

EFFECT OF OVULATORY FOLLICLE SIZE ON LUTEAL FUNCTION, PREGNANCY  
RATE, AND LATE EMBRYONIC/FETAL MORTALITY IN BEEF CATTLE.

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A Dissertation presented to the Faculty of the Graduate School  
University of Missouri-Columbia

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In Partial Fulfillment  
of the Requirements for the Degree  
Doctor of Philosophy

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by

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DECEMBER 2009

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

EFFECT OF OVULATORY FOLLICLE SIZE ON LUTEAL FUNCTION,  
PREGNANCY RATE, AND LATE EMBRYONIC/FETAL MORTALITY IN BEEF  
CATTLE.

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## DEDICATION

I owe so much to my husband and family for their support and unconditional love. Thank you Brandon for all you have done. I could not ask for someone more understanding, entertaining, and good natured- when you aren't being feisty that is. I love you so much! My immediate family, Dad (Jim), Mom (Loah), Jason and wife Petra, Angie and husband Derrick, John, Tom, niece Iris, and nephews Ian, Jasper, Calvin, and Jude are so much a part of who I am. Thank you for all the life lessons, fun times, and constant encouragement. I am blessed with fabulous in-laws, Kay, Gary, Penny, Justin, Sarah, and Caleb. You have truly made me feel like part of the family and I can't thank you enough for all the support. I am fortunate to be a descendant of James and Lucille Clement and Joe and Hazel Manning. I am so thankful for the years we have had together. Not only were you wonderful parents that raised such close-knit families but you have been wonderful grandparents. You have taught us with your infinite wisdom on life and love and spoiled us with your plethora of stories, jokes, and delicious comfort foods- Thank you.

Thanks to all my family and friends! You have made my life rich with laughter, love, and joy! I am truly blessed.

## ACKNOWLEDGEMENTS

I owe a big thank you to Dr. Michael Smith, my major professor. I have LOVED graduate school. I believe a large part of my enthusiasm for graduate education is thanks to Dr. Smith and the environment that he establishes with his graduate students, the laboratory, and the department. I have learned so much from him and will always feel fortunate to have worked with Dr. Smith during my graduate education. I would also like to thank Dr. Thomas Geary who served as my graduate advisor while I carried out research at the USDA/ARS experiment station in Miles City, MT. Thanks to Dr. Geary, I was able to conduct exciting research and also be involved in so many other projects- I truly learned so much during my time in Miles City. I would like to thank my dissertation committee members, Dr. David Patterson, Dr. Matthew Lucy, and Dr. Bryan Garton, who were wonderful to work with and learn from during the past years.

There were many people who helped with my research in Miles City including Sue Bellows, Whitney Lott, Mike Woods, Doug Armstrong, Rick Harris, Alan Doug Mason, Dave Phelps, Lance Gierke, Tyler Hamilton, Kathy Neary, Kenny Wells, Emma Jinks, Fernanda Abreu, Megan Minten, Sarah Hanson, Crystal Roberts, Libby Erickson, Page Beardsley, and the supporting staff at the USDA ARS Fort Keogh. Thank you so much- we could not have done this without your hard work!

I would like to thank Dr. Cliff Murphy and Lee Spate who were instrumental in teaching me about embryo transfer and handling embryos. Also, Dr. Cliff Lamb hosted us at his laboratory and shared his knowledge about embryo transfer techniques and embryo grading. I received a lot of guidance on the statistical analysis and would have been lost without the help of Drs. William Lamberson, Michael MacNeil, and Mark Ellersieck. Dr. Duane Keisler was so helpful in teaching me about radioimmunoassays, science, and philosophy. I will forever look at science and research in a different light thanks to Dr. Keisler. I would like to thank my fellow lab mates for keeping me sane during this process, Mallory Risley (could not have written my dissertation without your support), Emma Jinks (will miss our chute-side chats), and Ky Pohler (Pohhler). You have made graduate school and research a lot of fun!

There are many graduate students (past and present) that have helped me along this journey. Without these friends, graduate school would have been a very lonely experience- Thank you all for the fun times! Lastly I would like to thank all the people in the Reproductive Biology Group and the Division of Animal Sciences. This has been a great place to learn and wonderful people to work alongside. Thank you all for the past six years!

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## LIST OF ABBREVIATIONS

3 $\beta$ -HSD	3 $\beta$ -hydroxysteroid
A4	Androstendione
AI	Artificial insemination
ACL	Accessory corpus luteum
AMH	Anti-mullerian hormone
ARKO	Aromatase knock out
BMP	Bone morphogenetic protein
bFGF	Basic fibroblast growth factor
C	Celsius
CIDR	Controlled Internal Drug Release
CL	Corpus luteum/Corpora lutea
d	Day/Days
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
E2	Estradiol-17 $\beta$
ECM	Extra cellular matrix
EGF	Epidermal growth factor
ER	Estrogen receptor
ERKO	Estrogen receptor knock out

ET	Embryo Transfer
F	Fahrenheit
FIG $\alpha$	Factor in the germline $\alpha$
FSH	Follicle stimulating hormone
FSHr	Follicle stimulating hormone receptor
GC	Granulosa cells
GDF-9	Growth differentiation factor 9
GnRH	Gonadotropin releasing hormone
h	Hour
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IL	Interleukin
i.m.	Intramuscular
INF	Interferon
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
mg	milligram
MGA	Melengestrol acetate
MHz	Megahertz
mRNA	Messenger ribonucleic acid
min	Minute(s)

mL	Milliliter
mm	Millimeter
MMP	Matrix metalloproteinase
MZT	Maternal to zygotic transition
NAHMS	National animal health monitoring system
ng	Nanograms
PAPP-A	Pregnancy associated plasma protein-A
PGF <sub>2α</sub>	Prostaglandin F <sub>2α</sub>
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
pg	Picogram(s)
PGC(s)	Primordial germ cell(s)
P4	Progesterone
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SCNT	Somatic cell nuclear transfer
SEM	Standard error of the mean
SF-1	Steroidogenic factor-1
StAR	Steroidogenic acute regulatory protein
TAI	Fixed time artificial insemination
TDF	Thecal cell differentiation factor
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor metalloproteinases
trt	Treatment

VEGF	Vascular endothelial growth factor
μg	Microgram
UFO	Unfertilized oocyte
U.S.	United States
SAS	Statistical analysis system
SCNT	Somatic cell nuclear transfer

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**ABSTRACT**

Ovulation of small dominant follicles resulted in reduced pregnancy rates compared to cows that ovulated large follicles. Reasons for the presence of a small dominant follicle at the time of GnRH-induced ovulation and mechanisms by which follicle size affect pregnancy outcome have been a focus of our research. Two experiments were conducted to study the occurrence of small follicles at time of ovulation induction in estrous cycling ( $n = 60$ ) and anestrous ( $n = 55$ ) suckled beef cows. All cows were treated with the CO-Synch protocol (GnRH1 on d -9, PGF<sub>2 $\alpha$</sub>  on d -2, and GnRH2 on d 0). Follicle growth rate leading up to GnRH2 was similar between cows that ovulated a large or small follicle in both estrous cycling and anestrous cows ( $P = 0.75$  and  $0.99$ , respectively). Cyclic cows on d 5, 9, 13, or 18 of the estrous cycle that ovulated in response to GnRH1 had larger follicles at GnRH2 compared to cows that did not ovulate to GnRH1 (11.4 and 9.5 mm, respectively;  $P = 0.04$ ). Similarly, anestrous cows that ovulated following GnRH1 had

larger follicles at GnRH2 compared to cows that did not ovulate after GnRH1 (12.3 vs. 11.0 mm, respectively;  $P = 0.04$ ). In the third experiment, a reciprocal embryo transfer approach was used to differentiate between oocyte competence and uterine environment factors that affect establishment of pregnancy following induced ovulation of a large or small follicle. Embryos from cows that ovulated a small follicle ( $< 12.5$  mm) were transferred into cows that ovulated a large follicle ( $\geq 12.5$  mm) and vice versa resulting in the following treatment groups; small to small (S-S; negative control;  $n = 71$ ), small to large (S-L; primary effects of oocyte quality;  $n = 111$ ), large to small (L-S; primary effects of uterine environment;  $n = 122$ ) and large to large (L-L; positive control;  $n = 50$ ). The probability of recovery of a fertilized and live embryo seven days after breeding increased as the diameter of the ovulatory follicle (in the donor with single embryo recovery) increased ( $P = 0.01$ ). As ovulatory follicle diameter and serum concentrations of progesterone at embryo transfer increased in the recipient cow, the likelihood of pregnancy success also increased ( $P = 0.05$  and  $<0.001$ , respectively). Neither ovulatory diameter of the donor cows nor the serum concentrations of progesterone at ET were predictive of pregnancy after embryo transfer ( $P = 0.6$  and  $0.3$ , respectively). In summary, ovulation to GnRH1 increased follicle diameter at GnRH2. Ovulatory diameter at GnRH2 was positively associated with recovery of a live embryo (possibly indicating improved oocyte competence and (or) an early uterine environment that was more conducive to embryonic/fetal development in cows that ovulated a large follicle). Pregnancy establishment from seven to 72 days after breeding appears to relate to the uterine environment established by the ovulatory follicle independent of oocyte quality.

# CHAPTER I

## INTRODUCTION

Artificial insemination (AI) is a valuable technology to improve genetics and production in the beef cattle industry. According to Seidel (1995), AI and estrus synchronization are the most important farm ready technologies currently available to beef producers. At a recent conference, one producer shared his summary of net profit yield from AI or non-AI sired calves born to AI or non-AI sired dams (Sutphin, 2005). Sutphin's ranch realized a profit of almost \$40 more per AI-sired calf vs. non-AI sired calf, supporting the idea that the benefits of improved genetics and reproductive management with AI outweigh the costs associated with this technology. Additionally, Sutphin saw an additional benefit in improved performance (weaning weights and carcass data) of calves born to AI sired replacement dams compared to calves born from natural service sired dams. According to Harlan Hughes, an agricultural economist, cow-calf producers can increase profits by managing females calving later in the season forward to calve earlier. Use of estrus synchronization also improves reproductive management by increasing the number of cows and heifers in estrus early in the breeding season and thus increasing the proportion of heifers and cows calving earlier in the calving season. This

results in a higher cumulative percentage of calves born early, resulting in older and heavier calves at weaning and also provides postpartum cows a longer period to recover before the following breeding season. For example, cumulative calf crop was 64% by d 15, 70% by d 21, and 91% by d 42 of the calving season in herds after implementation of an estrus synchronization program (Schafer, 2005).

Understanding the bovine estrous cycle and development of estrus synchronization products that can control luteal lifespan and follicular waves has led to the development of accurate protocols that synchronize regression of luteal tissue and ovulation of a synchronized dominant follicle. Synchronization of the timing of ovulation has led to the ability to breed cows by appointment at a fixed time (TAI). Breeding cows with TAI reduces the time and labor associated with estrous detection which is the leading reason producers give for not using this technology (NAHMS, 2008). Yet, still fewer than 10% of the nation's beef cattle operations use AI in their herd (NAHMS, 2008).

Although protocols that precisely control the time of ovulation in beef cows are currently available, variation in pregnancy rates following TAI have been reported. The physiological maturity of the ovulatory follicle may be an important factor affecting the establishment and maintenance of pregnancy (Perry et al., 2005). Increasing evidence indicates that there is large variation in the diameter of the ovulatory follicle at the time of GnRH-induced ovulation and TAI. Furthermore, cows that were induced to ovulate a small dominant follicle had reduced pregnancy rates following TAI compared to cows that ovulated large follicles. Improvements in the control of TAI protocols and pregnancy rates following TAI are important goals to increase the application of estrus

synchronization and AI in the beef industry. Therefore, the objectives of this dissertation were 1) to understand why small dominant follicles are present at the time of GnRH-induced ovulation in cycling (chapter III) and anestrous (chapter IV) postpartum beef cows and 2) to understand how the size of the follicle may affect pregnancy by separating the contributions of the oocyte quality from the uterine environment using a reciprocal embryo transfer approach (chapter V).

## **CHAPTER II**

### **LITERATURE REVIEW**

#### **INTRODUCTION**

Fertilization rate was previously reported to be approximately 95% in beef cattle (Ayalon, 1978) but more recent reports suggest fertilization rates are lower (75% in beef cows [Breuel et al., 1993] and 88% in heifers; [Maurer and Chenault, 1993; Dunne et al., 2000; reviewed by Santos et al., 2004]). Regardless of the “true” fertilization rate, a large percentage of pregnancy loss occurs prior to calving resulting in a significant economic loss in beef and dairy operations. Many factors contribute to embryonic/fetal mortality including genetics, nutrition, heat stress, environmental influences, age, breed, twinning, and other unknown influences (Santos et al., 2004; BonDurant, 2007).

Increasing evidence links the physiological maturity of the dominant follicle at GnRH-induced ovulation with establishment and(or) maintenance of pregnancy (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005; Waldmann et al., 2006; Lopes et al., 2007; Meneghetti et al., 2009; Sa Filho et al., 2009; Dias et al., 2009). Cows induced to ovulate a follicle less than 11.3 mm had reduced pregnancy rates to AI at d 27 of gestation compared to cows induced to ovulate follicles larger than 11.3 mm in diameter (Perry et al., 2005). Cows induced to ovulate small follicles had a tendency to abort the pregnancy compared to cows ovulating large follicles (optimum follicle diameter was 14.8 mm; Perry et al., 2005). Cows and heifers that were induced to ovulate a small follicle had reduced concentrations of estradiol (Perry et al., 2005; Busch et al., 2008;

Atkins et al., 2008a) at the time of breeding and reduced concentrations of progesterone during the following luteal phase (Perry et al., 2005; 2007; Atkins et al., 2008b).

Interestingly, cows that spontaneously ovulated small follicles did not differ in pregnancy rates or late embryonic/fetal mortality regardless of ovulatory follicle diameter (Perry et al., 2005) suggesting that the factor(s) affecting establishment/maintenance of pregnancy are due to the physiological maturity of the follicle rather than simply diameter of the dominant follicle.

Other researchers have reported similar reduction in luteal function and embryo development when follicles are ovulated following a short vs. long proestrus period regardless of follicle diameter (Burke et al., 2001; Mussard et al., 2003, 2007; Bridges et al., 2006). Reducing the length of proestrus resulted in inadequate luteal function following ovulation independent of follicle diameter (Mussard et al., 2003). In the same study, pregnancy rates following embryo transfer were lower in cows with a shorter proestrus compared to cows with a longer proestrus (Mussard et al., 2003). Therefore, it is likely the physiological maturity of the follicle and not simply the size of the follicle that contributes to establishment and maintenance of pregnancy.

A relationship between follicle diameter and establishment of pregnancy is likely present in humans. McNatty et al. (1979) reported an increased number of granulosa cells aspirated from larger follicles than smaller follicles in women. Furthermore, follicular fluid concentrations of estradiol and the number of healthy oocytes increased in follicles with more granulosa cells (McNatty et al., 1979). Several researchers have attempted to find follicular fluid components that would predict pregnancy outcome in women. For example, more oocytes were successfully fertilized following

intracytoplasmic sperm injection when collected from follicles with increased follicular fluid concentrations of estradiol compared to follicles with reduced concentrations of estradiol in women (Lamb et al., 2007). A recent review summarizes the multitude of factors reported in follicular fluid that may affect human oocyte competence (Revelli et al., 2009). The authors argue that no one follicular factor is adequate to estimate pregnancy success but rather characterization of the follicular milieu, or the metabolomics of the follicle, may improve success (Revelli et al., 2009).

The reason for the reduced ability to establish and maintain a pregnancy following ovulation of immature dominant follicles is unknown but may involve compromised oocyte quality (Arlotto et al., 1996; Brevini-Gandolfi and Gandolfi, 2001), reduced luteal function (Perry et al., 2005; Vasconcelos et al., 2001; Murdoch and van Kirk, 1998; Busch et al., 2008; Atkins et al., 2008a), inadequate oviductal environment and(or) uterine environment (Murdoch and Kirk, 1998; Bridges et al., 2006; Moore, 1985). This review summarizes follicle maturation and the follicular determinants of oocyte competence, corpus luteum (CL) function, oviductal, and uterine environment

### **DOMINANT FOLLICLE MATURATION**

Formation of follicles in mammals begins in the fetal ovaries when a primordial germ cell becomes surrounded by squamous pre-granulosa cells (primordial follicle) and subsequently develops into a primary (single layer of cuboidal granulosa cells), secondary (multiple layers of granulosa cells and the start of the theca layers), and tertiary (continued development of theca and granulosa cells and formation of an antrum) follicle. Follicles grow in waves that consist of four phases: cyclical recruitment,

selection, dominance, and ovulation or atresia (Fortune et al., 1991; Lucy et al., 1992). During cyclic recruitment a cohort of small Graafian follicles are recruited for continued growth during a follicular wave. In monovular species, one follicle deviates from the remaining cohort of follicles (selection) and becomes the dominant follicle (dominance) while the remaining follicles become atretic. The dominant follicle is fated to either ovulate (during the follicular phase) or become atretic (during the luteal phase).

There are two main theories concerning how the dominant follicle gains a competitive advantage over the remaining subordinate follicles at follicular deviation. One theory is that the dominant follicle acquires LH receptors on the mural granulosa cells while the subordinate follicles do not (Bao and Garverick, 1998). The other theory is that the dominant follicle has increased bioavailability of insulin like growth factor-I (**IGF-I**) which increases the responsiveness of follicular cells to gonadotropins (Fortune et al., 2004). Beg and Ginther (2006) proposed a combination of the theories suggesting that both an increase in luteinizing hormone (**LH**) receptor (**LHr**) in the granulosa cells and an increase in free IGF-I control the selection of a dominant follicle in cattle. Another hypothesis is that the dominant follicle is already more advanced at the time of recruitment (Adams et al., 2008). Others have suggested that the oocyte of the future dominant follicle is more advanced and therefore accelerates the development of the follicle (Mermillod et al., 2008; Hendrickson et al., 2000). Although the exact reason why the selected follicle becomes dominant is under debate, most agree the dominant follicle is equipped to respond to LH for steroidogenesis in a low follicle stimulating hormone (**FSH**) environment while the remaining follicles cannot survive in a low FSH environment.

The dominant follicle will either ovulate (under low concentrations of progesterone) or undergo atresia (under high concentrations of progesterone). The growth and maturation of a dominant follicle has been reviewed recently (Webb and Campbell, 2007; Lucy 2007); therefore, I will summarize some key factors in the final development of the bovine dominant follicle including the role of gonadotropins and their receptors, estradiol and estrogen receptors, growth factors, vasculature, oocyte/granulosa interactions, and final changes leading to ovulation or atresia. For recent reviews on early follicle development and dominant follicle selection please refer to McNatty et al., (2007) and Beg and Ginther, (2006), respectively.

***Gonadotropins.*** The two cell-two gonadotropin theory states that the theca cells in response to LH stimulation synthesize androstendione which is transported to the granulosa cells where, in response to FSH stimulus, the granulosa cells aromatize androstendione to estradiol (Fortune and Armstrong, 1978; Fortune, 1986). As the dominant follicle matures, the amount of estradiol and inhibin increases which has a negative feedback on FSH secretion by the pituitary (Ireland et al., 1983; Burke et al., 2003). Around the time of follicular deviation, the mural granulosa cells of the dominant follicle acquire LHr (Xu et al., 1995; Bao et al., 1997), induced by FSH. It is not known how LH stimulation of the granulosa cells increases estradiol production but may relate to stimulation of the granulosa cell synthesis of the androgen precursor (pregnenolone) which in turn increases the supply of androstendione available to aromatize into estradiol (Fortune, 1986). The FSH receptor (**FSHr**) in the granulosa cells has been shown to stay

relatively constant from 12 h after recruitment through the end of follicle maturation (Xu et al., 1995).

The health and ovulatory capacity of the dominant follicle is dependent on LH although the pulsatile pattern of LH secretion can vary (Campbell et al., 2007). In sheep, there are four splice variants of the LHR resulting in four variations of LHR mRNA (A, B, F, and G; Bacich et al., 1999) and similar isoforms have been described in cattle (Kawate and Okuda, 1998). The specific isoform of LHR mRNA or post translational regulation of LHR may play a role in dominant follicle development. The A form of LHR is the only isoform that has been shown to be totally functional while the function of the remaining forms is still unclear. Binding proteins for LHR mRNA have been described as a possible post-translational regulation of LHR as LHR mRNA bound to its binding protein degraded faster than free LHR mRNA (Kash and Menon, 1999).

***Estradiol and estrogen receptors.*** Production of estradiol is a hallmark of a healthy dominant follicle and plays a role in inducing the preovulatory gonadotropin surge and stimulation, proliferation, and differentiation of follicular cells. Estradiol from the follicle directly affects the hypothalamus, pituitary, uterus, mammary gland, and the ovary itself. Aromatase knock out mice (**ARKO**) had precocious follicular development and no evidence of ovulation (no CL formation) but administration of estrogens partially restored follicular maturation although normal luteal development did not occur (Toda et al., 2001). Estradiol induced expression of both FSHr and LHR in granulosa cells (Richards et al., 1976; 1979). Estradiol is also thought to increase steroidogenesis (both androgens and progestins), increase gap junctions among the granulosa cells, and prevent

apoptosis of the follicular cells (reviewed by Rosenfeld et al., 2001). Estradiol administration also increased the expression of oxytocin receptors in bovine granulosa cells in vitro (Uenoyama and Okuda, 1997).

Estrogen bound to its receptor can act as a transcription factor by regulating transcription of genes via an estrogen response element (Kumar et al., 1987). Estrogen receptors (**ER**) can be in the cytoplasm or the nucleus and can act through regulation of gene transcription or by a faster mode of action through membrane bound receptors (Morley et al., 1992; Levin, 1999; Pietras and Szego, 1999; Nadal et al., 2000). Some recent studies have examined the role of genomic estrogen receptors  $\alpha$  and  $\beta$  in follicle development in mice (reviewed by Woodruff and Mayo, 2005). Knock out studies suggested estrogen receptor  $\alpha$  regulates follicle development through the hypothalamic-gonadal axis as gonadotropin replacement resulted in follicle development and ovulation similar to controls (Couse et al., 1999). Estrogen receptor  $\beta$  knock out ( **$\beta$ ERKO**) mice had morphologically normal follicle development but impaired ovulation and were subfertile compared to controls even with gonadotropin replacement (Couse and Korach, 1999). Couse and colleagues (2005) reported that  $\beta$ ERKO mice have impaired granulosa cell differentiation, cumulus expansion, and altered steroid profiles in response to gonadotropin stimulation compared to controls and  $\alpha$ ERKO mice. Therefore, ER $\alpha$  appears to play a role in the hypothalamic regulation of follicle stimulus and ER $\beta$  may have a more local regulatory role in follicle development.

***Metabolic hormones and growth factors.*** Metabolic hormones and intraovarian growth factors also affect the maturation of the dominant follicle (see Table 2.1). These factors will be discussed in the following section.

