulation occurs from follicles that are either small or large at the time of ovulation. The animal model utilized provided animals that could have ovulations from a large follicle with a spontaneous estrus and, therefore a spontaneous LH surge. This was compared with animals with ovulations from a small follicle (approximately 10 mm at the time of the LH surge) induced with GnRH. The size of 10 mm was chosen because a dominant follicle of this size should respond to an LH surge, but is much smaller than what normally occurs in a spontaneous estrus and ovulation. Ovulation from small follicles are often induced in animals with timed AI treatment regimens and the overall success of the breeding program is dependent on fertility of this class of animals. Many of the timed AI programs currently utilized are satisfactory for synchronizing stage of estrous cycles, but do a poor job of synchronizing stage of follicular wave development. As a result, at timed ovulation and insemination there are follicles from ovulation occurs with a wide range of sizes as well as animals that are in standing estrus and have already had a spontaneous LH surge. The first experiment was designed to mimic the largest difference in size of follicles from which ovulation occurs that may occur in a timed AI breeding scenario. If a difference exists in fertility

resulting when ovulation occurs from follicles of varying sizes, this animal model should be effective in elucidation underlying causes. Even with a relatively small group of animals a difference in fertility of 24 percentage points occurred among animals that had ovulations from a large follicle following a spontaneous estrus and ovulation as compared with animals in which ovulation was induced with GnRH from a follicle. An interesting and unexpected observation in this experiment was the large number of double ovulations. While this provided a new set of questions to address for other students, (Lucas Helser, Kate Colliflower, Chad Bailey, and Emma Jinks) in the laboratory where the present research was conduced it was something that needed to be regulated and controlled in future experiments. The success of this experiment was important in that incidence of double ovulations was not different between treatments. It was also interesting in this experiment that conception rates were extremely high. A conception rate of 100 % with the SPON treatment was unexpected. This treatment was incorporated in the study because it provided an optimum estrous synchrony regimen for a production setting and while it is not unusual to have exceptional conception rates (60 - 75 %), it is rare that all of the animals become pregnant following one service of AI. The conception rate of 76% with the GnRH-10 treatment was also very good. The unusual conception rate achieved in this experiment was attributed in part to the large number of double ovulations. While only half of the animals ovulating from a dominant follicles were observed with two fetuses, the extra oocyte and CL could certainly have had a beneficial impact on conception rates. It is difficult to take advantage of this scenario in an AI or natural breeding situation, however it may be beneficial to have two

CL's present for embryo transfer. By utilizing embryo transfer rather than AI, there is little chance of twin fetuses; however there could be advantages of having double ovulations in improving conception rates. We attributed greater than typical incidence of double ovulations to the timing of luteal regression with follicular wave emergence and future experiments were designed to try to suppress the incidence of double ovulations.

While results from this experiment were encouraging, results were somewhat confounded and needed to be addressed before reaching a conclusion. The most obvious confounding effect was the difference in spontaneous estrus and ovulation compared with an induced LH surge and ovulation. The second experiment was designed to compare fertility of animals induced to have ovulations from either small or large follicles. The model was designed to limit the opportunity that animals may have to exhibit spontaneous estrus to isolate effects of follicle size on fertility. In addition, to limit incidence of double ovulation the time of CL regression was delayed until after ovarian follicular wave emergence. As a consequence of this action, duration of proestrus was shortened. This was necessary, otherwise the percentage of animals exhibiting spontaneous estrus would increase. It was hypothesized that a difference would exist in fertility resulting after ovulations from ovarian follicles of different sizes, however we predicted this difference to be smaller than previously observed in the first experiment, therefore more animals were included in this experiment. This was an unnecessary action, however, because the difference in fertility in this experiment was even greater

than in the first experiment. A difference in fertility of 53 percentage points was observed and provided clear evidence that size of follicle from which ovulation occurs does indeed impact fertility.

With this evidence, we set forth to identify what mechanisms may affect fertility in animals having ovulations from a small follicle. The third experiment was conducted to start this process. Embryos from untreated cows were implanted into animals that had been treated with the same treatment regimen as animals utilized in Exp 2. By utilizing embryos from untreated animals we were able to isolate some of the mechanisms that impact fertility. The influence of oocyte quality, oviductal function and sperm transport were assessed. If fertility rates were similar between treatments, than it would be reasonable to hypothesize the reduction in fertility in previous experiments was due to factors which were assessed using the present animal model. The hypothesis in this experiment was rejected, as fertility rates were different to the extent observed when AI was performed in the second experiment. Results from the embryo transfer experiment suggested that oocyte quality, oviductal function and sperm transport were not the primary factors suppressing fertility with use of this animal model.