**Table 2.1** Metabolic hormones and growth factor actions on follicle maturation.

Growth Factor	Action	Source
Insulin	GC proliferation GC E2 production	Gutierrez et al., 1997 Butler et al., 2004
IGF-I	Increase GC FSHr and aromatase Prevent GC apoptosis GC proliferation	Zhou et al., 1997
IGF-II	Antral follicle growth	Yuan et al., 1998; Armstrong et al., 2000; Webb et al., 1999
Leptin	Inhibit E2 and A4 production	Armstrong et al., 2003
GDF-9	Early follicle formation Inhibition of DF maturation	Juengel et al., 2002 Campbell et al., 2005
BMP-15	Early follicle formation Inhibition of DF maturation	Juengel et al., 2002 Campbell et al., 2005
BMP-4	E2 production? Prevent P4 production	Glister et al., 2004; Spicer et al., 2006
BMP-6	Increased E2 production	Glister et al., 2004
BMP-7	Increased E2 production Prevent P4 production	Glister et al., 2004
Inhibin	Inhibit FSH Increase LH stimulated TC production of A4	Hsueh et al., 1987; Hillier and Miro 1993; Wrathall and Knight 1995; Campbell and Baird 2001; Reviewed by Knight and Glister, 2001
Activin	Increase FSH action Increase GC LHR Increase GC E2 GC proliferation Prevent luteinization Oocyte maturation	Reviewed by Knight and Glister, 2001
Follistatin	Bind activin and inhibit FSH	Reviewed by Knight and Glister, 2001

Abbreviations: Androstendione (A4), DF (dominant follicle), E2 (estradiol), FSH (follicle stimulating hormone), FSHr (follicle stimulating hormone receptor), GC (granulosa cells), IGF (insulin like growth factor), LH (luteinizing hormone), LHR (luteinizing hormone receptor), theca cells (TC).

*Insulin, leptin, and insulin like growth factor.* Insulin and leptin regulation of follicle maturation may represent physiological mechanisms for nutritional regulation of reproduction. Granulosa cells were dependent on insulin (Guitierrez et al., 1997) and estradiol production increased in response to insulin (Butler et al., 2004). Leptin may block the insulin induced increase in follicular steroidogenesis (Spicer and Francisco, 1997; Spicer et al., 2001). Bovine theca and granulosa cells cultured with leptin produced less androstendione and estradiol, respectively compared to cells cultured without leptin (Armstrong et al., 2003).

Growth hormone stimulates IGF-I secretion by the liver and the primary source of IGF-I in bovine follicular fluid is from circulation. Growth hormone deficient cattle have limited IGF-I and dominant follicles are not present with follicle growth arrested at 8 mm (Chase et al., 1998). Insulin like growth factor I increased the expression of FSHr and aromatase in murine granulosa cells and may prevent apoptosis of cells by enhancing the protective effects of FSH and estradiol (Zhou et al., 1997). Insulin like growth factor binding proteins (**IGFBP**)-2 and 4 are expressed in the granulosa and theca cells, respectively (Webb et al., 2003; 2004) and the selection of the dominant follicle, during a follicular wave, was associated with increased amount of free IGF-I, decreased IGFBP-2 (Armstrong et al., 1998; Ginther et al., 2002; Kojima et al., 2003) possibly due to an increase in IGFBP protease (PAPPA-1; Monget et al., 2003; Fortune et al., 2004). The theca cells are thought to be the main producer of ovarian IGF-II (Yuan et al., 1998; Armstrong et al., 2000; Webb et al., 1999) which binds to the IGF type 1 receptor (Lucy, 2000).

*Transforming Growth Factor  $\beta$  (TGF $\beta$ ) superfamily.* Members of the TGF $\beta$  superfamily are also important for maintenance of dominant follicle health including growth differentiation factors (**GDF**), bone morphogenic proteins (**BMPs**), activin, inhibin, and binding proteins (follistatin). The TGF $\beta$  superfamily is complex and differentially regulated in follicular cells (reviewed by Knight and Glister, 2006). Growth differentiation factor 9 and BMP-15 production appear to be oocyte specific (Sendai et al., 2001; Lonergan et al., 2003; Pennetier et al., 2004). The role of GDF-9 and BMP-15 in early follicular development is species dependent. Follicles in GDF-9 null mice (Dong et al., 1996) and GDF-9 or BMP-15 immunized ewes had few to no follicles progress beyond the primary follicle stage (Juengel et al., 2002). The role of BMP-15 in murine folliculogenesis is different than in sheep as mice lacking BMP-15 remained fertile (Yan et al., 2001). Growth differentiation factor 9 along with BMP-15 may continue to play a role in granulosa cell differentiation and proliferation in the dominant follicle (Eppig, 2001; Knight and Glister, 2006; Spicer et al., 2006). Culturing bovine granulosa cells and oocytes in the presence of GDF-9 and BMP-15 reduced granulosa cell proliferation and estradiol production suggesting that these factors may have an inhibitory effect on dominant follicle maturation (Campbell et al., 2005 and reviewed in Webb and Campbell, 2007). Heterozygous mutations in GDF-9 and BMP-15 genes increased ovulation rate in sheep (reviewed in McNatty et al., 2007) and Knight and Glister (2006) suggest this increase in ovulation is due to increased follicle development to the ovulatory stage rather than a direct action on ovulation.

Theca cells produce BMP-4 and -7 which are thought to increase estradiol, inhibin, and activin production by the granulosa cells as well as prevent progesterone

synthesis (reviewed by Knight and Glistler, 2006). Granulosa cells and the oocyte produce BMP-6 which may drive granulosa cell differentiation and prevent luteinization (Knight and Glistler, 2006). Cultured ovine granulosa cells produced more estradiol and inhibin when BMP-2, 4, and 7 were added to the media (Souza et al., 2002; Campbell et al., 2006) and BMP-4, 6, and 7 increased estradiol, inhibin-A, activin-A, and follistatin in cultured bovine granulosa cells (Glistler et al., 2004); However, Spicer and colleagues (2006) reported a decrease in estradiol production by bovine granulosa cells cultured with BMP-4.

Inhibin, activin, and follistatin also affect follicle maturation (reviewed by Knight and Glistler, 2001). Inhibin and follistatin (by binding activin) have been shown to inhibit FSH secretion while activin enhances FSH stimulation of the granulosa cells (Knight and Glistler, 2001). The proportion of inhibin:activin increases as follicles develop (Schwall et al., 1990; Yamamoto et al., 1992; Glistler et al., 2006). There is evidence that inhibin A from the granulosa cells may increase LH stimulated androgen production of the theca (Hsueh et al., 1987; Hillier and Miro 1993; Wrathall and Knight 1995; Campbell and Baird 2001).

The TGF family members have a variety of actions in the developing follicle and contain a number of molecules with similar functions thereby building redundancy in the system. The TGF family is vital to follicle health as members control oocyte and somatic cell communication, regulation of FSH (through anti-mullerian hormone [AMH], inhibin, and activin), and somatic cell proliferation and steroidogenesis.

***Vasculature.*** Angiogenesis refers to the development of new vasculature due to sprouting of existing blood vessels. Angiogenesis occurs in reproductive tissues in adult

animals during changes of the estrous cycle, ovarian function, and placentation (see reviews by Reynolds et al., 1992; Stouffer et al., 2001). Angiogenesis requires the following steps (reviewed by Felmeden et al., 2003): 1) activation of endothelial cells 2) basement membrane breakdown and migration of endothelial cells, 3) proliferation and differentiation of endothelial cells, 4) reformation of the basement membrane, and 5) vascular stabilization and maturation. Growth factors (such as vascular endothelial growth factor (**VEGF**), basic fibroblast growth factor (**bFGF**), and angiopoietin) and their receptors are required for angiogenesis. These growth factors are regulated by cytokines and factors related to oxygen supply like hypoxia inducible factor (**HIF**; reviewed by Felmeden et al., 2003).

Angiogenesis occurs in the thecal layer of antral follicles and the vasculature continues to develop as the follicle matures (Jiang et al., 2003; reviewed by Tamanini and De Ambrogi, 2004). Angiogenesis is spatially regulated in the follicle as angiogenesis occurred mainly in the apical portion of the theca in medium sized antral follicles and primarily in the middle or basal layer of the dominant follicles (Jiang et al., 2003). Seventy two hours prior to ovulation, blood flow to the dominant follicle increased dramatically to over 10 mL/min/g of tissue while there was a decrease in blood flow to the regressing CL and a relatively constant flow to the stroma (Bruce and Moor, 1976). Blood flow is correlated with increased estradiol leading up to ovulation although there is no direct evidence linking estradiol to vascular development (Acosta et al., 2003).

Expression of VEGF was restricted to the theca layer in antral follicles until near ovulation when VEGF mRNA was found in the cumulus granulosa cells (Berisha et al., 2000; Stouffer et al., 2001). Luteinizing hormone was associated with an increase in

VEGF secreted by the granulosa cells (Garrido et al., 1993) and VEGF expression in the granulosa cells increased as the dominant follicle matured. Tamanini and De Ambrogi (2004) speculated a role for VEGF in stimulating vessel permeability and that an increase in VEGF with approaching ovulation could improve the transport of LH to the follicle which may explain the correlation of blood flow and estradiol secretion.

Basic fibroblast growth factor (**bFGF**) is expressed in the dominant follicle and promotes endothelial cell proliferation (Bikfalvi et al., 1998). Theca interna levels of bFGF mRNA significantly increased during the final maturation of the bovine dominant follicle (Shimizu et al., 2002) and remained relatively unchanged in the granulosa cells (Berisha and Schams, 2005). Angiopoietin mRNA was detected in the theca and granulosa cells of bovine follicles but the expression reduced as follicles matured (Hayashi et al., 2003). In addition, several other angiogenic factors have been identified in the bovine ovary including nitric oxide, epidermal growth factor, and endothelin-1 (reviewed in Tamanini and De Ambrogi, 2004).

Proper vascularity of the ovulatory follicle may be important for establishment of pregnancy, as heifers that became pregnant had increased follicular blood flow at the time of AI compared to heifers that did not become pregnant (Siddiqui et al., 2009b). It is not known how follicular vascularity affects pregnancy establishment but may relate to the ability of a follicle to respond to endocrine signals or the vascularity of the subsequently formed CL. Experiments in rhesus monkeys suggested that all antral follicles could bind human chorionic gonadotropin (**hCG**) but when labeled hCG was administered through the vasculature, the dominant follicle bound the majority of the hCG while the remaining antral follicles bound little to no hCG (Zelevnik et al., 1981). This differential response

suggests the vasculature of the dominant follicle is optimized so that the dominant follicle is exposed to more of the hormones (Moor and Seamark, 1986). Adequate maturation of the vasculature of the follicle may have direct consequences on the development of a healthy CL. Tamanini and De Ambrogi (2004) suggested that proper capillary development of the follicle wall through coordinated efforts of both the theca and granulosa is essential not only to the follicle but also the developing CL.

***Oocyte-granulosa cell interactions.*** The follicular cells continue to proliferate, and differentiate as the dominant follicle matures which requires communication with the oocyte. The granulosa cells can be separated into mural cells (lining the basement membrane), the cumulus oophorus (along the stalk containing the oocyte), and the corona radiata (in direct contact with the zona pellucida). Cellular polarity is established in these granulosa cells based on nearness to the basement membrane or oocyte (Plancha et al., 2005). The mural granulosa cells take on a polarity similar to epithelial cells with an apical and basal side (Plancha et al., 2005). The granulosa cells form gap junctions between each other and also have direct communication with the oocyte. The corona radiata have transzonal projections which interface with the oolemma (Motta et al., 1994). The transzonal projections have two forms, one containing microtubules that play a role in paracrine communication and the other with actin in the projections which have gap-junctional communication (Albertini et al., 2001; Navarro-Costa et al., 2005). There is evidence that FSH can reduce the formation of these transzonal projections (Combelles et al., 2004) and in the human, mouse, and hamster it appears that as the follicle develops the number of transzonal projections decreases (Motta et al., 1994, Albertini et al., 2001; Plancha and Albertini, 1994).

Much evidence is accumulating on the bidirectional regulation and communication between the oocyte and the developing follicle (Driancourt and Thuel, 1998; Eppig, 2001; Sugiura and Eppig, 2005). Pincus and Enzmann (1935) reported that oocytes removed from follicles underwent spontaneous resumption of meiosis and El-Fouly et al. (1970) reported that follicular cells become luteinized after removal of the oocyte. The rate of primordial follicle development doubled when more advanced oocytes were associated with pre-granulosa cells compared to immature oocytes (Eppig et al., 2002).

Proximity to the oocyte is thought to be a main regulator of the differentiation of the mural vs. cumulus cell types. The mural granulosa cells are best equipped for steroidogenesis (contain aromatase) and response to gonadotropin stimulus (express gonadotropin receptors) while the cumulus cells are better equipped to provide nutrients to the oocyte as they express genes associated with amino acid uptake and glycolysis (reviewed Sugiura and Eppig, 2005). This differentiation can be reversed based on proximity of the oocyte. Removal of the oocyte from the cumulus mass results in increased expression of genes associated with mural cells (*lhr*, *kitl*) and replacement of the oocyte returns the phenotype to a cumulus cell (Eppig et al., 1997; Joyce et al., 1999). Similarly, mural cells cultured with mature oocytes had decreased expression of LHr mRNA (Eppig et al., 1997).

Oocyectomy experiments indicated that the oocyte controls the regulation of metabolism of the cumulus cells and requires cumulus cells for glycolysis and amino acid uptake (reviewed in Sugiura and Eppig 2005) although the degree of regulation may vary by species (Zuelke and Brackett 1992). In mice, evidence suggests the oocyte is

necessary for cumulus expansion. Cumulus cells secrete hyaluronic acid in response to gonadotropin stimulus but if the oocyte is removed, the cumulus cells do not expand. Replacement of a mature oocyte reinstated the ability of the cumulus to expand (Salustri et al., 1990). However, this is species specific as removal of the oocyte does not prevent cumulus expansion in the rat, pig or cow (Prochazka et al., 1991; Vanderhyden, 1993; Ralph et al., 1995) but oocytes from these species are able to induce expansion of the mouse cumulus so they likely secrete the same factor (Singh et al., 1993; Vanderhyden, 1993; Ralph et al., 1995).

***Ovulation or Atresia.*** The final fate of the dominant follicle is either atresia (during the luteal phase) or ovulation (during the follicular phase). A number of hormones and cytokines act as survival factors effectively inhibiting apoptosis in cells of a developing follicle including gonadotropins, estradiol, IGF-1, activin, EGF and FGF, interleukin (IL)-1 $\beta$ , and cGMP while other factors are proapoptotic including tumor necrosis factor  $\alpha$ , androgens, IL-6, GnRH, and reactive oxygen species (reviewed by Chun and Hsueh, 1998). The apoptotic pathway is present throughout folliculogenesis but the presence of survival factors can prevent apoptosis from occurring and there is a link between cellular proliferation and increased apoptosis (reviewed by Quirk et al., 2004). Cells that became quiescent and terminally differentiated escaped the apoptotic pathway while cells that continued to divide became apoptotic in the absence of stimulation (Quirk et al., 2004). Cells are more susceptible to apoptosis during the G1 to S phase of the cell cycle and both IGF-I and estradiol are thought to increase the rate of progression through this phase of the cycle thereby protecting the cells from apoptosis

(reviewed in Quirk et al., 2004). Granulosa cells of an aged follicle, with decreased survival factors, eventually become apoptotic and the follicle will undergo atresia.

In a low progesterone environment, a positive feedback loop between estradiol and LH eventually initiates the preovulatory gonadotropin surge which induces ovulation, resumption of meiosis, and luteinization of the thecal and granulosa cells. Following luteinization, reduced cellular proliferation and terminal differentiation aid in the prevention of apoptosis of the cells. Leading up to the ovulatory LH surge, estradiol production increases to twice the concentrations produced in the nonovulatory dominant follicles (Adams and Pierson, 1995). After the LH surge, aromatase expression is decreased and the genes associated with progesterone synthesis are upregulated (Richards, 1994). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production also increases after the LH surge (Hedin et al., 1987) and murine knock out models suggest that prostaglandins are required for ovulation (Lim et al., 1997). In addition to a rise in PGE<sub>2</sub> and a switch from estradiol to progesterone synthesis, EGF like growth factors increase during the periovulatory period (Park et al., 2004) and this rise is likely controlled by progesterone and PGE<sub>2</sub> (Shimada et al., 2006). Recently Kawashima et al. (2008) reported that mimicking the sequential hormonal changes leading up to ovulation in cultured porcine oocytes led to improved embryo development to blastocyst stage and the authors concluded that in vitro maturation of oocytes will be improved by mimicking the species specific hormonal pattern approaching ovulation.

Leading up to ovulation, there is an increase in follicle diameter, the follicle wall at the stigma becomes thinner, and the oocyte becomes free floating within the follicle. The superficial epithelium covering the follicular apex is sloughed off and the underlying

theca layers break apart (Espey and Lipner, 1994). The basement membrane breaks down between the theca and the granulosa compartments (except in humans) and the vasculature becomes leaky. The theca layers undergo changes akin to an inflammatory response including hyperemia, edema, extravascular coagulation, ischemia, infiltration of leukocytes, basophils, and eosinophils, vascular injury, and angiogenesis (Cavender and Murdoch, 1988). The gap junctions between granulosa cells breakdown and the granulosa cells begin to fold and luteinize. The cumulus cells produce hyaluronic acid that expands and creates a mucous filled space between the cumulus cells leading to cumulus cell expansion (Chen et al., 1993). The extracellular matrix of the follicle undergoes dramatic changes required for ovulation and formation of luteal tissue (reviewed by Curry and Smith, 2006 and Russell and Robker, 2007).

The final maturation of the dominant follicle is associated with changes in steroidogenesis, local growth factors, angiogenesis, follicle cell proliferation and maturation, and communication with the oocyte. Premature ovulation before final maturation of the follicle may detrimentally affect the oocyte, formation of the CL, or oviductal and uterine environments (through the altered endocrine profile associated with early ovulation). The following sections will review how premature ovulation may alter oocyte, CL, oviduct, and uterine physiology.

## **OOCYTE COMPETENCE**

Oocyte competence can be defined as the ability of an oocyte to 1.) resume meiosis following gonadotropin stimulation, 2.) undergo cleavage divisions after fertilization, 3.) develop to the blastocyst stage, 4.) result in birth of live young and 5.)

offspring with good health (defined by Sirard et al., 2006). Oocyte competence requires proper maturation of the oocyte which is intimately associated with follicle development (reviewed in cattle by Fair, 2003). Both nuclear and cytoplasmic oocyte maturation requires gonadotropin stimulation and communication from the granulosa. Inadequate oocyte development could result in failure to complete meiosis, ability to fertilize, and inadequate pre-implantation embryo development (Eppig, 1991; Eppig et al., 2002; Gosden, 2002; Matzuk et al., 2002). A minimum follicle diameter of 2 to 3 mm in cows is required for a fertilizable oocyte but the oocyte continues to develop and store proteins and mRNA in follicles up to 15 mm in diameter in cattle (Arlotto et al., 1996). The rate of blastocyst development was faster following fertilization of larger oocytes ( $> 115 \mu\text{m}$ ) than smaller oocytes ( $< 114 \mu\text{m}$ ; Arlotto et al., 1996) and as diameter of the oocyte increased so did the quality of the embryo. The following section will review the final maturation of the oocyte, follicle determinants of oocyte quality, oocyte production of mRNA and proteins, and epigenetic modification of the oocyte genome important to embryo development.

***Oocyte maturation.*** In mammals the oocyte begins meiosis in the fetal ovaries and meiosis is arrested at the late prophase stage (diplotene) in the primordial follicle. Early in follicle development (primary follicle) there is an increase in numbers of mitochondria and smooth and rough endoplasmic reticulum in the bovine oocyte (Fair et al., 1997). At the secondary follicle stage, the zona pellucida begins to form around the oolema, cortical granules begin to form in the cytoplasm, and RNA synthesis is initiated (Fair et al., 1997). Abundant transcriptional activity continues in the oocyte of a tertiary follicle until the oocyte reaches about  $110 \mu\text{m}$  in diameter (2 to 3 mm follicle; Fair et al.,

1995, 1996; Crozet et al., 1986). In cattle, the oocytes in tertiary follicles have already developed the ability to undergo meiosis and oocyte removal is followed by spontaneously resumption of meiosis (Pincus and Enzmann, 1935; Edwards, 1965) suggesting the follicular cells secrete a meiotic inhibitor. Growth in oocyte diameter slows after the tertiary follicle stage (reaching a maximal diameter of 120 to 130  $\mu\text{m}$ ) but the oocyte continues to undergo cytoplasmic, nuclear, and molecular maturation (defined by Sirard et al., 2006).

*Cytoplasmic maturation.* Piedrahita et al. (2002) used oocytes from small (1 to 3 mm) or large (6 to 12 mm) follicles for somatic cell nuclear transfer (SCNT) in cattle. The authors reported that the embryo development and final pregnancy rates were similar between embryos formed in cytoplasm of small and large oocytes; however, the allantois was smaller in the fetuses derived from SCNT with the oocytes from small follicles vs. those from large follicles. This suggests that proper cytoplasmic maturation in the oocyte could have downstream effects on placental development.

During cytoplasmic maturation, a space forms between the zona pellucida and the oolema, called the perivitelline space. There is a decrease in the number of rough and smooth endoplasmic reticulum, the number of mitochondria increases, and the mitochondria, Golgi complex, cortical granules, and nucleus move from a central location to the periphery of the cytoplasm (Fair et al., 1997). As the dominant follicle matured, there was an increase in lipid content in the ooplasm, the Golgi complex became smaller, the cortical granules migrated to the edge of the cytoplasm, vacuoles formed in the nucleolus, and the perivitelline space continued to expand (Assey et al., 1994; reviewed by Fair, 2003).

Another facet of ooplasm maturation is the restructuring of the cytoskeleton which may be affected by the follicular environment. The chromatin, nuclear lamina, microtubules, and centrosomal proteins ( $\gamma$ -tubulin and pericentrin) exhibited distinct spatial patterns between in vitro matured and in vivo matured murine oocytes (Sanfins et al., 2004). Furthermore, oocytes collected 5 h after the LH surge followed by a 16 hr culture period had similar cytoskeletal changes compared to oocytes that were in vivo matured while oocytes removed 1.5 h after the LH surge had distinctly different appearance of the cytoskeleton (Sanfins et al., 2004). This experiment suggested that in mice by 5 h after the LH surge, the cytoskeleton of the oocyte is adequately developed. After the LH surge, the transzonal projections between the oocyte and the corona radiata broke apart, the lipid content in the ooplasm continued to increase, the Golgi complex continued to decrease in size, and the cortical granules continued their migration to just underneath the oolemma (reviewed by Fair, 2003).

*Nuclear maturation.* Following the LH surge, the oocyte resumed meiosis after activation of the maturation promoting factor (a protein complex of cyclin B1 and P32cdc2) which initiated the cell cycle machinery needed to resume meiosis at which time a polar body was extruded (Sirard et al., 1989). The chromosomes condensed and under the influence of cytostatic factor, the cell cycle stopped at the metaphase stage of meiosis II until fertilization (Hyttel et al., 1986, 1989, 1997; Sirard et al., 1989; reviewed by Fair, 2003). The nuclear membrane degenerated (germinal vesicle breakdown) and nuclear contents were released into the cytoplasm. After germinal vesicle breakdown, the oocyte is no longer capable of mRNA transcription. Therefore, until the embryonic genome is able to undergo transcription, the oocyte, zygote, and early embryo (8 to 16

cell stage in cattle) is dependent on mRNA and proteins made before germinal vessel breakdown.

*Molecular maturation.* Molecular maturation (epigenetic modification and final production and modification of mRNA and proteins) of the oocyte is not as defined as the nuclear and cytoplasmic maturation. Sirard and colleagues (2006) argued that while many oocytes attain meiotic and cytoplasmic competence, the molecular milieu of the oocyte may determine the ability of the oocyte to become an embryo capable of establishing and maintaining a pregnancy. Although, the molecular changes in the cytoplasm are difficult to visualize and study, Sirard and colleagues (2006) suggested that these final changes in the last days before ovulation may be the “capacitators” that confer the ability to culminate in a normal pregnancy. Rodriguez and Farin (2004) reported a dramatic increase in transcription of the oocyte just before germinal vessel breakdown. It was suggested that these genes play a role in meiotic resumption but may also have an effect on early embryo development (Rodriguez et al., 2006).

The pattern of LH pulses may also affect the molecular maturation of the oocyte. Oussaid et al. (1999) reported normal ovulation and fertilization rates between ewes with normal pre-ovulatory LH pulses or ewes with no LH pulses (treated with a GnRH antagonist) prior to LH surge. However, ewes with no LH pulses leading up to the LH surge had fewer embryos survive to the blastocyst stage (Oussaid et al., 1999). The process of ovulation and fertilization were not affected by LH pulses but survival of the embryo after fertilization improved when ewes had increasing LH prior to ovulation suggests the nuclear and cytoplasmic maturation were similar but possibly the molecular

maturation of the oocyte was affected. For more details on molecular maturation, see sections on oocyte mRNA and protein and epigenetics below.

***Cumulus oocyte complex.*** The role of cumulus cells after ovulation is species dependant (reviewed by Van Soom et al., 2002). In cattle, the cumulus cells are dispersed within a few hours to 10 h after ovulation (Lorton and First, 1979; Hyttel et al., 1988) but oocytes without a cumulus complex were not fertilized (Van Soom et al., 2002). Furthermore removal of the cumulus mass prior to in vitro fertilization decreased fertilization rates in cattle (Zhang et al., 1995). Cumulus cells aid in the transport of the oocyte through the oviduct and may secrete a chemotactic factor that assists in sperm transport through the oviduct (Ito et al., 1991). Additionally the cumulus may facilitate sperm capacitation and the acrosomal exocytosis (reviewed by Van Soom et al., 2002).

***Follicular determinants of oocyte quality.*** As mentioned above, the cumulus granulosa cells and oocyte development are intimately associated and a communication loop between oocyte and granulosa cells is vital for provision of nutrition and energy to the developing oocyte and proper stimulus to the developing follicle. In fact, cumulus cell mRNA expression may potentially be used to screen for oocyte competence in IVF clinics (Anderson et al., 2009). Oocyte competence improved consistently when collected from more advanced stages of dominant follicles and oocytes from follicles exposed to an LH surge were of better quality compared to oocytes from cows without an LH surge (reviewed in Sirard, 2006). Blondin and colleagues (2002) reported improved formation of blastocysts (80%) from oocytes collected after a 48 h “coasting” period (gonadotropin withdrawal) followed by LH stimulus six hours before oocyte aspiration. Oocytes collected from early atretic follicles have improved developmental competence

compared to oocytes collected from growing follicles (Fair, 2003) maybe due to similar follicular changes between final maturation of the follicle and changes in early atretic follicles (Kruip and Dieleman, 1982; Dieleman et al., 1983; Assey et al., 1994). The following section will review specific follicular factors that influence oocyte competence including hormones, vascular supply, and extra-cellular matrix remodeling.

*Transforming Growth Factor  $\beta$  superfamily.* Members of the TGF $\beta$  superfamily including inhibin, follistatin, activin and AMH from the follicle affect oocyte maturation. Follistatin improved bovine oocyte development (Silva and Knight, 1998) and oocyte abundance of follistatin mRNA was positively associated with time to first cleavage in zygotes (Patel et al., 2007). The role of inhibin and activin is a little confused in the literature possibly due to species variation. Inhibin was reported to stimulate nuclear maturation in cultured bovine oocytes (Stock et al., 1997) and similar results were reported in pigs (Miller et al., 1991). Franchimont et al., (1990) linked high concentrations of inhibin in the follicular fluid with better quality oocytes in humans. O et al. (1989) reported no stimulation in rat oocyte development in the presence of inhibin. Activin receptors were reported in bovine oocytes (Hulshof et al., 1997) but similar discrepancy exists as to the role of activin in oocyte maturation. In rats activin increased the number of oocytes undergoing germinal vesicle breakdown (Itoh et al., 1990; Sadatsuki et al., 1991) but activin did not affect nuclear maturation in oocytes from pigs (Coskun and Lin, 1994) or cattle (Van Tol et al., 1994; Stock et al., 1997). Inhibin and activin promoted cytoplasmic maturation (Stock et al., 1997) although the evidence is weak (reviewed by Driancourt and Thuel, 1998). In rats, AMH had a dose dependent inhibition on the resumption of meiosis (Takahashi et al., 1986; Ueno et al., 1988).

*Steroids.* Follicular fluid concentrations of estradiol may play a direct role in the competence of the oocyte. More oocytes were able to develop to blastocysts that came from follicles with higher concentrations of estradiol in cattle (Mermillod et al., 1999) and humans (Carson et al., 1982; Botero Ruiz et al., 1984; Fishel et al., 1983), while other studies found no correlation between oocyte development and follicular fluid concentrations of estradiol in humans (Yding-Andersen, 1990; Tarlatzis et al., 1993; Suchanek et al., 1994) and cattle (Hazeleger et al., 1995) or even a negative correlation (cattle; Siddiqui et al., 2009a). Murine (Wu et al., 1992) and human (Wu et al., 1993) oocytes were positive for estrogen receptor  $\alpha$  while this has not been true of livestock species (Driancourt and Thuel, 1998). Estradiol may promote nuclear maturation as administration of an aromatase inhibitor (which increased androgens and decreased estrogens in the follicular fluid) decreased the number of oocytes that advanced to metaphase II in primates (Zelinski Wooten et al., 1993); however it is unclear whether the effect was due to lower estradiol or increased androstendione. Increasing doses of androgens in the culture media of murine oocytes led to a dose dependent decrease in oocyte cytoplasmic maturation (Andriesz and Trounson, 1995). Studies with patients deficient in steroidogenic enzymes suggest estradiol is not required for nuclear maturation in humans as long as gonadotropins are administered (Rabinovici et al., 1989; Pellicer et al., 1991). The exact role of steroids in oocyte development is further complicated by the presence of steroid binding hormones in the follicular fluid which were also implicated in oocyte health (Yding-Andersen, 1990). While multiple studies in humans reported no effect of follicular fluid concentrations of progesterone on oocyte quality (Yding-Andersen et al., 1990; Tarlatzis et al., 1993; Suchanek et al., 1994), a

negative correlation between concentrations of progesterone and oocyte quality was concluded in cattle (Hazeleger et al., 1995).

*Growth factors.* Growth factors secreted by the follicle may directly affect the oocyte. The oocyte has many growth factor receptors including EGF (Chia et al., 1995; Rappolee et al., 1988), IGF-I and -II (Teissier et al., 1994) and platelet derived growth factor (Watson et al., 1992). Epidermal growth factor may play a role in both cytoplasmic (Kobayashi et al., 1994; Lonergan et al., 1994) and nuclear maturation since more bovine oocytes cultured with EGF reached the metaphase II stage than oocytes cultured without EGF (Harper and Brackett, 1993; Im and Park, 1995; Lonergan et al., 1996). The EGF promoted maturation was also reported in other species including mice, pigs, and primates (reviewed by Driancourt and Thuel, 1998); however, follicular fluid concentrations of EGF did not correlate with the percentage of fertilizable oocytes in humans (Artini et al., 1994). It is unlikely that IGF-I had a direct effect on oocyte maturation as IGF- I did not have a measureable effect on nuclear maturation in cultured bovine oocytes (Lorenzon et al., 1995) and follicular fluid concentrations of IGF-I did not correlate with fertilization rate of human oocytes (Artini et al., 1994).

*Vascularity.* The advancement of Doppler ultrasonography has led to experiments linking follicular vascularity with the quality of the oocyte. Recently Siddiqui and colleagues (2009a) reported improved oocyte competence associated with the vascular development of a dominant follicle in heifers. More oocytes cleaved following fertilization and embryos were more advanced (> 8 cell vs. < 8 cell) when the oocytes were collected from dominant follicles with increased vascularity (Siddiqui et al., 2009a) compared to dominant follicles with reduced vascularity. A similar link between

follicle vascular supply and embryo development was reported in women (Coulam et al., 1999) where the establishment of pregnancy was improved when embryos came from fertilized oocytes contained within follicles with increased vascular perfusion. The reason for the improvement in oocyte competence due to vascular development of the follicle is unknown but Siddiqui et al. (2009a) suggested that increased oxygen to the follicle may improve mitochondrial function in the oocyte.

*Extra-cellular matrix remodeling.* The role of extra-cellular matrix (**ECM**) remodeling is complex and an often overlooked area of physiological changes and regulation. The ECM not only provides structural support for cells and tissues but also plays a role in many physiological events including cell adhesion, migration, proliferation, bioavailability of growth factors, and communication from extracellular environment to the nucleus (reviewed in Alberts et al., 2002). Remodeling of the ECM is an important part of ovulation and CL formation which is largely controlled by several classes of proteinases including: matrix metalloproteinases (**MMPs**) which are subdivided into collagenases, gelatinases, stromelysins, and membrane-type enzymes, MMP inhibitors (tissue inhibitor metalloproteinases [**TIMP**]), plasmin/plasminogen activator system, and the a disintegrin and metalloproteinase with thrombospondin motifs (**ADAMTS**; reviewed by Curry and Smith, 2006).

Many components of these proteinase systems are upregulated in response to the LH surge (reviewed by Curry and Smith, 2006 and Russell and Robker, 2007). For example, TIMPs have been detected in both the theca (Shores and Hunter, 2000) and granulosa cells in the developing follicle (Smith et al., 1993; McIntush et al., 1996; Shores and Hunter, 2000). Tissue inhibitor of metalloproteinase -1 was not present in

follicles prior to the LH surge but granulosa cells contained TIMP-1 protein soon after the LH surge in ewes (McIntush et al., 1996). There may be species variation in the presence of TIMPs as Shores and Hunter (2000) reported TIMP-1 in the theca and granulosa cells of small and large follicles but that the expression increased in the granulosa after the LH surge. The TIMPs may directly affect oocyte competence as Funahashi et al. (1999) reported an increase in the percentage of cleaved oocytes and embryos that developed to the blastocyst stage when porcine oocytes were matured in culture media containing TIMP-1 compared to control oocytes. Interestingly, the inclusion of TIMP-1 to the media after fertilization had no effect on embryo development suggesting that TIMP-1 improved embryo development by a direct action on the oocyte (Funahashi et al., 1999).

***Oocyte mRNA and proteins.*** The oocyte makes mRNA and proteins important to final maturation and ovulation of the oocyte. In the mouse, RNA transcription increases 300 fold while the oocyte is at its peak growth phase (Piko and Clegg, 1982). The translation of these RNAs is temporally controlled as some are transcribed throughout oogenesis (*zp3*), only around final maturation and ovulation (*mos* and *tissue type plasminogen activator*), while others are not translated until early embryogenesis (*stem loop binding protein*; reviewed by Gosden, 2002). After fertilization and prior to the activation of translation in the embryo, the developing embryo depends on mRNA and proteins from the cytoplasm of the oocyte. The embryo is unable to transcribe genes until the 8 to 16 cell stage in sheep and cattle (Brevini-Gandolfi and Gandolfi, 2001). The mRNA in the oocyte undergoes post transcriptional modification that controls the timing of translation and protein synthesis. Therefore, the embryo is dependent on RNA

transcripts synthesized in the oocyte prior to ovulation. Premature ovulation of the follicle may lead to the expulsion of an oocyte before the final transcriptional activity is complete. The following section will summarize the current research on mRNA and proteins in the oocyte and their role in oocyte maturation, fertilization and embryo development.

In recent studies, multiple researchers have examined the specific mRNA patterns in oocytes and early embryos in cattle (Sendai et al., 2001; Brevini et al., 2004; Hwang et al., 2005; Pennetier et al., 2004; 2005; Valee et al., 2005; reviewed by Cui and Kim, 2007). Some genes have been relatively well characterized in oocytes including *Mos*, *Zp1*, *Zp2*, *Zp3*, *Gdf9*, *Figla*, *Bmp15*, *Hlfoo*, and *Zar1* (reviewed by Vallee et al., 2005). Hwang and colleagues (2005) reported that *dncl1*, *zp2*, *gtl3*, and *fank1* were all expressed in immature oocytes and increased in expression in metaphase II oocytes followed by a gradual decrease in the amount of transcript until the 8 cell stage at which time the transcripts were no longer detected. The authors concluded that these genes were maternally derived mRNAs needed for embryo development.

Pennetier and colleagues (2005) used subtractive and suppressive hybridization to study transcripts upregulated in the oocyte and early embryo compared to somatic cells. These authors found 8 novel genes expressed in the oocyte of which 5 were identified as B cell translocation gene 4 (BTG4; antiproliferation), cullin 1 (cell cycle regulation), MCF.2 transforming sequence (cell cycle regulation), and a locus similar to snail soma ferritin (iron storage). The transcripts were high in the oocyte coupled with a gradual degradation to the 8 cell stage and gone by the time of morula and blastocyst development (except for BTG4 which remained in the blastocysts). A reduction in

transcripts at the maternal to zygotic transition was also reported for maternally derived *mater*, *zar1*, *gdf9*, *bmp15*, and *nalp9* (Sendai et al., 2001; Brevini et al., 2004; Pennetier et al., 2004). One of the genes (CUL1) in the oocytes and embryos reported by Pennetier et al. (2005) had a peculiar pattern. It consistently had a transient increase from the 2 to 4 cell embryo and 4 cell and 5 to 8 cell embryos. Although most report transcription inactivity in the bovine embryo until the 8 cell stage, Memili and First (2000) reported transcription activity in 4 cell bovine embryos; therefore, the CUL1 transcript increase might be from the embryonic genome. Vallee and colleagues (2005) also used subtractive techniques to study transcripts upregulated in bovine, murine, and xenopus oocytes. These authors reported an increase in *GDF9* (described in previous section), *MLF1-interacting protein* (possible role in cell division), *polyadenylate-binding protein-interacting protein1* (translational initiation and protein synthesis), *B cell translocation gene* (described above), and *protein tyrosine phosphatase receptor type Q* (regulates cell survival and proliferation).

In a review on zygotic gene activation in mice, Minami (2007) described ten “maternal effect genes” that may play a role in the transition from maternal to zygotic transcription in the mouse including *Mater*, *Hsf1*, *Dnmt1o*, *Pms2*, *Zar1*, *Npm2*, *Stella*, *Zfp3612*, *Basonuclin*, and *Brg1*. These genes are suggested to be required for embryo transcription as null mutation embryos do not develop past the 1 to 2 cell stage in mice (when the embryonic genome is transcriptional activated; reviewed by Minami, 2007). For example, *Mater* is exclusively expressed in oocytes (Tong and Nelson, 1999) and female *mater* knock out mice are sterile while male knock out models are fertile (Tong et al., 2000). *Mater* null female mice had follicles capable of ovulation and seemingly

normal luteinization but embryos did not progress beyond the 2 cell stage (Tong et al., 2000).

The oocyte produces the zona pellucida glycoproteins (ZPs) that make up the zona pellucida matrix. In mice, the zona pellucida matrix is composed of ZP-1, -2, and -3. An interesting experiment compared folliculogenesis, oogenesis, fertilization, and embryo development among wildtype, ZP1, 2, and 3 mouse knock out models (Rankin et al., 2001). The ZP1 knock out mice still developed a zona pellucida but the ZP2 and ZP3 knock out mice had abnormal or no zona pellucida formation, respectively. The ZP1 knock outs had reduced litter size but were still able to produce pups while the ZP2 and ZP3 null mice were sterile. The ZP2 null mice had reduced numbers of antral follicles, number of ovulated oocytes in the oviduct, and no 2 cell embryos formed. Oocytes from mice lacking a zona pellucida (ZP2 and ZP3 null) can be matured and fertilized in vitro. Although the embryos developed to blastocysts stage, the pregnancies did not make it to term after transfer into wild type recipients (Rankin et al., 2001; reviewed by Zhao and Dean, 2002). The authors conclude that not only is the zona pellucida important to folliculogenesis and fertilization but also the ZP facilitated communication between granulosa cells and the oocyte is necessary for oocyte competence (Rankin et al., 2001).

Protein translation or degradation may be controlled by the hormonal milieu in the follicle. An aberrant protein pattern in oocytes was reported when sheep were treated with exogenous gonadotropin stimulus compared to oocytes from ewes after a natural cycle (Moor et al., 1985; reviewed in Moor et al., 1998). Many transcripts in the oocyte are masked to prevent translation until a later time. Shortening of the poly(A) tail allows mRNA transcripts to be stored rather than translated. Masked transcripts have a

conserved sequence with many uracil residues called the cytoplasmic polyadenylation signal (Fox et al., 1989). A translational repressor protein has also been described which may actively prevent translation of transcripts (Stutz et al., 1998; Richter, 1999).

Translation may also be prevented by combining mRNA with ribonucleoprotein particles (Matsumoto et al., 1998; Jansen, 1999). Micro-RNAs are an additional form of regulating mRNA translation. For an in-depth review of mRNA translational controls, refer to Kuersten and Goodwin (2003) and Farley and Ryder (2008).

***Epigenetic modification.*** The alteration of the genome to regulate expression of specific genes is referred to as epigenetics (above genetics). The DNA and histones can be modified through covalent methylation or acetylation to change gene transcription. Nutrition and environment can affect epigenetic changes and these alterations can be transgenerational (reviewed by Jirtle and Skinner, 2007).

Genomic imprinting refers to differential methylation of specific alleles in the paternal and maternal genomes of gametes for preferential transcription of alleles from either the dam or sire. For example the *H19* gene (with unknown function, although it may regulate fetal growth) is preferentially expressed by the maternal allele; whereas, the paternal allele is silent (Walsh et al., 1994). Conversely, the IGF-II transcript is preferentially expressed by the paternal allele (DeChiara et al., 1991). Many genes have been implicated as imprinted genes and a variety of physiological functions are associated with these genes such as embryonic and postnatal growth, placentation, behavior, and metabolism (reviewed by Tycko and Morison, 2002 and Miyoshi et al., 2006). Abberant epigenetic modification is likely the cause of fetal abnormalities and lower rate of pregnancy success from embryos associated with assisted reproductive

technologies such as in vitro fertilization or cloning (reviewed by Moore and Reik, 1996 and Farin et al., 2006). Therefore, the epigenetic modification of the oocyte is another factor that could affect the ability of the oocyte to result in live healthy offspring.

The follicular environment may affect the imprinting in the oocyte. Sirard and colleagues (2006) postulated that in vitro matured oocytes aspirated from small follicles had not completed proper epigenetic imprinting. The signals marking certain genes for imprinting are unknown but paracrine regulators from the follicle may be candidate regulators of deoxyribonucleic acid (**DNA**) methylation patterns (Murray et al., 2008). Oocytes from women and mice that were superstimulated had altered DNA imprinting (Sato et al., 2007). Manipulation of the steroidal and gonadotropin concentrations in cultured mouse follicles resulted in altered oocyte competence and altered global DNA methylation (Murray et al., 2008).

The epigenetic modification of the genome progresses as the oocyte matures. Nuclear transfer of nongrowing immature oocytes (diplotene stage of first meiosis; 15 to 20  $\mu\text{m}$ ) into an ooplasm from a fully matured germinal vesicle stage de-nucleated oocyte resulted in normal fertilization and blastocyst development but reduced number of pups born suggesting that in mice, oocytes continue to undergo nuclear or molecular modification that is important for embryo survival (Kono et al., 1996). A follow up experiment from the same laboratory looked at developmental competence of embryos formed following nuclear transfer of progressively more mature oocytes from juvenile and adult mice (Bao et al., 2000). The authors reported a progressive increase in the number of live pups born when the nuclear transfers came from progressively more advanced oocytes (Bao et al., 2000).

The epigenetic marking of genes is reversible and transferrable. During gametogenesis the parental imprints are erased and new imprints are propagated into the somatic cells of the embryo. The erasing of the imprints appears similar in both gametes but reestablishment of the imprints is sex specific (reviewed by Miyoshi et al., 2006 and Feil, 2009). Imprinted genes have imprinted control regions that become methylated. These regions can be up to several kilobases long and contain CpG islands (Hutter et al., 2006; Kobayashi et al., 2006). Histones can also be modified resulting in epigenetic imprinting of the genome (Grewel and Moazed, 2003) and other molecules have been associated with gene silencing like polycomb group protein Eed (Mager et al., 2003), Lsh (Fan et al., 2005) and possibly microRNAs (O'Neill, 2005).

A major enzyme involved in methylation is DNA methyltransferase (**DNMT**) which is required for normal development and X inactivation (Li et al., 1993). In fact, *DNMT* is one of the maternal effect genes described in mice (Minami, 2007) and transcript for *DNMT* was expressed in mature oocytes and early embryos (Howlett and Reick, 1991). Mouse oocytes and early embryos express a variant of DNMT called DNMT1 $\alpha$ . Mice with homozygous mutations in *DNMT1 $\alpha$*  were phenotypically normal except the females were infertile (Howell et al., 2001). The heterozygous offspring born from the homozygous knock out mice died during the final trimester (after d 14 of pregnancy; Howell et al., 2001). The pregnancy failure was likely due to oocyte incompetence as wild type embryos transferred to the DNMT1 $\alpha$  null mice had normal pregnancy rates (Howell et al., 2001). The heterozygote embryos had partial to complete biallelic expression of normal imprinting genes (*H19 snprn*, and *igfr2*) suggesting that the

DNMT1o was required for appropriate oocyte gene imprinting and fetal survival (Howell et al., 2001).

Epigenetic modification of the oocyte genome is likely affected by the follicular environment. Inadequate epigenetic imprinting of the oocyte could lead to complications in embryo development and altered fetal/offspring development. Imprinted genes regulated many vital processes in embryo/fetal development including growth and X inactivation; therefore inadequate oocyte maturation could result in deleterious effects on pregnancy establishment and maintenance.

### **CORPUS LUTEUM FUNCTION**

Soon after the LH surge, the follicular cells differentiate into luteal cells in a process called luteinization (reviewed in Stocco et al., 2007). Progesterone synthesized by the corpus luteum is required for establishment and maintenance of pregnancy in cattle and inadequate luteal function may be a major cause of pregnancy loss (Rhinehart et al., 2008; reviewed by Inskeep, 2004 and Spencer et al., 2004). Therefore, proper luteinization of the ovulatory follicle is required for establishment of pregnancy.