Given the results of the third experiment it was important to eliminate the one remaining confounding effect that persisted throughout the previous experiments. In addition to comparing follicle size at time of ovulation, the animal model utilized also was used to compare duration of proestrus. Because we were not able to conclusively determine which effect was impacting fertility to the greatest extent, these two variables needed to be separated. To accomplish this, the animal model was adjusted such that

both treatments would ovulate follicles of a similar size, however the duration of proestrus would be different between treatments. The animal model was effective with this assessment as the difference in ovulatory follicle size was only 0.3 mm while length of proestrus differed by 1 full day. The hypothesis was not rejected in this experiment as fertility rates were suppressed by 47 percentage points with the treatment which resulted in a shorter period of proestrus. An interesting part of this experiment was the greater than expected incidence of short cycles, particularly in the animals with a short proestrus. While other research has described similar findings, we were surprised by this due to the low incidence of short estrous cycles in the three previous experiments. While we have hypothesized several reasons for this finding, the fact is we still do not know why this experiment resulted in a greater incidence of short estrous cycles. When fertility rates of cows that had normal luteal phases were compared the difference in fertility was 61 percentage points favoring the long proestrus treatment.

To answer the original question; Does size of the ovulatory follicleimpact fertility? The answer is yes, sort of. As depicted in table 6.1 it appears that ovulatory follicle size is of less importance than is duration of proestrus. The longer the duration of proestrus, the more likely it is that an animal will become pregnant. As duration of proestrus increased from 1.0 to 4.7 days, fertility increased by 96 percentage points. A similar trend is not identifiable when looking at follicle size. Other studies have suggested that follicle size is a predictor of fertility, while this may be true, it is more likely that as follicle size increases so does the duration of proestrus. In other studies, where follicle size and duration of proestrus are not as closely regulated as in the current

experiments, it is likely these two effects are confounded. The majority of related studies looking at the influence of follicle size on fertility are retrospective in nature and because follicle size is easier to measure than duration or proestrus, leads many researchers to believe that ovulatory follicle size impacts fertility, when actually duration of proestrus is the primary determinant of fertility. The mechanisms through which duration of proestrus are influencing fertility have become the primary interest of other graduate students in this laboratory (Allen Bridges, Lucas Souto and Leandro Cruppe). While further studies have revealed 17β estradiol is greater in animals that have a longer duration of proestrus, the pathways and mechanisms by which greater concentrations of circulating estradiol are impacting fertility remain unclear and are the subject of ongoing experiments.

| Conception Rate (%) | Follicle diameter at Ovulation (mm) | Age of follicle (days) | Duration of Proestrus (days) | n | Experiment |
|---|---|---|---|---|---|
| 4 | 11.1 ± 0.2 | 5.4 | 1.0 ± 0.1 | 45 | Mussard et al., 2003a |
| 8 | 11.1 ± 0.2 | 5.1 | 1.0 ± 0.1 | 12 | Mussard et al., 2003b |
| 10 | 12.6 ± 0.2 | 8.0 | 1.25 ± 0.0 | 38 | Mussard Dissertation Chap 5 |
| 57 | 13.6 ± 0.2 | 6.6 | 2.2 ± 0.1 | 54 | Mussard et al., 2003a |
| 67 | 13.7 ± 0.2 | 6.1 | 2.0 ± 0.1 | 12 | Mussard et al., 2003b |
| 71 | 12.9 ± 0.2 | 8.0 | 2.25 ± 0.0 | 40 | Mussard Dissertation Chap 5 |
| 76 | 10.7 ± 0.1 | 5.1 | 3.3 ± 0.1 | 29 | Mussard et al., 2007 |
| 100 | 12.0 ± 0.3 | 6.0 | 4.7 ± 0.2 | 24 | Mussard et al., 2007 |

Table 6.1. A summary of conception rates observed in the experiments contained in this dissertation. Fertility rates in relationship to ovulatory follicle size and age and duration of proestrus. As fertility rates improve, duration of proestrus is extended. There is no detectable trend for fertility and ovulatory follicle size**.**

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