Small dominant follicles induced to ovulate formed CL that produced lower serum concentrations of progesterone in cows (dairy: Vasconcelos et al., 2001 and beef: Perry et al., 2005; Busch et al., 2008) and beef heifers (Atkins et al., 2008a). A similar reduction in luteal progesterone production occurred when cows ovulated similar size follicles after a short (1.2 to 1.5 d) compared to long (2.2 to 2.5 d) proestrus (Bridges et al., 2006). These studies suggest that ovulation of a dominant follicle after inadequate follicular maturation could result in a less functional CL. At present, the precise link

between dominant follicle maturity and the subsequent CL function is unknown. The following section will summarize possible follicular determinants of CL function.

McNatty (1979) suggested that the development of a healthy and productive CL is dependent on the proper development of the follicle. This development would include having a follicle with 1) sufficient numbers of granulosa cells, 2) granulosa cells capable of progesterone secretion following luteinization 3) and sufficient LH receptors in the theca and granulosa cells for luteal stimulation (McNatty, 1979). A link between follicular cells and luteal function has been reported in humans. The ability of human granulosa cells to proliferate and secrete progesterone in culture was affected by the follicular environment prior to culture (McNatty and Sawers, 1975). Progesterone production from human luteinized granulosa cells varied depending on the gonadotropin stimulus of the follicle before granulosa cell collection (Lobb et al., 1998). Furthermore, progesterone production was highest in cultured luteinized granulosa cells when collected from follicles of spontaneously ovulating women compared to women treated with FSH ± LH (Lobb and Younglai, 2001).

Corpora lutea in ewes induced to ovulate 12 h after luteal regression (short proestrus) were smaller in diameter and weight, had fewer large luteal cells, and produced less progesterone compared to ewes induced to ovulate 36 h after luteal regression (long proestrus; Murdoch and van Kirk, 1998) suggesting that ovulation of a follicle after a longer maturation period (proestrus) resulted in a more functional CL. Ewes with the shorter proestrus had lower serum concentrations of estradiol at the time of ovulation compared to the ewes with a longer proestrus which could be due to a decreased number of granulosa cells or lower production of estradiol by the granulosa

cells (Murdoch and van Kirk, 1998). Follicular secretions of estradiol may affect the number of future large luteal cells and their ability to respond to LH. The large luteal cells are thought to originate from the granulosa cells (O'Shea, 1987), although there is some dispute over luteal cell lineage (Fritz and Fitz, 1991), and the granulosa cells do not increase in number after the LH surge (McClellan et al., 1975). As mentioned in a previous section, estrogen promotes granulosa cell division and also increases LHR expression. Given this line of evidence, it is possible that reduced concentrations of pre-ovulatory estradiol may lead to reduced numbers of granulosa cells and thus reduced numbers of large luteal cells (which produce the majority of the progesterone secreted by the CL).

It is logical that fewer granulosa cells could result in a reduced population of large luteal cells and impaired luteal progesterone production following luteinization; However, cows that ovulated small follicles spontaneously (not induced with GnRH) had increased serum concentrations of progesterone compared to cows that were induced to ovulate similarly sized follicles (Perry et al., 2005). Cows that ovulated after a short proestrus also had reduced subsequent rise in concentrations of progesterone compared to cows that ovulated a similar size follicle following a long proestrus (Bridges et al., 2006). Assuming that similar size follicles have similar numbers of granulosa cells, the previous studies would suggest that reduced luteal production of progesterone is not simply due to reduced granulosa cell numbers and subsequently reduced luteal cell numbers.

Follicular secretions of estradiol may play an important role in subsequent luteal secretion of progesterone as well as timing of prostaglandin  $F_{2\alpha}$  (**PGF<sub>2 $\alpha$</sub>** ) release by the uterus in ruminants. Ewes given an aromatase inhibitor prior to hCG-induced ovulation

had a delayed rise in circulating concentrations of progesterone (Benoit et al., 1992). Pre-ovulatory estradiol may control the timing of prostaglandin release through three mechanisms: 1) estradiol-induced progesterone receptors in the uterus (Stone et al., 1978; Zelinski et al., 1982; Ing and Tornesi, 1997); progesterone is thought to control the timing of PGF<sub>2α</sub> release (reviewed by Silvia et al., 1991), 2) estradiol (from a follicular wave) -induced oxytocin receptors in the uterus; oxytocin bound to its receptor is thought to increase cyclooxygenase activity and PGF<sub>2α</sub> synthesis (McCracken hypothesis, reviewed by Silvia et al., 1991), and 3) estradiol affected the ability of uterine cells to synthesize PGF<sub>2α</sub> (reviewed in Silvia et al., 1991). The hypothesis of estradiol regulation of PGF<sub>2α</sub> secretion is supported by Mann and Lamming (2000) who reported an earlier (d 6) rise in PGF<sub>2α</sub> metabolite in cows with low pre-ovulatory estradiol compared to high concentrations of estradiol.

Dramatic changes in the blood supply occur during the luteinization of the follicle and CL development. Angiogenesis in the CL has been the topic of many research studies not only to understand luteal function but understanding the physiology of angiogenesis has given insight to tumor blood supply development. Many authors have reviewed the importance and regulation of angiogenesis in the CL (reviewed by Smith et al., 1994, Davis et al., 2003, Fraser and Wulff, 2003, Tamanini and De Ambrogi, 2004, and Berisha and Shams, 2005) but less is known about how the maturity of the dominant follicle may affect the CL vasculature.

Davis et al. (2003) and Tamanini and De Ambrogi (2004) speculated that the follicular cells may influence the angiogenesis of new vasculature in the subsequent CL. The theca and granulosa cells are thought to secrete angiogenic factors important to

developing the microvasculature in the thecal layer (Redmer et al., 1985). For instance, renin which cleaves the pre-cursor angiotensinogen to angiotensin I, was found in both the theca and granulosa of bovine pre-ovulatory cells (Shauser et al., 2001). Angiotensin I is cleaved to the active form (angiotensin II) by the enzyme, angiotensin I-converting enzyme, and angiotensin II acts as a vasoconstrictor. Angiotensin II receptors have been localized to the theca externa of antral and pre-ovulatory follicles (Shauser et al., 2001) and the amount of Angiotensin II receptors was positively correlated with the diameter of the follicle (Nielsen et al., 1994). These results suggest that the renin-angiotensin system contributes to angiogenesis of the maturing follicle which could affect the vascular foundation for luteal cells following luteinization.

## **OVIDUCTAL ENVIRONMENT**

The oviduct is a dynamic organ and is important to many steps in fertilization and early cleavage of the embryo including gamete transport (both spermatozoa and ova), creating a sperm reservoir, final maturation of the gametes, fertilization, and early development of the embryo (for reviews see Hunter, 1988; Buhi et al., 1997; Buhi et al., 2000; Killian, 2004; specifically in the pig: Brussow et al., 2008; specifically in the cow: Ellington, 1991). The oviduct consists of three anatomically distinct regions: the infundibulum, ampulla, and isthmus with fertilization occurring at the ampullary-isthmic junction in most mammals. The significance of the oviductal environment to embryo development is emphasized by the many reports of improved in vitro embryo development when embryos are cultured with oviductal secretions, epithelial cells, sections, or whole oviducts (reviewed in Buhi et al., 1997).

In many species a sperm reservoir is created in the numerous crypts created by the folds in the lining of the epithelium of the isthmus (reviewed by Suarez, 1998). The sperm undergo final maturation (including capacitation, hyperactivation, and the initiation of acrosomal exocytosis) in the isthmus. The reservoir may also function to prevent polyspermy (reviewed by Suarez, 1998). Upon arriving at the reservoir, sperm take approximately 4 to 5 h to undergo capacitation in the bovine oviduct (Hunter, 1988). The newly released ovum is transported from the infundibulum to the ampullary-isthmic junction within minutes after ovulation in many mammals; however these sperm are generally not viable (Hunter, 1988). After fertilization the bovine embryo remains in the oviduct for 72 to 84 h until it enters the uterus (Black and Davis, 1962; El-Banna and Hafez, 1970; reviewed for many species in Hunter, 1988). The early embryo remains close to the ampullary-isthmic junction for the majority of this time and then rapidly moves to the uterus (about the 8 to 16 cell stage embryo; Hunter, 1988).

The oviduct undergoes dynamic changes in morphology, ciliary movement, gene expression, and oviductal fluid secretions during the estrous cycle. Many of these changes are hypothesized to be controlled by ovarian steroids and possibly the presence of gametes and(or) embryos. Bauersachs et al. (2004) reported upregulation of 37 genes which function in regulation of protein secretion, protein modification, and mRNAs for secreted proteins in the oviduct around estrus. Forty genes were up-regulated in the oviducts of diestrous cows that mainly played a role in transcription regulation (Bauersachs et al., 2004). Differential gene expression (35 different genes) was also reported in the ipsilateral vs. contralateral oviducts in cows 3.5 d after estrus (Bauersachs et al., 2003). The ipsilateral oviduct had 27 up-regulated genes compared to the

contralateral oviduct with known functions in cell to cell interaction, signal transduction, and immune function (Bauersachs et al., 2003). Estrogen and progesterone receptor expression in the oviduct also varied throughout the cycle (Ulbrich et al., 2003) where expression of receptors for progesterone and ER $\alpha$  was elevated during the follicular phase and the protein abundance of these receptors peaked in the early luteal phase. Estrogen receptor  $\beta$  mRNA and protein expression was highest during the luteal phase. These expression patterns were replicated in vitro with hormone administration (Ulbrich et al., 2003).

The above evidence suggests the oviduct changes dynamically with the hormonal and local changes during the estrous cycle. The following section reviews the literature pertaining to cyclic changes in the bovine (unless otherwise specified) oviduct and its regulation by ovarian steroids and possibly by follicle maturation. The section summarizes changes in the oviductal morphology, secretions, and gene expression that may be important to gamete transportation, maturation, fertilization, and early embryogenesis.

Yaniz et al. (2000) studied anatomical changes in the bovine oviduct at 4 different time points during the estrous cycle (estrus, metestrus [d 2 to 4 after estrus], diestrus [d 5 to 15 after estrus], and proestrus [d 16 to 21 after estrus]). The authors reported that the number of ciliated cells in the infundibulum was cyclical with peak numbers occurring during estrus. The cranial portion of the ampulla displayed similar cyclic changes in the number of ciliated cells but the caudal portion of the ampulla and the apical areas of the isthmus had less evident morphological changes throughout the cycle (Yaniz et al., 2000). The pocketed “crypts” in the isthmus also had cyclic changes with more ciliated

cells during the estrus and proestrus phases and the secretory cells seemed more prevalent during estrus and diestrus. Bauersachs et al. (2004) suggested that the cyclic changes in the number of ciliated and secretory cells occurred because individual epithelial cells changed their functional status rather than replacing a secretory cell with a ciliated cell, for example. The changes in the population of ciliated and secretory cells could translate into changes in gamete transport and oviductal fluid volume and composition throughout the estrous cycle and at specific locations in the oviduct.

Gamete and embryo transport through the oviducts is due in part to ciliary movements in the epithelium and also smooth muscle contractions of the oviduct. It is interesting that prior to fertilization the ciliary and contractile movements are in opposite direction within the ampulla and the isthmus (i.e., from the infundibulum towards the ampullary-isthmic junction and from the isthmus towards the ampullary-isthmic junction). Oviduct contractions were low in frequency and amplitude during the luteal phase and peaked at estrus followed by a rapid decrease in contractions (Bennett et al., 1988). Contractions of the oviduct of rabbits were reported to be under the control of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , where  $\text{PGF}_{2\alpha}$  stimulated contractions and  $\text{PGE}_2$  quieted muscle contractions (Saksena and Harper, 1975). Estradiol increased  $\text{PGF}_{2\alpha}$ -induced contractions while progesterone blocked  $\text{PGF}_{2\alpha}$ -induced contractions in rabbits (Saksena and Harper, 1975). Embryos from superovulated cows entered the uterus earlier than embryos from single ovulations possibly facilitated by the earlier rise in progesterone associated with the formation of multiple CL (El-Banna and Hafez, 1970). In cattle, LH stimulated PGF release from oviductal tissue and increased contractions of oviducts in vitro, collected from cows in the ovulatory or early luteal phase of the cycle.

Alternatively, oxytocin completely blocked LH stimulated contractions (Wijayagunawardane et al., 2001). Interestingly, oviducts collected from cows during the luteal phase of the cycle were not stimulated to secrete PGF or contract when incubated with LH (Wijayagunawardane et al., 2001).

Sperm underwent hyperactivation when incubated with oviductal explants (Pollard et al., 1991) and oviductal fluid (during non-luteal phase; McNutt et al., 1994). Sperm cells incubated with oviductal fluid collected from cows with low serum concentrations of progesterone (non-luteal phase) underwent capacitation earlier, maintained motility longer, and bound better to oocytes compared to sperm cells incubated in oviductal fluid from cows with high progesterone (luteal phase; McNutt and Killian, 1991). Differences in the final maturation and the fertilization rate of spermatozoa were not only affected by the stage of the cycle at oviductal fluid collection but also the region of the oviduct (Grippio et al., 1995; Way et al., 1997; Topper et al., 1999; reviewed by Killian, 2004). Killian (2004) concluded that the isthmus functions to maintain the spermatozoa in a relatively quiet state and to induce capacitation of the sperm cells but that the ampulla may provide the appropriate environment for fertilization as the fertilization rate was improved in sperm incubated in ampullary fluid.

Way et al., (1997) studied the sequential importance of exposure of sperm and ovum to isthmic and ampullary oviductal environments. The best fertilization and cleavage rates came from incubating sperm in isthmic fluid and the ovum in ampullary fluid prior to co-incubation. These experiments suggest that the oviductal milieu is prime for gamete maturation and fertilization under the influence of low progesterone (and

possibly high estradiol) and that the environment in varying regions of the oviduct is physiologically different and differentially affects the competence of sperm and ova.

Oviductal fluid is a combination of transudate from the serum as well as factors synthesized by the oviductal epithelial cells (predominantly the secretory cells). The amount of fluid increases around estrus (Hunter, 1988; Killian et al., 1989) and the fluid components also vary throughout the estrous cycle with peak protein, cholesterol, and phospholipids being produced in oviductal fluid from cows with low progesterone (non-luteal; Killian et al., 1989). Cows with persistent follicles had an altered protein profile in oviductal fluid compared to cows with a healthy dominant follicle (Binelli et al., 1999) suggesting that the follicular secretions were able to alter oviductal environment.

Oviductal fluid contains many growth factors and cytokines (reviewed in Buhi et al., 1997; 2000) which have been reported to improve embryo development in culture (Gandolfi, 1995; Watson et al., 1994). The most abundant protein in the oviductal fluid around the time of ovulation is estrogen dependent glycoprotein (reviewed by Buhi, 2002). The exact function of the estrogen dependent glycoprotein is unknown but it can bind to the zona pellucida of the ovum and embryo (Brown and Cheng, 1986; Hedrick et al., 1987; Boice et al., 1992; Weger and Killian, 1991). Estrogen dependent glycoprotein secretion has been detected in both the isthmus and ampulla (Boice et al., 1990) of the cow oviduct and is positively correlated with concentrations of estradiol. Oocytes preincubated with estrogen dependent glycoprotein had improved fertilization rates compared to oocytes not exposed to estrogen dependent glycoprotein (Martus et al., 1998). Furthermore, the improvement in fertilization was negated when anti-estrogen dependent glycoprotein antibody was added (Martus et al., 1998; Kouba et al., 2000).

Estrogen dependent glycoprotein bound to sperm and may increase capacitation (King and Killian 1994) and increased mobility of sperm (Abe et al., 1995). However, Martus et al. (1998) did not report an improvement in fertilization rate in sperm pretreated with estrogen dependent glycoprotein. While evidence exists for the possible improvement of fertilization rate due to estrogen dependent glycoprotein, the requirement for this molecule is unlikely as mice lacking the estrogen dependent glycoprotein are still fertile (Araki et al., 2003).

The extra cellular matrix and cell to cell communication in the bovine oviduct also changes during the cycle. Urokinase-type plasminogen activator (uPA) mRNA expression was elevated leading up to ovulation followed by a threefold decrease subsequent to ovulation (Gabler et al., 2001). The highest net activity for uPA occurred during the early to mid-luteal phase (Gabler et al., 2001). Oviductal expression of TIMP-1 and MMP-2 mRNA was highest around ovulation and MMP-1 had peak mRNA expression during the luteal phase (Gabler et al., 2001). Some have reported an improvement in embryo development with TIMP-1 in culture media (Sato et al., 1994). Gabler et al. (2001) proposed the above proteins in extra-cellular matrix remodeling may aid in the protection of gametes and(or) embryos in the oviduct possibly through the release of growth factors from the matrix.

Differential expression of osteopontin and integrins has been reported during the estrous cycle (Gabler et al., 2003). Osteopontin is made by the oviductal epithelium and is present in the fluid secretions (Gabler et al., 2003). Sperm binding and fertilization rates increased when ova were incubated with osteopontin compared to controls (Goncalves et al., 2001; Way et al., 2002; Killian and Goncalves, 2002). However

osteopontin null mice remain fertile (Rittling et al., 1998); therefore, the requirement for osteopontin is unknown. Additional factors in the oviductal secretions have been reported to vary in a cyclic pattern including retinol binding protein (Eberhardt et al., 1999), antioxidant genes (Lapointe and Bilodeau, 2003; Lapointe et al., 2005), and nitric oxide synthases (Lapointe et al., 2006; Ulbrich et al., 2006).

### **UTERINE ENVIRONMENT**

The bovine embryo enters the uterus 3.5 to 5 d after estrus at the 8 to 16 cell stage (Black and Davis, 1962; El-Banna and Hafez, 1970; Guillomot, 1995). Tight junctions form between the blastomeres around d 5 to 6. The embryo develops into a blastocyst by d 7 to 8 after estrus and hatches from the zona pellucida around d 9 to 10 after estrus. The embryo changes from a spherical shaped blastocyst to an elongated (filamentous) embryo by d 13 to 16 (Grealy et al., 1996; Morris et al., 2000). During this time, the embryo secretes IFN $\tau$  which is required for maternal recognition of pregnancy (Roberts et al., 1999). Around d 19, the embryo begins to attach to the uterus in the cow with early placentomes detected by d 21 and complete placental attachment by d 42 (Hunter, 1980).

Wiebold (1988) reported that the uterine environment differed between dairy cows with excellent to good embryos vs. fair to poor embryos 7 d after breeding and that increased concentrations of glucose, total protein, calcium, magnesium, and potassium and decreased concentrations of zinc and phosphorous in luminal fluid were associated with poorer quality embryos. The uterine environment is affected by preovulatory concentrations of estradiol and postovulatory (luteal) concentrations of progesterone. Progesterone supplementation alone following ovariectomy at breeding can maintain

pregnancy in cattle (MGA: Zimbelman and Smith, 1966) and ewes (Foote et al., 1957). Interestingly, if ovariectomy occurs prior to breeding then a period of progesterone priming followed by estradiol supplementation before progesterone supplementation (mimicking luteal progesterone) is required to maintain a pregnancy following embryo transfer in ewes (reviewed in Moore, 1985). Ovariectomized ewes with no period of progesterone priming but exogenous estradiol supplementation followed by progesterone supplementation sufficient to maintain a pregnancy were unable to maintain an embryo following embryo transfer (Moore, 1985) but progesterone priming for 1, 4, or 8 d resulted in 6/20, 14/21, and 15/22 ewes with normal embryos 17 to 18 d after transfer (Moore, 1985). Similar studies in ovariectomized ewes that did not receive the estradiol bolus to simulate pre-ovulatory concentrations of estradiol found a decrease in total protein in the lumen of the uterus at embryo transfer, progesterone and estrogen receptors in the endometrium, and uterine weight (Miller et al., 1977). These elegant studies using an ovariectomized ewe model suggest the importance of ovarian steroids on the uterine environment in the establishment and maintenance of pregnancy (reviewed by Moore, 1985).

When dominant follicles were induced to ovulate prematurely or the proestrus period was shortened, the pre-ovulatory concentrations of estradiol and post-ovulatory concentrations of progesterone were reduced which may compromise the uterine environment. There was a positive correlation between pre-ovulatory concentrations of estradiol and the size of the dominant follicle induced to ovulate in beef heifers (Perry et al., 2007; Atkins et al., 2008b). Extending the proestrous period from 1.5 to 2.5 d and thus increasing the duration of elevated preovulatory estradiol concentrations resulted in

increased luteal progesterone production after luteinization and increased pregnancy rates regardless of the size of the follicle that ovulated (Bridges et al., 2006). Lengthening the proestrous period in ewes resulted in increased glandular development in the endometrium 12 d after ovulation compared to ewes induced to ovulate after a short proestrus (Murdoch and van Kirk, 1998). Cows exposed to a short proestrous period had increased oxytocin receptor and cyclooxygenase-2 mRNA in the uterus 5 d after GnRH-induced ovulation compared to cows ovulating after a long proestrus (Bridges et al., 2006), which could affect timing of uterine  $\text{PGF}_{2\alpha}$  release and CL maintenance. Collectively, these studies suggest the importance for preovulatory concentrations of estradiol for preparing the uterine environment for the establishment and maintenance of pregnancy.

Multiple studies suggest that concentrations of progesterone are compromised subsequent to the induced ovulation of a small dominant follicle (Vasconcelos et al., 2001; Perry et al., 2005; Busch et al., 2008; Atkins et al., 2008a). Taken together, the premature ovulation of a dominant follicle may affect the uterine environment largely due to the reduced preovulatory circulating concentrations of estradiol and postovulatory circulating concentrations of progesterone. The following section summarizes the affects of pre-ovulatory concentrations of estradiol and post-ovulatory concentrations of progesterone on the uterine environment.

*Pre-ovulatory concentrations of estradiol.* As described previously, a rise in preovulatory concentration of estradiol was necessary for establishment of pregnancy in ewes (Moore, 1985). Administration of estrogens around the time of estrus and ovulation has yielded mixed results. Some reported that cows administered estradiol cypionate

administration at estrus or ovulation had increased pregnancy rates compared to controls (Ahmadzadeh et al., 2003; Cerri et al., 2003) while others reported no benefits of estrogen administration (Hillegass et al., 2008). Preovulatory concentrations of estradiol may aid in sperm transport through the uterus, affect timing of PGF<sub>2α</sub> release (see the CL section), and indirectly alter the activity of progesterone by inducing progesterone receptors in the endometrium. These functions of estradiol will be reviewed below.

Sperm transport through the uterus is affected by estradiol and is optimized at estrus or when ovariectomized animals were administered estrogen (reviewed in Hawk, 1983). Ovariectomized ewes required estradiol for appropriate sperm transport (Allison and Robinson, 1972). Similarly, ovariectomized rabbits required estrogen for proper sperm transport but large quantities of estrogen were detrimental to sperm transport (Noyes et al., 1959). One potential explanation for how estradiol affects sperm transport is by altering the uterine pH around estrus. Uterine pH decreases at estrus (Elrod and Butler, 1993) and cows that displayed estrus had a lower pH compared to cows that were induced to ovulate (Perry and Perry, 2008a, 2008b) suggesting that concentrations of estradiol may affect the pH in the uterus which in turn could affect the sperm longevity and transport. Additionally, expression of SERPINA14, a member of the uterin serpins/uterine milk protein family, was elevated in the bovine endometrium around estrus (Bauersachs et al., 2005; Mitko et al., 2008). While the exact role of SERPINA14 at estrus is unknown, it was speculated that SERPINA14 may affect sperm transport or competence. The SERPINA14 protein was detected in luminal fluid around estrus (Ulbrich et al., 2009). Ulbrich and colleagues (2009) concluded that the estradiol induced

secretion of SERPINA14 may play a functional role in sperm transport through the uterus.

Administration of estradiol to ovariectomized ewes altered uterine protein secretion, weight, and progesterone and estrogen receptors independent of either progesterone priming prior to estradiol or progesterone administration after estradiol (Miller et al., 1977). This suggests that the rise in serum concentrations of estradiol before ovulation affects the uterine environment days later. An interesting gene called NUDT16 was described in the pregnant ewe. The NUDT16 gene is upregulated in the epithelial tissue from d 5 to 9 of pregnancy and expression decreased by d 13 (Ing et al., 2006). This expression pattern was obtained by steroid replacement of ovariectomized ewes (similar to those described by Moore, 1985) and required estrogen replacement prior to progesterone replacement for similar NUDT16 expression (Ing et al., 2006). While the functional role of this gene product in pregnancy is unknown, this experiment suggests that pre-ovulatory concentrations of estradiol can affect the uterine environment days later.

The altered uterine environment might be indirectly related to pre-ovulatory estradiol through altered progesterone activity. Follicular concentrations of estradiol have been associated with enhanced CL progesterone synthesis following gonadotropin stimulation (McNatty, 1979; see section on CL function) and with induction of endometrial progesterone receptors (Stone et al., 1978; Zelinski et al., 1982; Ing and Tornesi, 1997). Decreased ovulatory concentrations of estradiol may result in fewer progesterone receptors coupled with decreased progesterone production by the CL which

could result in a uterine environment unable to support pregnancy (see the following section on the role of progesterone in the uterine environment).

*Post-ovulatory concentrations of progesterone.* Progesterone is absolutely required to maintain pregnancy in most species. Progesterone stimulates the production of many growth factors and uterine secretions (Geisert et al., 1992; Spencer and Bazer, 2002) needed to nourish the early conceptus. A delayed rise in progesterone is implicated in asynchrony between the embryo signal for maternal recognition of pregnancy and luteal regression. Interferon- $\tau$  (INF $\tau$ ) is released by the trophoblast of the bovine and ovine embryo which reduces uterine PGF release by blocking expression of oxytocin receptors (reviewed by Spencer et al., 2007). Embryos (d 16 after breeding) collected from cows with a delayed rise in progesterone (6 d following estrus) had reduced INF $\tau$  production and were less developed compared to embryos recovered from cows with an advanced increase in concentrations of progesterone (Mann and Lamming, 2001; Kerbler et al. 1997). Whether progesterone has direct effects on the embryos is under debate. Recently, Clemente et al. (2009) reported progesterone receptor mRNA in the bovine embryo. Culturing IVP embryos with progesterone had little effect (Fukui and Ono, 1989) on embryo development while others have reported improved embryo development after progesterone supplementation in culture (inconsistencies reviewed by Lonergan, 2009).

Progesterone supplementation for 4 d (beginning 36 h after ovulation) increased protein secretion by the uterus 5 and 14 d after ovulation (Garret et al., 1988).

Progesterone stimulates histotrophe secretion by the uterine glands (Geisert et al., 1992; Spencer et al., 2004) and uterine gland knock out ewe models are unable to maintain

pregnancy past d 12 to 14 (Gray et al., 2002). Recent studies have examined gene expression differences between cyclic and pregnant sheep and cattle as well as progesterone treated vs. control or progesterone antagonist treatment to better understand the actions of progesterone in early pregnancy (reviewed in part by Spencer et al., 2008). While the systems and data remain a complex puzzle, steps toward understanding the actions of progesterone are being made by comparing differential gene expression in the endometrium during the cycle with progesterone supplementation, and between pregnant and nonpregnant animals (Spencer et al., 2008; Satterfield et al., 2009; Mitko et al., 2008; Bauersachs et al., 2005, 2006; Forde et al., 2009).

Forde and colleagues (2009) examined differential gene expression in the uterus of early pregnant heifers with supplemental progesterone or normal concentrations of progesterone on d 5, 7, 13, and 16 of pregnancy (d 0 = estrus). These authors reported temporal and treatment associated changes in gene expression and progesterone supplementation advanced the timing of gene expression profiles in the uterus (Forde et al., 2009). Some of the genes that were upregulated by progesterone were associated with triglyceride synthesis and glucose transport (Forde et al., 2009) which may be important sources of energy for the embryo (Cases et al., 2001; Ferguson and Leese, 2006). A number of IFN $\tau$  stimulated genes were also upregulated by d 13 and continued to d 16 (Forde et al., 2009). Mitko and colleagues (2008) reported upregulation of genes associated with tissue remodeling and cell growth around estrus in cattle while immune related genes and specific metabolic pathways were upregulated around diestrus.

Progesterone receptors in the luminal and glandular epithelium are down regulated after prolonged progesterone exposure yet the stromal cells continue to express

progesterone receptors (Spencer and Bazer, 1995). Therefore, the action of progesterone on the uterine environment must act through modifying stromal cells which secrete biologically active factors that act on the uterine epithelium. A recent study in ewes, examined the role of progesterone regulation of a number of growth factors and their receptors in the endometrium of ewes in an attempt to understand potential factors secreted by the stroma that may affect embryo development (Satterfield et al., 2008). The authors suggested a model for progesterone stimulation of growth factors resulting in stromal-induced changes in epithelial cell gene expression and secretion. The model suggests that progesterone binds to the stroma, stimulates stromal secretion of FGF10 which binds its receptor (FGFR2) on the uterine epithelium. Progesterone may also increase MET (HGF receptor) expression on the epithelial cells which bind stromal derived HGF. Epithelial cells also contain IGF1-R; therefore, IGF-I and IGF-II from the stroma may bind to the epithelial IGF-R. Multiple growth factors may act to stimulate gene expression and uterine secretions that stimulate embryonic development. The authors concluded that progesterone acts to initiate a cascade of events through several growth factors that lead to conceptus growth, differentiation, and uterine receptivity (Satterfield et al., 2008).

A few studies have examined specific factors regulated by progesterone in the endometrium. Progesterone stimulated the expression of retinol and folate binding protein mRNA (McNeill et al., 2006a); however, no changes were detected at the protein level in the uterus of cattle for retinol binding protein at d 3, 7, and 11 of the estrous cycle (Costello et al., 2006). Others reported a decrease in retinol binding protein in the gravid uterus (Ledgard et al., 2009). Ledgard and colleagues (2009) reported differential protein

content of uterine fluid between pregnant and non-pregnant cows. Nine proteins had increased concentrations in the pregnant uterine fluid of which five are involved in biosynthetic pathways (carbonic anhydrase, isocitrate dehydrogenase, nucleoside diphosphate kinase, purin nucleoside phosphorylase, and triosephosphate isomerase), two are antioxidant enzymes (peroxiredoxin 1 and thioredoxin), and one cell adhesion protein (ezrin), and heat shock protein 70. Four proteins had decreased amounts in the gravid compared to the non-gravid uterine horn (cystatin, legumain, retinol-binding protein, and TIMP-2).

Many reports of progesterone supplementation suggest an improvement in embryo development with an early rise in progesterone (for example Garret et al., 1988, Kerbler et al., 1997, Mann and Lamming, 2001, and Carter et al., 2008) while others report no consistent improvement in pregnancy following progesterone supplementation (for example, Funston et al., 2005, Hanlon et al., 2005, Galvao et al., 2006, Stevenson et al., 2007; 2008). Meier et al. (2009) used an interesting approach to study progesterone secretion patterns in dairy cattle. The authors identified subpopulations of cattle based on profile of circulating concentrations of progesterone during the estrous cycle. These subpopulations had distinct progesterone profiles based on the rate of the rise in progesterone, timing of peak progesterone, length of progesterone plateau, and rate of decrease in progesterone. The authors suggested that these subpopulations may result in varying fertility due to progesterone actions among otherwise normal cycling dairy cattle (Meier et al., 2009).

Mann and Lamming (1999) conducted a meta-analysis of 17 reports of progesterone supplementation and concluded that progesterone supplementation resulted

in a 5% improvement in pregnancy rates. Furthermore, the authors suggested that progesterone supplementation may result in conflicting results as day of treatment and relative fertility of cattle may alter the end results (Mann and Lamming, 1999). Progesterone supplementation improved pregnancy rates when cows had low concentrations of progesterone on d 5 (1 to 2 ng/ml; d 0 = AI) but did not improve pregnancy rates when cows had high progesterone on d 5 (Starbuck et al., 2001). While pregnant cows frequently have increased serum concentrations of progesterone following breeding compared to cows that failed to conceive (Perry et al. 2005, Lopes et al., 2007), there is a wide range in individual progesterone concentrations and progesterone cannot be used to predict pregnancy success (reviewed by Mann and Lamming, 1999). Stronge and colleagues (2005) reported a linear and quadratic relationship between rate of change of concentrations of progesterone (in milk) from d 5, 6, and 7 (d 0 = AI) and pregnancy rates. The optimum embryo survival occurred when concentrations of milk progesterone increased 4.7 ng/mL per day but there was a decrease in the probability of embryo survival if progesterone increased too quickly (Stronge et al., 2005). A similar relationship was reported by McNeill et al. (2006b) and Starbuck et al. (2001). Collectively, these studies suggest that while progesterone is certainly important, we do not know the minimum concentrations of progesterone needed to establish and maintain a pregnancy.

## **CONCLUSION**

In summary, the final maturation of the dominant follicle is intimately tied to the final maturation of the oocyte, proper preparation of the future luteal cells, and endocrine

control of the oviductal and uterine environment for gamete and embryo development. Premature ovulation of a dominant follicle led to reduced pregnancy rates although the mechanism for altered pregnancy establishment is unknown. Advancements in the study of the maturation of a dominant follicle in relation to pregnancy establishment will likely improve the efficiency of assisted reproductive technologies in a variety of species including cattle and humans.

## CHAPTER III

### FACTORS AFFECTING PRE-OVULATORY FOLLICLE DIAMETER AND OVULATION RATE TO GnRH IN POSTPARTUM BEEF COWS PART I: CYCLING COWS

**ABSTRACT.** Cows induced to ovulate small dominant follicles were reported to have reduced pregnancy rates compared to cows that ovulated large follicles. The reason for the presence of small dominant follicles at the time of GnRH-induced ovulation in timed AI protocols is unknown. Objectives of this experiment were to examine the role of day of the estrous cycle at initiation of treatment on ovulation following the first GnRH injection (GnRH1) and associated effects on growth rate and final size of the ovulatory follicle at the second GnRH injection (GnRH2), serum concentrations of estradiol at GnRH2, and subsequent luteal concentrations of progesterone in suckled beef cows. Estrous cycles of cows were manipulated to be at one of five specific days of the cycle (d 2, 5, 9, 13, and 18, d 0 = estrus; n = 12 per treatment group) at the start of the CO-Synch protocol (GnRH1 on d -9, PGF<sub>2α</sub> on d -2, and GnRH2 on d 0). Day of the estrous cycle at GnRH1 did not affect the size of the preovulatory follicle or the proportion of cows ovulating at GnRH2 ( $P = 0.65$  and  $0.21$ , respectively). When all cows were included in the analysis, cows that ovulated after GnRH1 had similar follicle size at GnRH2

compared to cows that did not ovulate after GnRH1 (11.4 and 10.4 mm, respectively;  $P = 0.23$ ). When only cows that could ovulate after GnRH1 (excluding Day 2 cows) were included in the analysis, cows that ovulated to GnRH1 had a larger follicle at GnRH2 than cows that did not ovulate after GnRH1 (11.4 and 9.5 mm, respectively;  $P = 0.04$ ). Follicle growth from d -5 to d 0 (d 0 = GnRH2) was similar between cows that ovulated after GnRH1 and cows that did not (1.01 vs. 0.89 mm/d, respectively;  $P = 0.75$ ). There was a tendency for faster follicle growth rate in cows that ovulated a large follicle ( $> 11$  mm) compared to cows that ovulated a small follicle ( $\leq 11$  mm; 1.01 vs. 0.86 mm/d, respectively;  $P = 0.07$ ). Serum concentrations of estradiol at GnRH2 and progesterone following ovulation were reduced in cows that ovulated small follicles compared to cows that ovulated large follicles ( $P = 0.006$  and  $< 0.05$ , respectively). In summary, day of the estrous cycle at initiation of synchronization did not affect ovulatory follicle size, but both synchronization of the follicular wave and follicle growth rates affected the size of the follicle at GnRH2. Cows that ovulated a small follicle had reduced serum concentrations of estradiol at GnRH2 and progesterone following ovulation.

## INTRODUCTION

Ovulation of a small and presumably physiologically immature dominant follicle reduced pregnancy rates (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005) and increased late embryonic/fetal loss (Perry et al., 2005) in beef and dairy cattle. Factors affecting ovulatory follicle size, or the mechanisms by which ovulatory follicle size affect fertility have not been determined. Administration of GnRH (**GnRH1**) 7 d before PGF<sub>2 $\alpha$</sub>  and a second GnRH injection (**GnRH2**) with fixed timed AI 48 h after

PGF<sub>2α</sub> has been used to breed beef cattle by appointment (CO-Synch; Geary et al., 1998). The GnRH1 injection is expected to ovulate a dominant follicle and initiate a new follicular wave so that a viable pre-ovulatory follicle is present at timed AI. It is logical that small dominant follicles present at the time of GnRH2 could result from failure to ovulate a dominant follicle and initiate a new follicular wave with GnRH1. Failure to initiate a new follicular wave with GnRH1 did not affect follicle diameter at GnRH2 or fertility in beef heifers (Atkins et al., 2008a). Alternatively, slower growth rate of the follicle could result in a small dominant follicle at GnRH2. In the present study, estrous cycles of beef cows were manipulated so that cows were on specific days of the estrous cycle at the start of the study. These days were selected based on prediction of the presence of a dominant follicle that either would or would not respond to GnRH1 and ovulate a 1<sup>st</sup>, 2<sup>nd</sup>, or 3<sup>rd</sup>, wave dominant follicle in response to GnRH2.

Our hypothesis was that day of the estrous cycle at GnRH1, and thus, ovulatory response to GnRH1 would affect growth rate, health, and diameter of the ovulatory follicle at the GnRH-induced ovulation for timed AI. Our objective was to determine how day of the estrous cycle at GnRH1 affected ovulation following GnRH1 and the associated effects on size and physiological maturity of the dominant follicle at GnRH2 in cycling beef cows.

## MATERIALS AND METHODS

***Animal handling.*** All protocols and procedures were approved by the Fort Keogh Livestock and Range Research Laboratory (LARRL) Animal Care and Use Committee (ACUC approval no. 020104-9). Postpartum suckled beef cows (n = 60) that

had resumed estrous cyclicity were assigned to one of five treatment groups based on age and days postpartum. The treatment groups (n = 12 per group) were defined as the day of the estrous cycle at the start of the CO-Synch protocol (d 2, 5, 9, 13, and 18 [d 0 = estrus] groups referred to as Day 2, 5, 9, 13, and 18, respectively). These days were selected based on prediction of the presence of a dominant follicle that either would or would not respond to GnRH1 and ovulate a 1<sup>st</sup>, 2<sup>nd</sup>, or 3<sup>rd</sup> wave dominant follicle in response to GnRH2 (Ginther et al., 2001). All cows were treated with the CO-Synch protocol except cows were not bred (GnRH [GnRH1] on d -9 followed by PGF<sub>2α</sub> [d -2] and GnRH [GnRH2] on d 0 *without* timed AI).

***Pre-synchronization.*** Cows were synchronized to be on their assigned day of the cycle using a controlled internal drug-releasing device (EAZI BREED CIDR containing 1.38 g progesterone; Pfizer Animal Health, New York, NY) for 7 d with an injection of GnRH at insertion and PGF<sub>2α</sub> at CIDR removal. Cows that displayed estrus ( $\pm$  12 h) on the same day were included in the treatment groups.

***Estrous Detection.*** The HeatWatch Estrous Detection System (DDx, Inc., Denver, CO) was used to monitor estrus during the pre-synchronization period and throughout the experiment. Estrus was defined as three mounts lasting longer than 2 s per mount within a 4 h period.

***Transrectal ultrasonography.*** Ovarian structures were monitored using an Aloka 500V ultrasound with a 7.5 MHz transducer (Aloka, Wallingford, CT). Follicles  $\geq$  5 mm in diameter and the presence of a corpus luteum (CL) were recorded. Follicle diameter was measured at the widest point and at a right angle to the first measurement. The follicular diameter was calculated as the average of the two measurements. Transrectal

ultrasonography was performed on d -9 (GnRH1) and d 0 (GnRH2) to determine the diameter of the dominant follicle. Ovulatory follicles less than or equal to 11 mm were considered small dominant follicles while follicles greater than 11 mm were considered large follicles. This cut off was determined based on pregnancy rates in cows ovulating various follicle sizes reported previously in this herd (Perry et al., 2005). Ovarian ultrasound exams were performed daily from d -9 to d 0 and on d 2 to characterize follicular waves and growth of dominant follicles and to confirm ovulation after GnRH1 and 2, based on the disappearance of a dominant follicle and the formation of luteal tissue. All ultrasound scans were recorded to video. Individual follicles were tracked starting on d -5 to d 0 to determine the long term follicle growth rate. These days were chosen to monitor long term growth because most cows had a follicle that could accurately be tracked during this time period. In addition, ovaries were scanned daily starting 9 (Day 18 group), 4 (Day 13 group), 2 (Day 9 and Day 5 group), or 1 d (Day 2 group) before GnRH1 to characterize the dominant follicle before GnRH1 [i.e., follicle wave number and stage of growth (increasing, plateau, or regressing)]. Stage of growth at GnRH2 was assessed by fitting a polynomial curve to the follicle growth pattern of each cow. The first derivative of the polynomial was solved for zero to find the point on the curve where the follicle was no longer growing ( $\pm 0.5$  d; plateau). The follicle was considered increasing in size before the time of plateau and decreasing after the plateau.

***Blood collection and radioimmunoassay (RIA).*** Blood samples were collected via tail or jugular venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA) daily from d -9 until d 21 (d 0 = GnRH2). After collection, the blood was incubated for 24 h at 4°C followed by centrifugation at 1,200 x g for 25 min. Serum

was collected and stored at -20°C until RIA. Serum concentrations of progesterone were measured in all samples using a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA; Kirby et al., 1997). Intra and inter-assay coefficients of variation were 2.9 and 9.8%, respectively for the progesterone RIAs. Two distinct luteal stages were characterized: 1) early luteolysis referred to cows that had undergone luteal regression prior to PGF<sub>2α</sub> and 2) incomplete luteal regression referred to cows that had incomplete luteal regression after administration of PGF<sub>2α</sub>. Cows were determined to have undergone early luteolysis when serum concentrations of progesterone dropped below 1.0 ng/mL before PGF<sub>2α</sub>. Cows were determined to have incomplete luteal regression when serum concentrations of progesterone decreased following PGF<sub>2α</sub> but did not drop below 1.0 ng/mL and then increased earlier than cows that had undergone complete luteal regression. Serum concentrations of estradiol-17β were measured using RIA (Kirby et al., 1997) in samples collected from d -9 to d 0 (d 0 = GnRH2). Intra and inter-assay coefficients of variation were 13.1 and 17.6%, respectively for the estradiol RIAs.

***Statistical analysis.*** One-way ANOVA with day as the independent variable was used to test differences among treatment groups in average follicular diameter and serum concentrations of estradiol at GnRH1 and 2 with SAS (SAS Inst. Inc., Cary, NC). Differences in the proportion of cows ovulating among treatment groups at GnRH1 and 2, undergoing premature luteolysis, and in estrus early were tested using GENMOD with SAS. Differences in average follicular diameter in follicular diameter at GnRH1 and 2 between cows that did or did not ovulate after GnRH1 were analyzed with the two-sample T test. The percentage of cows with a class III follicle (> 9 mm; Moreira et al., 2000) from d -9 through d 0 was analyzed using the GENMOD procedure in SAS for

repeated measures. Changes in serum concentrations of progesterone over time during the linear increase in progesterone (d 2 to 12 after GnRH2) were analyzed by ANOVA for repeated measures (PROC MIXED; Littell et al., 1998) between cows that ovulated different size follicle groups after GnRH2. Differences in long term follicle growth rate (d -5 to d 0) among treatment groups (day of the cycle), between cows that did or did not ovulate to GnRH1, and cows with a small or large follicle at GnRH2 was analyzed by weighted ANOVA for repeated measures (PROC MIXED; Littell et al., 1998) in which time points were weighted based on the number of observations. A multiple regression with GLM in SAS was used to analyze the affect of ovulatory follicle diameter and display of estrus on serum concentrations of estradiol at the time of GnRH2. The correlation between ovulatory follicle diameter, follicle growth rate, and serum concentrations of estradiol were analyzed using the PROC CORR in SAS. Cows that underwent incomplete luteal regression after PGF<sub>2α</sub> were not included in the progesterone analysis after GnRH2 but were included in all other analyses.

## RESULTS

***Ovulatory response and follicle diameter.*** The percentage of cows that ovulated to GnRH1 and GnRH2 was 50 and 76%, respectively. Percentage of cows ovulating at GnRH2 was greater when the largest follicle was increasing in size (32/36;  $P = 0.0003$ ) or had reached a plateau in growth (9/10;  $P = 0.0115$ ) compared to cows with a decreasing follicle diameter (4/7; cows that were in estrus before GnRH2 were not included in this analysis). The proportion of cows ovulating to GnRH2 was not different

between cows with an increase in follicle growth and cows that had reached a plateau in follicle growth ( $P = 0.92$ ).

The size of the follicle at GnRH1 and the proportion that ovulated following GnRH1 was decreased in Day 2 cows ( $P < 0.003$  and  $0.005$ , respectively) compared to the remaining treatment groups. There was no difference in the proportion ovulating to GnRH1 among Day 5, 9, 13, or 18 cows ( $P > 0.2$ ; Table 3.1). Neither the proportion ovulating nor size of the dominant follicle at GnRH2 was affected by day of the cycle at GnRH1 ( $P = 0.21$  and  $0.65$ , respectively; Table 3.1). Day of the cycle at GnRH1 affected the percentage of cows with a class III follicle between d -9 and d 0 of the treatment period (day of cycle by time interaction  $P < 0.05$ ; Figure 3.1a); However the percentage of cows with a class III follicle at GnRH2 was not affected by day of the cycle at the start of treatment ( $P > 0.2$  Figure 3.1a).

The range in ovulatory follicle diameter at GnRH2 was 7.7 to 18.2 mm with 33% ovulating small ( $\leq 11$  mm) follicles. Ovulation to GnRH1 did not affect the proportion ovulating to GnRH2 or the size of the follicle at GnRH2 ( $P = 0.22$  and  $0.23$ , respectively; Table 3.2). Considering that cows on Day 2 of the cycle were unable to have a follicle capable of ovulating when administered GnRH1, a second analysis was performed to compare only cows capable of ovulating following GnRH1 (Day 2 treatment group was removed). When cows on Day 2 of the estrous cycle were excluded, cows that ovulated following GnRH1 had larger follicle diameters at GnRH2 than cows that did not ovulate ( $P = 0.04$ ; Table 3.2). Among cows that ovulated after GnRH2, the proportion of cows that ovulated a small follicle was not affected by ovulatory response to GnRH1 (7/25 and 10/21 of cow that did or did not ovulate to GnRH1 ovulated a small follicle at GnRH2,

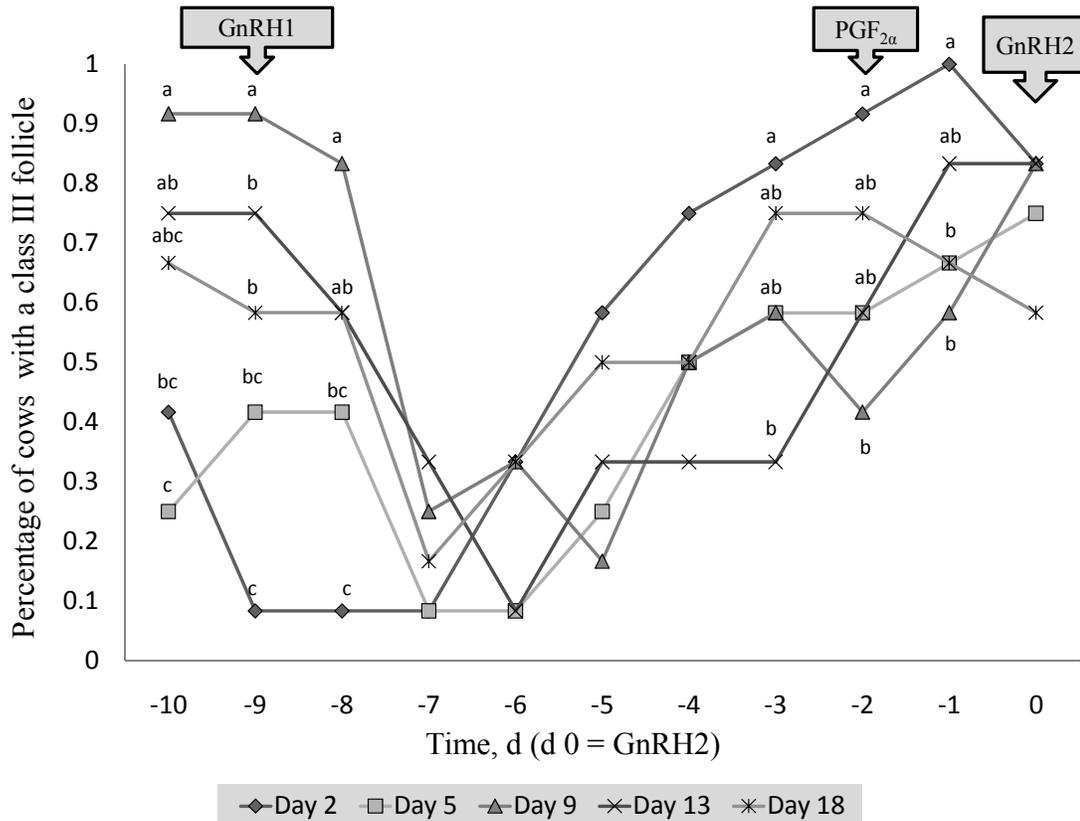
**Table 3.1** Mean diameter (mm) of the largest follicle ( $\pm$  SEM) at GnRH1 and GnRH2, number (%) of cows ovulating to GnRH1 and 2, serum estradiol at GnRH2 (pg/mL,  $\pm$  SEM), number (%) of cows undergoing luteolysis before prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ), and in estrus from GnRH1 to PGF<sub>2 $\alpha$</sub> .

Trt <sup>1</sup>	GnRH1 <sup>1</sup>		GnRH2 <sup>1</sup>		Serum estradiol at GnRH2	Luteolysis before PGF <sub>2<math>\alpha</math></sub> <sup>2</sup>	In estrus before PGF <sub>2<math>\alpha</math></sub>
	Largest follicle diameter	Ovulating	Largest follicle diameter	Ovulating			
Day 2	6.5 <sup>a</sup> $\pm$ 0.6	0/12 <sup>d</sup> (0)	11.7 $\pm$ 0.7	9/12 (75)	4.7 $\pm$ 1.0	0/12 <sup>a</sup> (0)	0/12 <sup>d</sup> (0)
Day 5	9.0 <sup>b</sup> $\pm$ 0.3	9/12 <sup>c</sup> (75)	10.8 $\pm$ 0.5	9/12 (75)	3.2 $\pm$ 0.5	0/12 <sup>a</sup> (0)	0/12 <sup>d</sup> (0)
Day 9	11.5 <sup>c</sup> $\pm$ 0.4	8/12 <sup>c</sup> (67)	10.8 $\pm$ 0.4	12/12 (100)	3.5 $\pm$ 0.5	0/12 <sup>a</sup> (0)	0/12 <sup>d</sup> (0)
Day 13	10.0 <sup>bc</sup> $\pm$ 0.4	6/12 <sup>c</sup> (50)	11.4 $\pm$ 0.9	9/12 (75)	4.3 $\pm$ 0.6	11/12 <sup>b</sup> (92)	2/12 <sup>dc</sup> (17)
Day 18	10.0 <sup>bc</sup> $\pm$ 0.5	7/12 <sup>c</sup> (58)	9.8 $\pm$ 1.6	7/12 (58)	5.0 $\pm$ 1.0	5/12 <sup>c</sup> (42)	4/12 <sup>c</sup> (33)

<sup>1</sup>Treatment groups were based on the day of the estrous cycle at the start of the CO-Synch protocol (GnRH [GnRH1] followed 7 d later with an injection of prostaglandin F<sub>2 $\alpha$</sub>  [PGF<sub>2 $\alpha$</sub> ] and 48 h after PGF<sub>2 $\alpha$</sub>  a second injection of GnRH [GnRH2] is administered). Means or percents having different superscripts within a column were different (<sup>abc</sup>P < 0.01, <sup>dc</sup>P < 0.05).

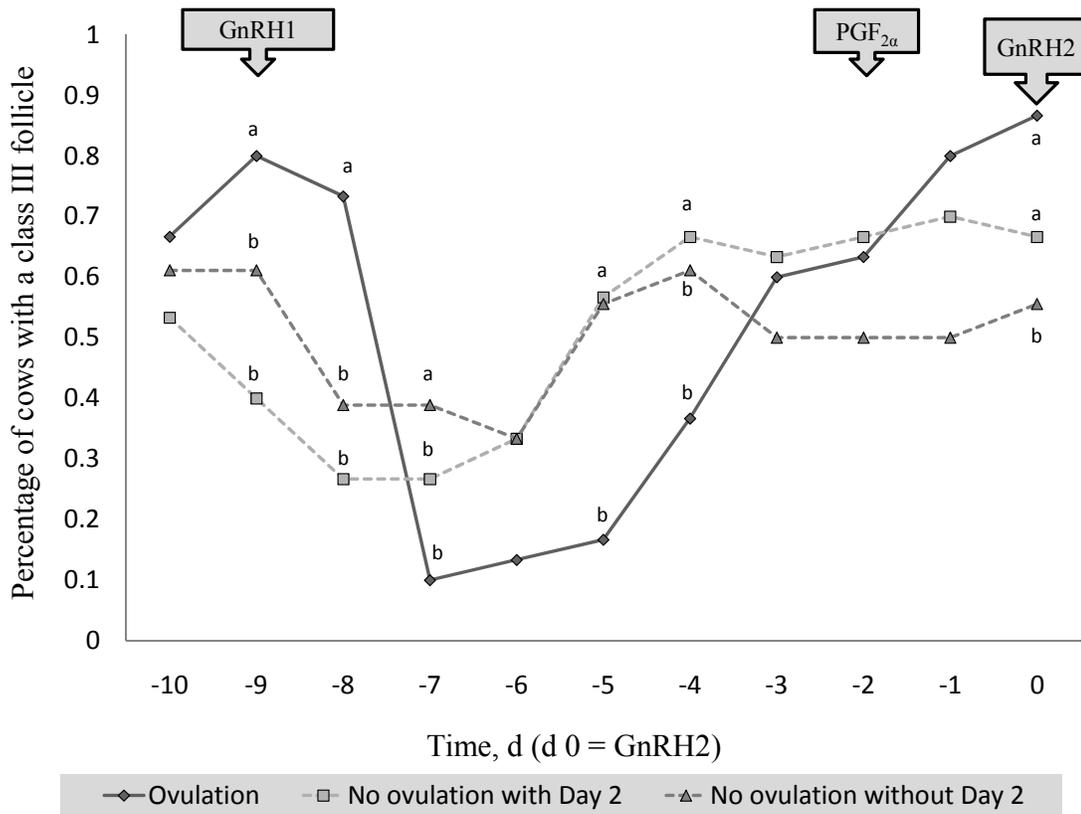
<sup>2</sup>Luteolysis was defined as the day when serum progesterone levels fell below 1.0 ng/mL.

a.)



**Figure 3.1a** Percentage of cows with a class III (> 9 mm) follicle by day of the cycle at GnRH1. Cows (n = 12 per treatment group) were on Day 2, 5, 9, 13, or 18 of their estrous cycle at the start of the CO-Synch protocol (GnRH [GnRH1] followed 7 d later with PGF<sub>2α</sub> and a second GnRH [GnRH2] 48 h after PGF<sub>2α</sub>). Day of the cycle at GnRH1 affected the proportion of cows with a class III follicle between d -9 and d 0 (time by cycle day interaction;  $P < 0.05$ ); however, on d 0 (GnRH2) there was no affect of day of the cycle on the percentage of cows with a class III follicle ( $P > 0.2$ ). Treatment groups with different letters had statistically significant differences in percentage of cows with a class III follicle ( $^{abc}P < 0.05$ ).

b.)



**Figure 3.1b** Percentage of cows with a class III (> 9 mm) follicle by ovulatory response to GnRH1 (b) during the treatment period. Cows (n = 12 per treatment group) were on Day 2, 5, 9, 13, or 18 of their estrous cycle at the start of the CO-Synch protocol (GnRH [GnRH1] followed 7 d later with PGF<sub>2α</sub> and a second GnRH [GnRH2] 48 h after PGF<sub>2α</sub>). The analysis compared cows that ovulated to GnRH1 (ovulation) to cows that did not ovulate to GnRH1 either with (no with Day 2) or without the Day 2 cows (no without Day 2) but did not compare the latter two groups. Treatment groups with different letters had statistically significant differences in percentage of cows with a class III follicle (<sup>abc</sup>*P* < 0.05).

**Table 3.2** Effect of ovulatory response at GnRH1 in the CO-Synch protocol<sup>1</sup> on mean  $\pm$  SEM follicular diameter (mm) at GnRH1 and 2, serum estradiol (pg/mL)  $\pm$  SEM at GnRH2, number and (percentage) ovulating after GnRH2, undergoing luteolysis before PGF<sub>2 $\alpha$</sub>  and in estrus before PGF<sub>2 $\alpha$</sub> .

Ovulation	GnRH1	n	Follicle diameter GnRH1	Follicle diameter GnRH2	Estradiol at GnRH2	Ovulating after GnRH2	Luteolysis <sup>2</sup> before PGF <sub>2<math>\alpha</math></sub>	Estrus before PGF <sub>2<math>\alpha</math></sub>
Yes		30	10.6 $\pm$ 0.3	11.4 <sup>a</sup> $\pm$ 0.5	4.46 <sup>c</sup> $\pm$ 0.5	25/30 (83)	6/30 <sup>e</sup> (20)	1/30 <sup>e</sup> (3)
No with Day 2		30	8.4 $\pm$ 0.3	10.4 <sup>a</sup> $\pm$ 0.7	3.85 <sup>c</sup> $\pm$ 0.4	21/30 (70)	10/30 <sup>e</sup> (33)	5/30 <sup>e</sup> (17)
No without Day 2		18	9.7 $\pm$ 0.4	9.5 <sup>b</sup> $\pm$ 0.8	3.25 <sup>d</sup> $\pm$ 0.3	12/18 (67)	10/18 <sup>f</sup> (56)	5/18 <sup>f</sup> (28)

<sup>1</sup>The CO-Synch protocol includes an injection of GnRH (GnRH1) followed 7 d later with an injection of PGF<sub>2 $\alpha$</sub>  and 48 h after PGF<sub>2 $\alpha$</sub>  a second injection of GnRH (GnRH2) is administered.

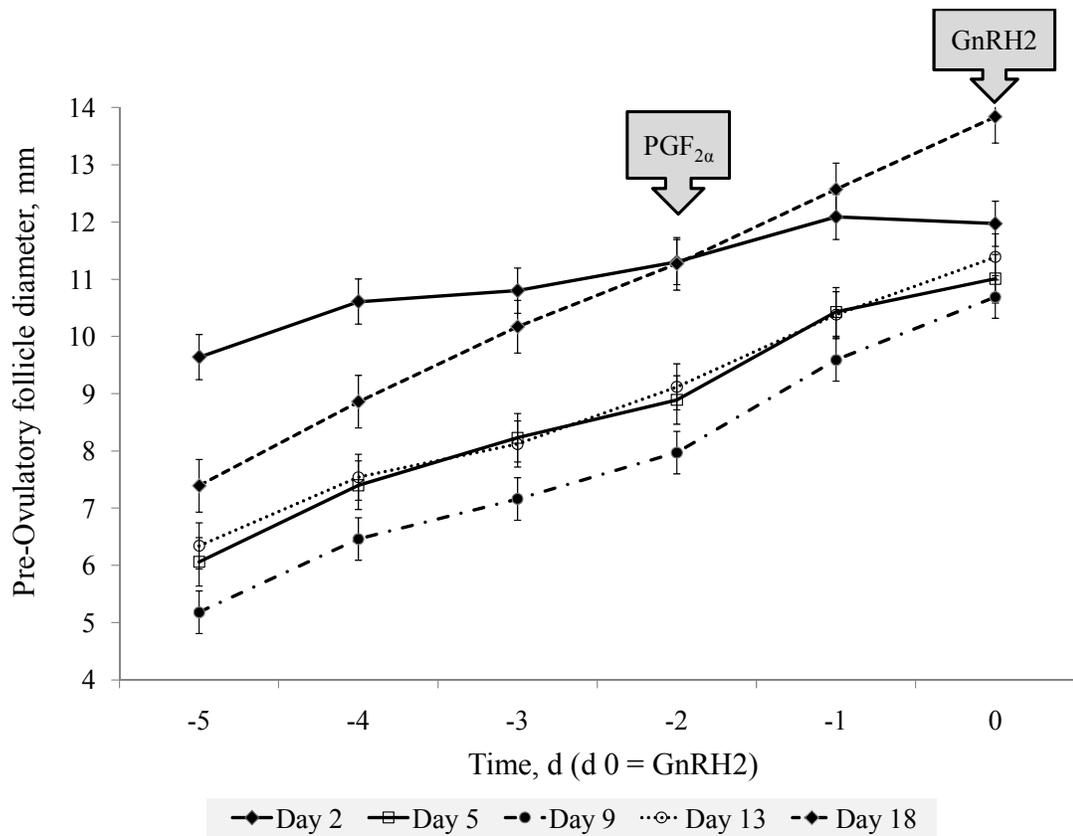
Means having different superscripts within columns were different between cows that ovulated (yes) or cows that did not ovulate (either No with or without cows at Day 2 of the estrous cycle at GnRH1; <sup>ab</sup>*P* = 0.04, <sup>cd</sup>*P* < 0.1, <sup>ef</sup>*P* < 0.01).

<sup>2</sup>Luteolysis was defined as the day the serum progesterone levels fell below 1.0 ng/mL.

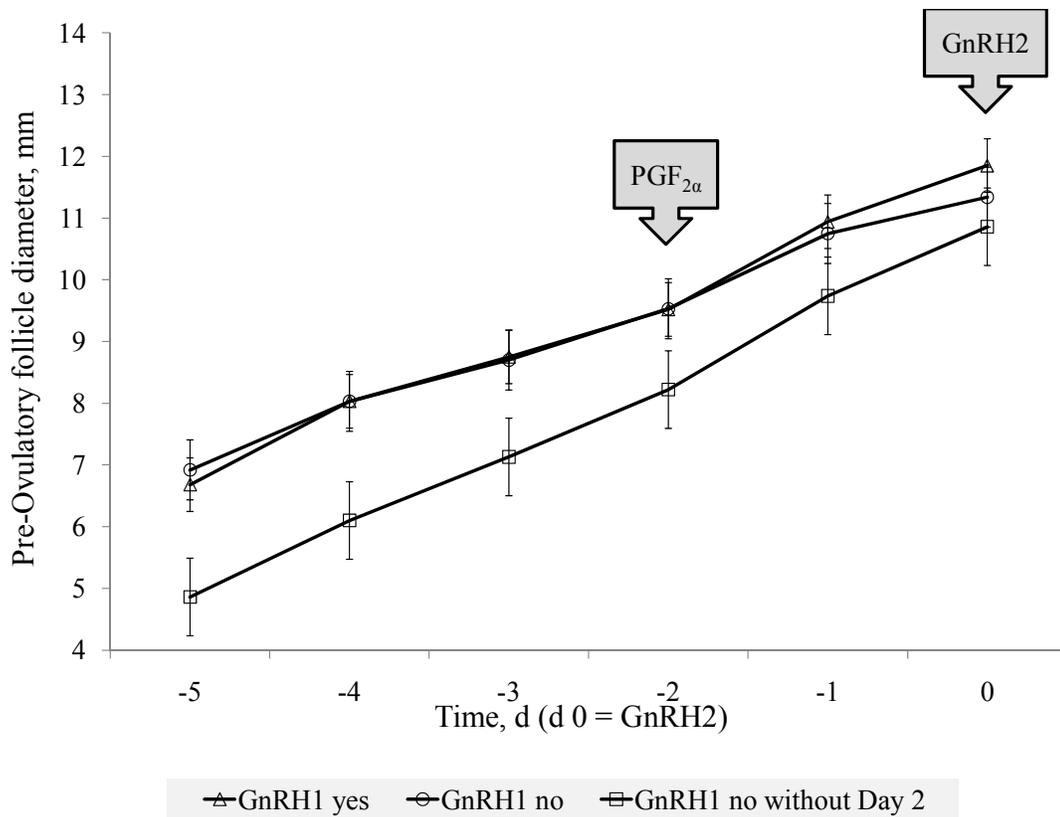
respectively;  $P = 0.17$ ). When Day 2 cows were removed from the analysis, there was still no difference in the proportion of cows that ovulated a small follicle in respect to ovulatory response at GnRH1 (7/25 and 6/12 of the cows that did or did not ovulate to GnRH1 ovulated a small follicle at GnRH2;  $P = 0.19$ ). Ovulation to GnRH1 affected the percentage of cows with a class III follicle throughout the synchronization period ( $P < 0.05$ ; Figure 3.1b). When Day 2 cows were excluded from the analysis, there was a greater percentage of cows with a class III follicle at GnRH2 that ovulated to GnRH1 than cows that did not ovulate to GnRH1 ( $P = 0.04$ ; Figure 3.1b).

**Luteolysis and estrus.** An increased proportion of cows in the later part of the estrous cycle at the start of the CO-Synch protocol underwent luteolysis before PGF<sub>2α</sub> administration ( $P < 0.01$ ; Table 3.1). An increased proportion of cows in the Day 18 treatment group were in estrus before PGF<sub>2α</sub> compared to cows in the Day 2, 5, or 9 treatment groups ( $P < 0.05$ ; Table 3.1). When all cows were included in the analysis, the proportion of cows undergoing luteolysis or in estrus before PGF<sub>2α</sub> was not different between cows that ovulated or did not ovulate after GnRH1 ( $P = 0.24$  and  $P = 0.09$ , respectively; Table 3.2). When cows in the Day 2 treatment group were excluded from the analysis, fewer cows that ovulated to GnRH1 underwent luteolysis or displayed estrus before PGF<sub>2α</sub> administration than cows that did not ovulate following GnRH1 ( $P < 0.001$  and  $P < 0.001$ , respectively; Table 3.2).

**Follicle growth.** Only cows ovulating following GnRH2 were used to analyze follicle growth rate. Follicle growth from d -5 to 0 was not affected by day of the cycle at GnRH1 ( $P = 0.82$ ; Figure 3.2). When all cows were included in the analysis, there was no significant time by ovulation to GnRH1 interaction ( $P = 0.75$ ; Figure 3.3). When Day



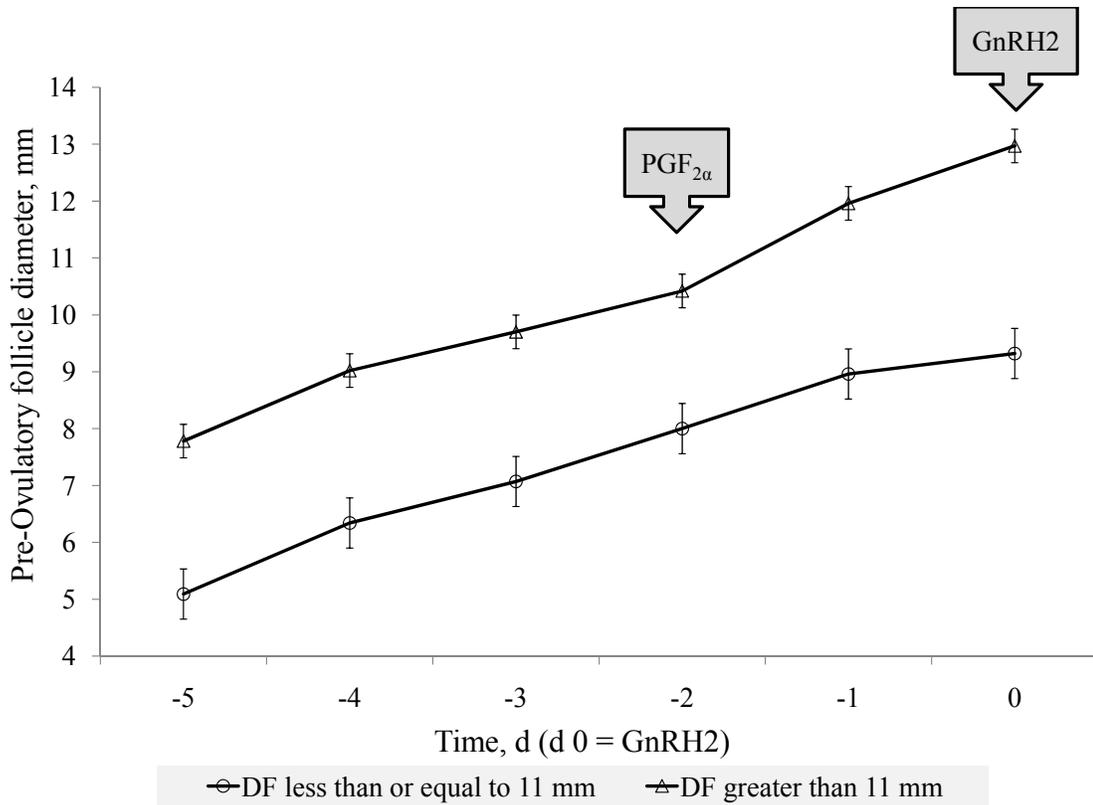
**Figure 3.2** Largest follicle diameter (y axis) leading up to GnRH2 (d 0) by treatment group (day of the cycle at the start of the CO-Synch protocol [GnRH followed 7 d later with  $\text{PGF}_{2\alpha}$  and a second GnRH 48 h after  $\text{PGF}_{2\alpha}$ ]). Follicle growth was similar among cows on different days of the cycle at the start of the CO-Synch protocol ( $P = 0.83$  for the day of the cycle by time interaction). Only cows that ovulated following GnRH2 are included.



**Figure 3.3** Growth of the pre-ovulatory dominant follicle leading up to GnRH2 (d 0) between cows that did (GnRH1 yes; n = 25) or did not (GnRH1 no; n = 21) ovulate in response to GnRH1. Cows that ovulated to GnRH1 had a similar growth pattern to cows that did not ovulate to GnRH1 when all treatment groups were included (GnRH1 yes and GnRH1 no;  $P = 0.75$ ). However, when the Day 2 cows were excluded from the analysis (GnRH1 no without Day 2; n = 12), cows that ovulated to GnRH1 had a similar growth rate from d -5 to d 0 ( $P = 0.57$ ) but the size of the ovulatory follicle was larger at d -5 compared to cows that did not ovulate ( $P = 0.05$ ). Only cows that ovulated following GnRH2 are included.

2 cows were removed from the analysis, there was also no time by ovulation to GnRH1 interaction ( $P = 0.57$ ) but cows that ovulated following GnRH1 had a larger follicle by d -5 than cows that did not ovulate after GnRH1 ( $P = 0.05$ ; Figure 3.3). There was no interaction in the long term follicle growth between ovulation after GnRH1 and the size of the ovulatory follicle ( $P = 0.25$ ; data not shown). Mean follicle diameter  $\pm$  SEM for small follicles ( $\leq 11$  mm) was  $9.3 \text{ mm} \pm 0.2$  with a range of 7.7 to 10.7 mm and mean follicle diameter  $\pm$  SEM for large follicles ( $> 11$  mm) was  $12.9 \text{ mm} \pm 0.3$  with a range from 11.1 to 18.2 mm. Follicle growth rate tended to be faster ( $P = 0.07$ ) from d -5 to d 0 in cows ovulating large compared to small follicles (Figure 3.4) and follicle size was larger ( $P < 0.001$ ) from d -5 to d 0 in these cows. Short term (from d -2 to d 0) growth rate and follicle diameter at GnRH2 were positively correlated ( $r = 0.467$ ;  $P < 0.0005$ ). Follicle growth rate was slower from d -2 to 0 in Day 2 cows than Day 9 cows ( $0.339$  vs.  $1.29$  mm/d, respectively;  $P = 0.048$ ) but was similar among all other treatment groups ( $1.06$ ,  $1.13$ , and  $1.28$  mm/d for Day 5, 13, and 18 cows, respectively;  $P > 0.06$ ; Figure 3.2).

***Serum concentrations of progesterone and estradiol.*** Serum concentrations of estradiol on the day of GnRH2 were not different among cows on different days of the cycle at GnRH1 ( $P = 0.328$ ; Table 3.1). Serum concentrations of estradiol on the day of GnRH2 were not different among cows that ovulated to GnRH1 compared to cows that did not ovulate following GnRH1 ( $P = 0.3462$ ; Table 3.2). When the Day 2 cows were removed from the analysis, there was a tendency for cows that ovulated to GnRH1 to have increased serum concentration of estradiol at GnRH2 compared to cows that did not ovulate to GnRH1 ( $P = 0.07$ ). In cows that ovulated to GnRH2, the serum



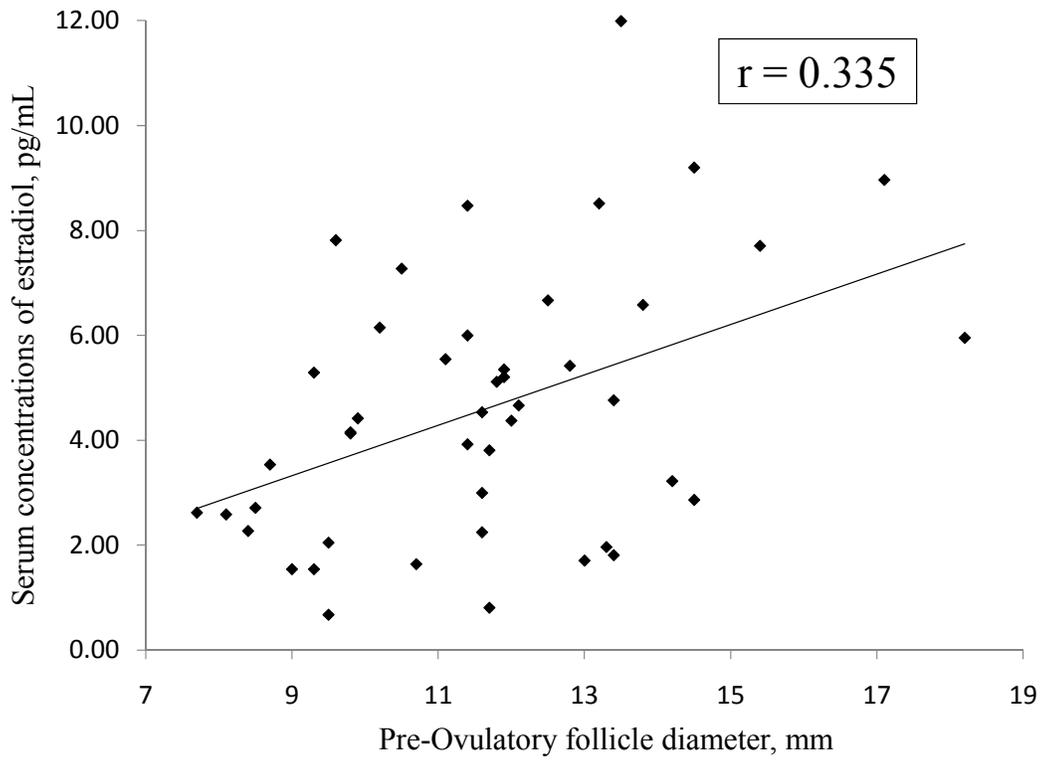
**Figure 3.4** Growth of the pre-ovulatory dominant follicle leading up to GnRH2 (d 0) among cows that ovulated a large (> 11 mm; n = 29) or small (≤ 11 mm; n = 17) dominant follicle at GnRH2. Cows with a large follicle at GnRH2 had a faster rate of follicle growth leading up to GnRH2 than cows with a small dominant follicle at GnRH2 ( $P = 0.07$ ) and the large ovulatory follicles were larger at d -5 than the small ovulatory follicles ( $P < 0.001$ ). Only cows that ovulated following GnRH2 are included.

concentrations of estradiol were positively correlated with size of the dominant follicle at GnRH2 ( $r = 0.335$ ;  $P = 0.006$ ; Figure 3.5) independent of estrus.

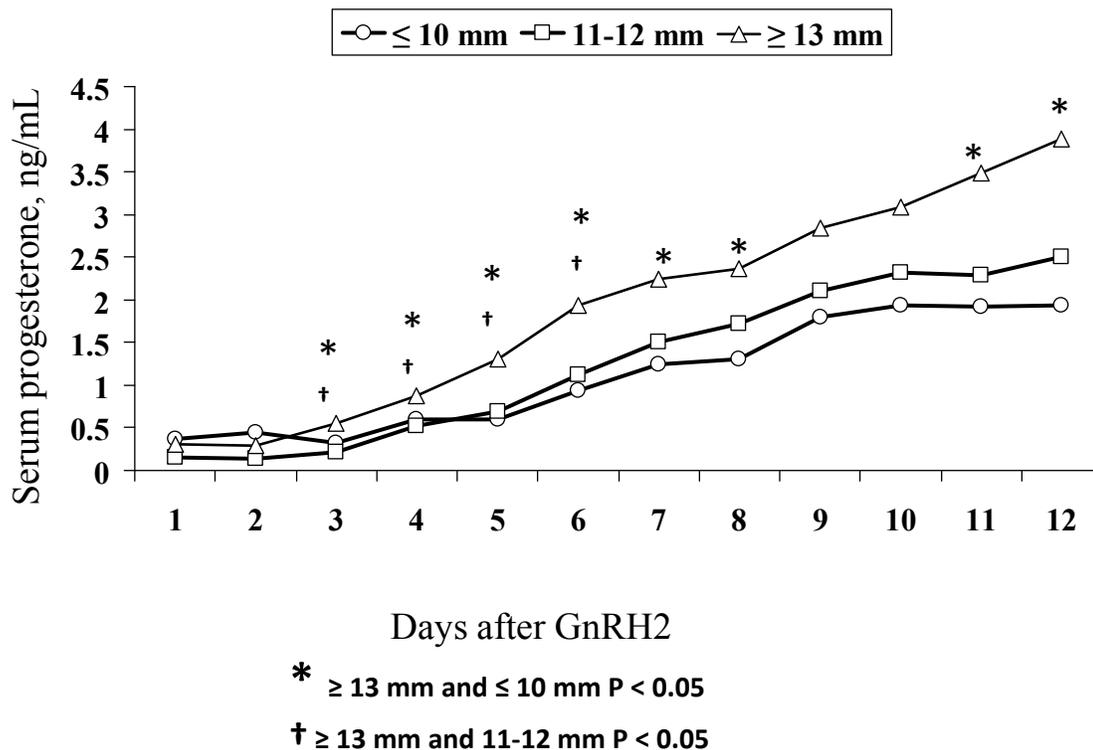
The variance of the mean serum concentrations of progesterone was not equal over time so the concentrations of progesterone were log transformed for the analysis. Actual values are graphed in Figure 3.6. Ovulation of follicles  $\leq 12$  mm led to reduced concentrations of serum progesterone (Figure 3.6;  $P < 0.05$ ) compared to cows that ovulated follicles  $\geq 13$  mm in diameter. Some cows from the Day 2 (6/12) and 5 (5/12) treatment groups underwent incomplete luteal regression after  $\text{PGF}_{2\alpha}$  (Figure 3.7) and were not included in the progesterone analysis. Of these cows that underwent incomplete luteolysis 2/6 and 2/5 of the Day 2 and Day 5 cows, respectively, were considered to have ovulated after GnRH2 based on disappearance of a dominant follicle and formation of luteal tissue.

## DISCUSSION

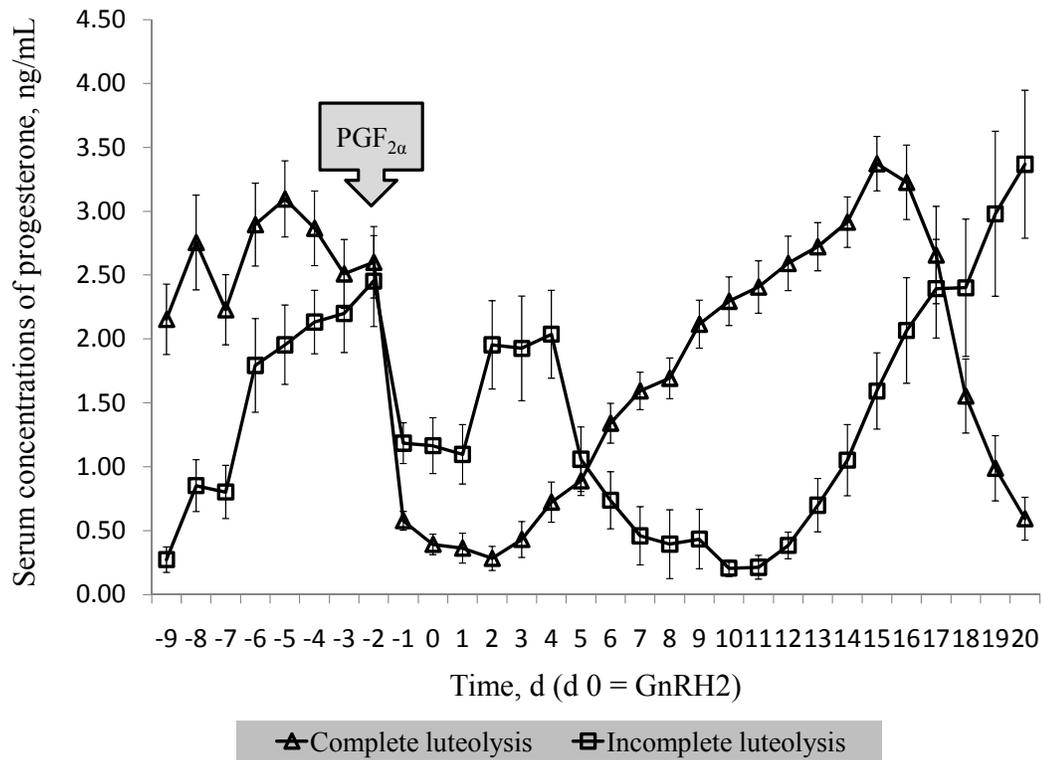
In the present study, day of the estrous cycle at the start of the synchronization program did not affect follicle size or proportion ovulating at the second GnRH injection. In cows that were capable of ovulating to GnRH1 (i.e., excluding cows on Day 2 of the estrous cycle), cows that ovulated in response to GnRH1 had a larger follicle at GnRH2 than cows that did not ovulate to GnRH1. In anestrous beef cows, those that ovulated following the first GnRH injection also had larger follicles at the second GnRH injection than cows that did not ovulate following GnRH1 (Atkins et al., unpublished). Vasconcelos et al. (1999) reported that day of the estrous cycle at the start of the Ovsynch



**Figure 3.5** Scatter plot of serum concentration of estradiol (pg/ml) and diameter of the pre-ovulatory follicle at GnRH2. Serum concentration of estradiol was positively correlated ( $r = 0.335$ ;  $P = 0.006$ ) with diameter of the dominant follicle at GnRH2. Only cows that ovulated following GnRH2 are included ( $n = 46$ ).



**Figure 3.6** Mean serum concentrations of progesterone from d 1 to 12 after GnRH2 (GnRH2 = d 0) in cows that ovulated follicles  $\leq 10$  mm (n = 14; circle), 11 to 12 mm (n = 18; square), or  $\geq 13$  mm (n = 9; triangle). Due to unequal variance over time the mean concentrations of progesterone were log transformed for the analysis but the non-transformed data are presented in this graph. Cows that ovulated larger follicles had greater ( $P < 0.05$ ) serum concentrations of progesterone than cows that ovulated small follicles. Only cows that ovulated following GnRH2 and underwent complete luteal regression are included.



**Figure 3.7** Mean serum concentrations of progesterone (ng/mL; error bars = SEM) during the treatment period and the subsequent estrous cycle. Some cows on day 2 (6/12) and day 5 (5/12) of the cycle at the start of the CO-Synch protocol (GnRH followed 7 d later with  $\text{PGF}_{2\alpha}$  and a second GnRH 48 h after  $\text{PGF}_{2\alpha}$ ) underwent incomplete luteolysis following  $\text{PGF}_{2\alpha}$  administration (open squares) while the remaining cows (from all treatment groups) had complete luteal regression (open triangles) based on serum concentrations of progesterone.

program did not affect the percentage of dairy cows that ovulated at GnRH2 but cows either in the early (cycle d 1 to 4) or latter (cycle d 17 to 21) stage of the estrous cycle at the start of synchronization had larger follicles at GnRH2 than cows in the middle of the estrous cycle. These results differ slightly from the current study but the current study used defined days of the estrous cycle instead of a range in days.

Similar studies conducted in estrous cycling beef (Atkins et al., 2008a) and dairy (Stevenson, 2008) heifers reported nearly the opposite observations from the current study. In beef heifers, ovulation to the first GnRH did not affect follicle size or ovulatory response at the second GnRH injection but day of the estrous cycle at the start of the synchronization program did affect follicle size, percent of heifers with a class III follicle at GnRH2, and ovulatory response after the second GnRH injection (Atkins et al., 2008a). In dairy heifers, inclusion of the first GnRH injection did not improve response at the second GnRH or pregnancy rates (Stevenson, 2008) but day of the cycle did affect ovulation rate and follicle size at the second GnRH. The reason for the discrepancy between the heifer and cow responses is unknown.

In the current study nearly half of the cows in the early part of the estrous cycle at the start of the CO-Synch protocol underwent incomplete luteolysis following PGF<sub>2α</sub> (6/12 and 5/12 of the cows on d 2 and 5 of the cycle at treatment initiation, respectively). Twagiramungu and colleagues (1994) reported that 4/18 cows treated with GnRH followed 7 d later with PGF<sub>2α</sub> underwent incomplete luteolysis. Similarly, Burke and colleagues (1996) reported 8% of lactating dairy cows had incomplete luteal regression following PGF<sub>2α</sub> administered 7 d after GnRH. It is interesting that in the present study

only the cows in the early part of the estrous cycle had incomplete luteal regression and all of the cows on Day 9, 13, or 18 underwent complete luteolysis.

Recent reviews (Webb et al., 2004 and Lucy 2007) have described a number of factors controlling growth of the dominant follicle in cattle including gonadotropins, locally produced growth factors, (members of the TGF $\beta$  superfamily, insulin like growth factors [IGF] I and II, IGF binding proteins, FGF, EGF, and cytokines), angiogenic factors, and nutritional effects through growth hormone, insulin, IGF, and leptin. Fortune et al. (1988) reported the growth rate of the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> wave dominant follicle to be 1.6, 1.1, and 1.7 mm/day, respectively in Holstein heifers. In the present study, follicle growth rate (0.89 mm/d; 5 d prior to GnRH2) was a little slower compared to the expected 1 to 2 mm per day. Follicle growth rate increased slightly after PGF $_{2\alpha}$  administration (0.99 mm/day) compared to the long term growth rate. The increase in follicle growth rate after PGF $_{2\alpha}$  may be due to the loss of negative feedback from progesterone on LH (Beck et al., 1976) and therefore an increase in LH pulse frequency could drive follicle growth and estradiol production (Fortune, 1994). Although follicle growth rate from d -5 to 0 (d 0 = GnRH2) was similar between cows that did or did not ovulate after GnRH1, cows that ovulated following GnRH1 had a larger follicle by d -5 than cows that did not ovulate after GnRH1. The larger follicular diameter by d -5 may have been due to the synchronized growth of a follicular wave in cows ovulating to GnRH1.

The reason for the reduced fertility associated with ovulation of a smaller dominant follicle may be related to the altered hormone profile in the cows after ovulation of an immature follicle. Britt and Holt (1988) described the following five

critical periods of changing hormone profiles that are important to fertility in lactating cows: 1) the luteal phase during the estrous cycle before insemination, 2) the period from the onset of luteolysis to estrus, 3) the preovulatory period, 4) the period from ovulation until progesterone rises, and 4) the luteal phase following insemination. Cows that are induced to ovulate a small dominant follicle may have altered endocrine profiles at several of these important periods which could affect fertility. In the current study, cows that ovulated a small dominant follicle had reduced serum concentrations of estradiol during the preovulatory period and reduced serum concentrations of progesterone in the subsequent luteal phase. Cows that were induced to ovulate a small dominant follicle had lower serum concentrations of estradiol at the time of ovulation compared to cows that ovulated large or small follicles spontaneously (Busch et al., 2008; Vasconcelos et al., 2001). Our results are supported by several others who reported reduced serum concentrations of progesterone in the subsequent luteal phase after induced ovulation of small (Perry et al., 2005; Atkins et al., 2008a; Busch et al., 2008) or immature (Burke et al., 2001 and Mussard et al., 2007) dominant follicles. Luteal secretion of progesterone is vital to embryo survival. Inadequate luteal function may impair interferon  $\tau$  production (Mann and Lamming, 2001) or uterine secretions important to embryo development (Garret et al., 1988). The mechanism of how induced ovulation of small dominant follicles affects establishment and maintenance of pregnancy is unclear but may be related to inadequate uterine environment supported by altered endocrine profiles of these cows or due to poor oocyte quality, gamete transport, or oviductal environment. Current studies are underway using reciprocal embryo transfer experiments to attempt to resolve this question.

In summary, day of the estrous cycle at initiation of the CO-Synch protocol did not affect ovulatory follicle size or ovulatory response at induced ovulation for timed AI. Ovulatory response to the first GnRH injection also did not affect ovulatory response to the second GnRH injection and affected ovulatory size only when Day 2 cows (which did not have a follicle capable of ovulating at GnRH1) were removed from the analysis. Follicle growth rate leading up to GnRH2 tended to be faster in cows that ovulated a large follicle compared to cows that ovulated a small follicle. Ovulatory follicle diameter was positively correlated with serum concentrations of estradiol at GnRH2 and cows that ovulated small follicles had a reduced rise in serum concentrations of progesterone 12 d after GnRH.

## CHAPTER IV

### FACTORS AFFECTING PRE-OVULATORY FOLLICLE DIAMETER AND OVULATION RATE FOLLOWING GNRH IN POSTPARTUM BEEF COWS PART II: ANESTROUS COWS

**ABSTRACT.** There is large variation in dominant follicle diameter at the time of GnRH-induced ovulation in the CO-Synch protocol (GnRH on d -9 [GnRH1] followed by PGF<sub>2α</sub> on d -2 and GnRH with timed AI on d 0 [GnRH2]) and the reason for the presence of small dominant follicles at GnRH2 is not known. Our hypothesis was that ovulatory response to GnRH1 and progesterone exposure (controlled intravaginal drug releasing insert [CIDR]) would affect ovulatory follicle size at GnRH2 in anestrous cows. This study used a two by two factorial design in which anestrous suckled beef cows (n = 55) either ovulated (Ov1+) or failed to ovulate (Ov1-) after GnRH1 and either received (CIDR+) or did not receive (CIDR-) a 7 d CIDR treatment (from GnRH1 to PGF<sub>2α</sub>) resulting in the following treatment groups: Ov1+CIDR+, Ov1-CIDR+, Ov1+CIDR-, and Ov1-CIDR- (n = 9, 17, 11, and 18, respectively). The Ov1+ had larger follicles at GnRH2, a decreased proportion of small follicles within cows that ovulated to GnRH2, and a similar growth rate of the ovulatory follicle from d -5 to d 0 (d 0 = GnRH2) compared to Ov1- cows (12.3 vs. 11.0 mm, 2/16 vs. 14/23, and 1.1 vs. 1.1 mm/d,

respectively;  $P = 0.04, 0.003, \text{ and } 0.99$ , respectively). Administration of a CIDR had no affect on follicle diameter at GnRH2, proportion of small ovulatory follicles at GnRH2, and follicular growth rate from d -5 to d 0 (d 0 = GnRH2; 11.8 vs. 11.2 mm, 7/19 vs. 9/20, and 1.2 vs. 1.1 mm/d for the CIDR+ vs. CIDR- cows, respectively;  $P = 0.3, 0.6, \text{ and } 0.76$ , respectively). Administration of a CIDR, but not ovulation to GnRH1, increased follicle growth from d -2 to d 0 (d 0 = GnRH2;  $P = 0.03 \text{ and } 0.9$ , respectively). Large follicles (> 11 mm) had a similar growth rate from d -5 to d 0 (d 0 = GnRH2;  $P = 0.44$ ) compared to small follicles (1.1 vs. 1.2 mm/d) but the large ovulatory follicles were larger at d-5 compared to small ovulatory follicles ( $P < 0.001$ ). Follicle diameter was positively correlated with serum concentrations of estradiol at GnRH2 ( $r = 0.622$ ;  $P < 0.0001$ ). In summary, ovulation to GnRH1 but not CIDR administration resulted in increased dominant follicle diameter at GnRH2 in anestrous suckled beef cows. Large follicles were already larger five days before GnRH2 but grew at a similar rate to small follicles and follicle size was positively correlated with serum concentrations of estradiol at the time of GnRH-induced ovulation.

## INTRODUCTION

Multiple reports indicated that cows induced to ovulate small dominant follicles have reduced pregnancy rates (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005). There is a large variation in the diameter of the largest follicle (Perry et al., 2005; Lamb et al., 2001) at the time of GnRH-induced ovulation in the CO-Synch protocol (GnRH on d-9 [**GnRH1**], PGF<sub>2α</sub> on d-2, and GnRH on d0 [**GnRH2**]; Geary et al., 1998) and other timed AI protocols (CO-Synch plus CIDR [Lamb et al., 2001]). Additionally,

cows that were induced to ovulate a small dominant follicle had an increased occurrence of late embryonic/fetal mortality (Perry et al., 2005). The reason for the presence of small dominant follicles at GnRH2 is unknown but may be due to failure to control the initiation of a new follicular wave prior to GnRH2. This lack of control of the follicle wave could result in more variation in the age and size of the follicle at GnRH2. Alternatively, varied follicle growth rates leading up to GnRH2 could contribute to variation in pre-ovulatory follicle diameter.

In anestrus cows, the pulse frequency and mean concentrations of circulating luteinizing hormone (**LH**) were less than cycling cows which could reduce the rate of dominant follicle growth (reviewed by Yavas and Walton, 2000). Administration of a progestin increased the frequency of LH pulses in postpartum beef cows (Garcia-Winder et al., 1986 and Williams et al., 1983). Addition of exogenous progesterone to the CO-Synch protocol may affect follicle growth rate and the pre-ovulatory follicle diameter at GnRH2.

We hypothesized that failure to initiate a new follicular wave after GnRH1 and absence of progesterone would increase the occurrence of small pre-ovulatory follicles at the GnRH-induced ovulation. The objectives of the study were to determine the effect of ovulation to GnRH1 and exogenous progesterone treatment on the size and growth rate of the largest follicle at GnRH2 in anestrus beef cows.

## **MATERIALS AND METHODS**

***Animal handling.*** All protocols and procedures were approved by the Fort Keogh Livestock and Range Research Laboratory (**LARRL**) Animal Care and Use

Committee (ACUC approval no. 020104-9). Anestrous suckled beef cows (n = 55) at the Fort Keogh LARRL were administered the CO-Synch protocol with the exception that no cows were bred. Anestrous status was based on serum concentrations of progesterone 10 d before the start of treatment and the absence of a corpus luteum (**CL**) at treatment initiation. Approximately one-half of the cows were administered exogenous progesterone (EAZI BREED CIDR containing 1.38 g progesterone; Pfizer Animal Health, New York, NY; n = 26) from GnRH1 to PGF<sub>2α</sub> resulting in a two by two factorial design based on ovulation (**OV1+**) or failure (**OV1-**) to ovulate to GnRH1 and the presence (**CIDR+**) or absence (**CIDR-**) of a CIDR (Ov1+CIDR+, Ov1-CIDR+, Ov1+CIDR-, Ov1-CIDR-; n = 9, 17, 11, and 18, respectively). There were no differences in average days postpartum, age, or body condition score (**BCS**; 1 to 9 where 1 = emaciated and 9 = obese) among the treatment groups (Table 4.1). Estrus was detected visually twice daily from d -9 to d 20 (d 0 = GnRH2) and was aided with the use of Estrus Alert (Western Point Inc., Apple Valley, MN) estrous detection patches.

***Transrectal ultrasonography.*** Ovarian structures were monitored using an Aloka 500V ultrasound with a 7.5 MHz transducer (Aloka, Wallingford, CT). Follicles  $\geq 5$  mm in diameter and the presence of a CL were recorded. Follicle diameter was measured at the widest point and at a right angle to the first measurement and the average of these measurements was recorded as the follicle diameter. Transrectal ultrasonography was performed on d -9 (GnRH1) and d 0 (GnRH2) to determine the diameter of the ovulatory follicle. Ovulatory follicles less than 11 mm were considered small dominant follicles and follicles greater than or equal to 11 mm were considered large follicles based on previous research defining the optimal follicle diameter for pregnancy in this herd (Perry

**Table 4.1** The average ( $\pm$  SEM) days postpartum (DPP, d), age (yr), and body condition score (BCS) for each treatment group.

Treatment <sup>1</sup>	n	DPP	Age	BCS <sup>2</sup>
Ov1+/CIDR+	9	42 $\pm$ 2.3	3.4 $\pm$ 0.8	4.3 $\pm$ 0.1
Ov1+/CIDR-	11	42 $\pm$ 1.8	3.9 $\pm$ 0.8	4.6 $\pm$ 0.1
Ov1-/CIDR+	17	44 $\pm$ 1.5	2.9 $\pm$ 0.4	4.2 $\pm$ 0.1
Ov1-/CIDR-	18	44 $\pm$ 1.7	2.7 $\pm$ 0.3	4.3 $\pm$ 0.1

<sup>1</sup> Ovulation to GnRH1 (Ov1+) or failure to ovulate (Ov1-) and presence (CIDR+) or absence (CIDR-) of a controlled internal drug releasing (CIDR) insert.

<sup>2</sup>Body condition score was based on a 1 to 9 scale where 1 = emaciated and 9 = obese. There were no treatment differences ( $P > 0.6$ ).

et al., 2005). Ovulation after GnRH1 and GnRH2 was determined on d -7 and 2, respectively, and was based on the disappearance of a dominant follicle and in some cases formation of new luteal tissue. Daily ultrasound exams from d -9 to d 0 were performed and recorded to monitor growth of dominant follicles during the treatment period. The long term (d -5 to d 0) follicle growth pattern required back tracking specific follicles using the recorded ovarian sonograms. Most cows had an individual follicle from a single follicular wave that was tracked starting on d -5 so the growth of the ovulatory follicle was calculated from d -5 to d 0. A polynomial equation was fit to the follicle growth curve and the first derivative of the polynomial equation was determined for each cow. The first derivative was solved for zero to determine the day the follicle had reached a plateau in growth ( $\pm 0.5$  d). Follicles were considered to be increasing in size prior to the plateau and decreasing in size after the plateau.

***Blood collection and radioimmunoassay (RIA).*** Blood samples were collected daily from d -9 to d 20 (d 0 = GnRH2) by tail or jugular venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). After collection, the blood was stored for 24 h at 4°C followed by centrifugation at 1,200 x g for 25 min. Serum was harvested and stored at -20°C until RIA. Serum concentrations of progesterone were measured in all samples by using a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA; Kirby et al., 1997). The intra and inter-assay coefficients of variation for the progesterone RIAs were 3.7 and 8.4%, respectively. Serum concentrations of estradiol-17 $\beta$  were measured by using RIA (Kirby et al., 1997) in samples collected from d -9 to d 0 (d 0 = GnRH2). The intra and inter-assay coefficients of variation were 9.5 and 18.8%, respectively.

***Resumption of the estrous cycle.*** Based on the serum concentrations of progesterone from d 0 to 20 and estrus data, cows were classified as cycling or remaining anestrus (serum concentrations of progesterone remained below 1.0 ng/mL for the duration of the experiment). The cycling cows were further separated into cows with a normal estrous cycle (>18 d) or cows with a short estrous cycle (< 16 d; Rantala et al., 2009).

***Statistical analysis.*** Throughout the analyses the main effects of CIDR administration and ovulation to GnRH1 were analyzed followed by interaction of the main effects. The proportions of cows that ovulated to GnRH, had a small ovulatory follicle, and resumed estrous cycling were analyzed using the GENMOD procedure in SAS (SAS Inst. Inc., Cary, NC). The percentage of cows with a class III follicle (defined as > 9 mm; Moreira et al., 2000) during the treatment period was analyzed using GENMOD for repeated measures over time. The main affect of CIDR administration and ovulation to GnRH1 on the average follicle diameter at GnRH1 and GnRH2, short term follicle growth rates (from d -2 to 0), and serum concentrations of estradiol was analyzed by one-way ANOVA using the two sample T-test in SAS while the interaction of the treatments was analyzed using a general linear model with treatment as the independent variable. Long term follicle growth (from d -5 to 0) was analyzed by a weighted ANOVA for repeated measures over time (PROC MIXED; Littell et al., 1998) in which time points were weighted according to the number of observations recorded at the time points. The correlation between concentrations of estradiol and ovulatory follicle diameter was analyzed with PROC CORR in SAS. Additionally, a multiple regression

model was used to analyze estrus and follicle size as the independent variables and serum concentrations of estradiol as the dependent variable (PROC GLM in SAS).

## RESULTS

***Ovulatory response and follicle diameter.*** Ovulatory response to GnRH1 and GnRH2 were 36 and 71%, respectively. A larger proportion of cows with a follicle that was increasing in diameter at GnRH2 ovulated after GnRH2 than cows with a follicle that had reached a plateau in growth or cows with a decreasing follicle diameter at GnRH2 (20/22, 1/9, and 12/23 for cows with an increase, plateau, or decrease in follicle growth, respectively;  $P < 0.05$ ). Neither ovulation to GnRH1 nor CIDR administration affected the proportion of cows ovulating to GnRH2 ( $P = 0.27$  and  $0.73$ , respectively; Table 4.2).

The range in ovulatory follicle diameter was 8.6 to 16.1 mm with 41% of the follicles smaller than 11 mm. Ovulation to GnRH1, but not CIDR treatment resulted in a larger follicle at GnRH2 ( $P = 0.04$  and  $0.3$ , respectively; Table 4.2; for each treatment group see Table 4.3) and there was more variation in ovulatory follicle diameter at GnRH2 in the Ov1- cows compared to OV1+ cows (variance was 4.0 vs. 2.9, respectively;  $P < 0.05$ ). When only cows that ovulated to GnRH2 were analyzed, cows from the OV1+ treatment group had a decreased proportion of cows that ovulated small follicles in response to GnRH2 compared to Ov1- cows (Ov1+ had 2/16 small ovulatory follicles and Ov1- had 14/23 small ovulatory follicles;  $P = 0.0025$ ). CIDR administration did not affect the proportion of small ovulatory follicles at GnRH2 (CIDR+ had 7/19 small ovulatory follicles and CIDR- had 9/20 small ovulatory follicles;  $P = 0.61$ ) or the variation in ovulatory follicle diameter ( $P > 0.10$ ).

**Table 4.2** Main treatment effects of ovulation to GnRH1 (Ov1+) or failure to ovulate (Ov1-) and presence (CIDR+) or absence (CIDR-) of a controlled internal drug releasing (CIDR) insert on the proportion (%) ovulating after GnRH2, size of the largest follicle (mm  $\pm$  SEM), and serum concentrations of estradiol (pg/mL  $\pm$  SEM) at GnRH2.

Treatment	n	Ovulation after GnRH2	Dominant follicle diameter	Estradiol
<b>GnRH1</b>				
Ov1+	20	16/20 (80)	12.3 <sup>a</sup> $\pm$ 0.5	3.4 <sup>a</sup> $\pm$ 0.5
Ov1-	35	23/35 (66)	11.0 <sup>b</sup> $\pm$ 0.3	2.3 <sup>b</sup> $\pm$ 0.3
<b>CIDR</b>				
CIDR+	26	19/26 (73)	11.8 $\pm$ 0.4	2.8 $\pm$ 0.4
CIDR-	29	20/29 (69)	11.2 $\pm$ 0.4	2.5 $\pm$ 0.3

Differing superscripts were different between treatments ( $P < 0.05$ ).

**Table 4.3** The proportion (%) ovulating, size of the largest follicle (mm  $\pm$  SEM), and serum concentrations of estradiol (pg/mL  $\pm$  SEM) at GnRH2 in each individual treatment group.

Treatment <sup>1</sup>	n	Ovulation after GnRH2	Dominant follicle diameter	Estradiol
Ov1+/CIDR+	9	7/9 (78)	12.3 $\pm$ 0.9	3.9 <sup>a</sup> $\pm$ 0.8
Ov1+/CIDR-	11	9/11 (82)	12.3 $\pm$ 0.7	3.6 <sup>ab</sup> $\pm$ 0.6
Ov1-/CIDR+	17	12/17 (71)	11.6 $\pm$ 0.4	2.4 <sup>ab</sup> $\pm$ 0.4
Ov1-/CIDR-	18	11/18 (61)	10.5 $\pm$ 0.5	2.1 <sup>b</sup> $\pm$ 0.3

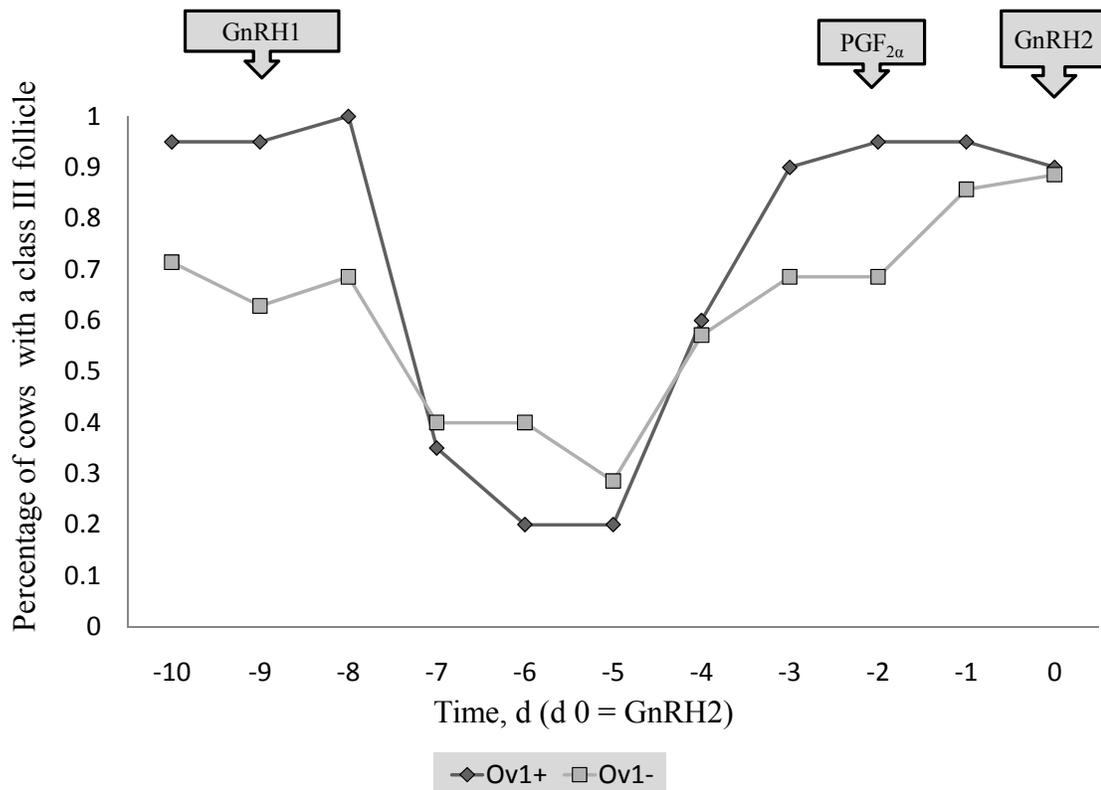
<sup>1</sup> Ovulation to GnRH1 (Ov1+) or failure to ovulate (Ov1-) and presence (CIDR+) or absence (CIDR-) of a controlled internal drug releasing (CIDR) insert.

Differing superscripts were significantly different at  $P = 0.07$ .

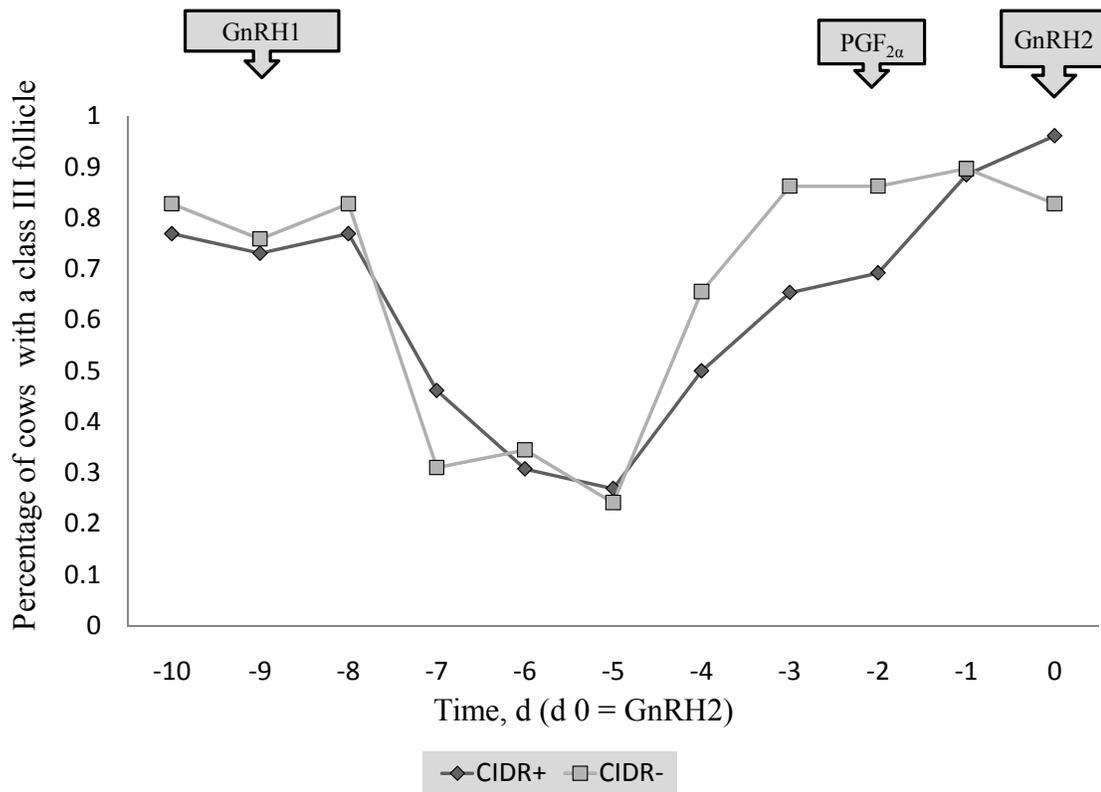
The percentage of cows with a class III follicle (> 9 mm) was affected by treatment day ( $P = 0.0005$ ). Ovulation to GnRH1 and CIDR treatment did not affect the percentage of cows with a class III follicle during the treatment period (Figure 4.1 a and b;  $P = 0.9$ ) nor was there an interaction of these treatments (Figure 4.1c;  $P = 0.21$ ).

**Follicle growth.** The average long term (d -5 to 0) and short term (d -2 to 0) follicle growth rate across all cows was 1.1 and 0.89 mm/d, respectively. Among cows that ovulated to GnRH2, Ov1+ cows had similar follicle growth from d -5 to 0 (1.1 and 1.1 mm/d, respectively; Figure 4.2a;  $P = 0.99$ ) and from d -2 to 0 compared to Ov1- cows (0.77 and 0.70 mm/d, respectively;  $P = 0.9$ ). The ovulatory follicle was already larger on d -5 in Ov1+ cows compared to Ov1- cows (Figure 4.2a;  $P = 0.003$ ). Among cows that ovulated to GnRH2, CIDR+ cows had a faster growth rate from d -2 to 0 (0.97 and 0.50 mm/d, respectively;  $P = 0.03$ ) compared to CIDR- cows but the growth rate was similar from d -5 to 0 (1.2 and 1.1 mm/d, respectively;  $P = 0.99$ ; Figure 4.2b). There was no interaction between CIDR administration and ovulation after GnRH1 in either long term ( $P = 0.97$ ) or short term ( $P = 0.17$ ) follicle growth. Follicle growth was similar ( $P = 0.44$ ) from d -5 to d 0 in cows ovulating large (> 11 mm) compared to small follicles at GnRH2 (1.1 and 1.2 mm/d, respectively; Figure 4.3) but cows that ovulated a large follicle at GnRH2 already had a larger follicle at d -5 compared to cows with a small ovulatory follicle (Figure 4.3;  $P < 0.001$ ). The short term follicle growth rate was positively correlated with the size of the follicle at GnRH2 ( $r = 0.44$  and  $P = 0.005$ ).

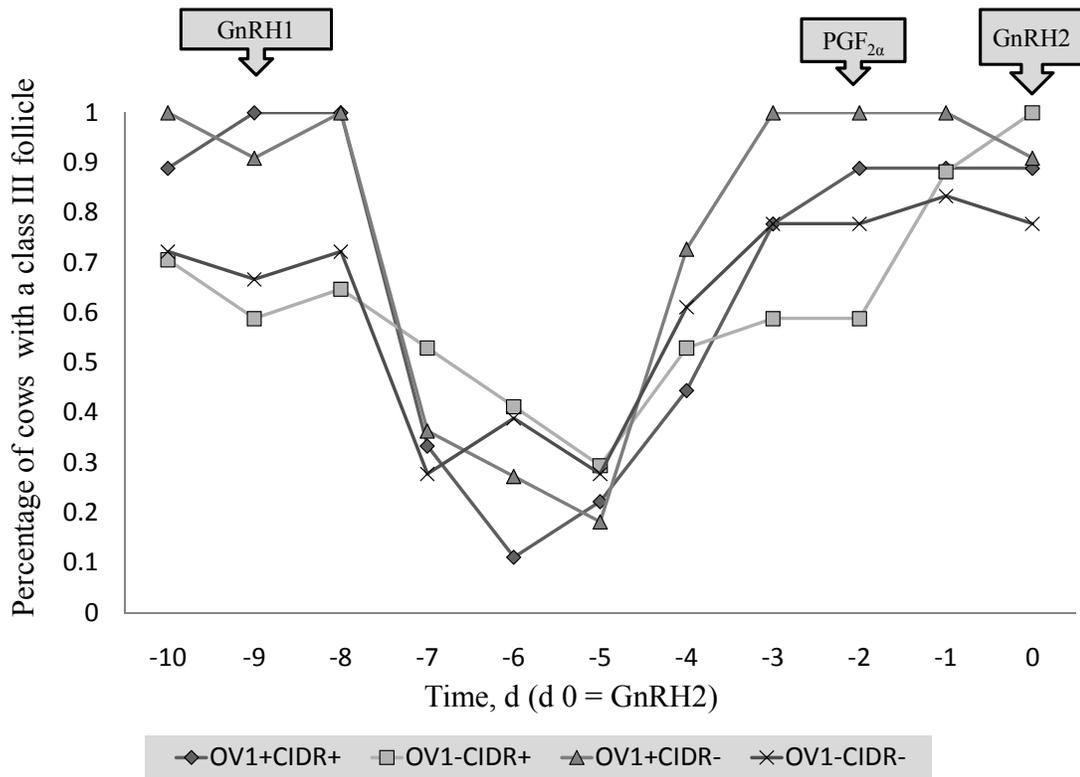
**Serum concentrations of estradiol and estrus.** Ovulation to GnRH1, but not CIDR treatment resulted in increased serum concentrations of estradiol at GnRH2 ( $P = 0.004$  and  $0.59$ , respectively; Table 4.2). The serum concentrations of estradiol were



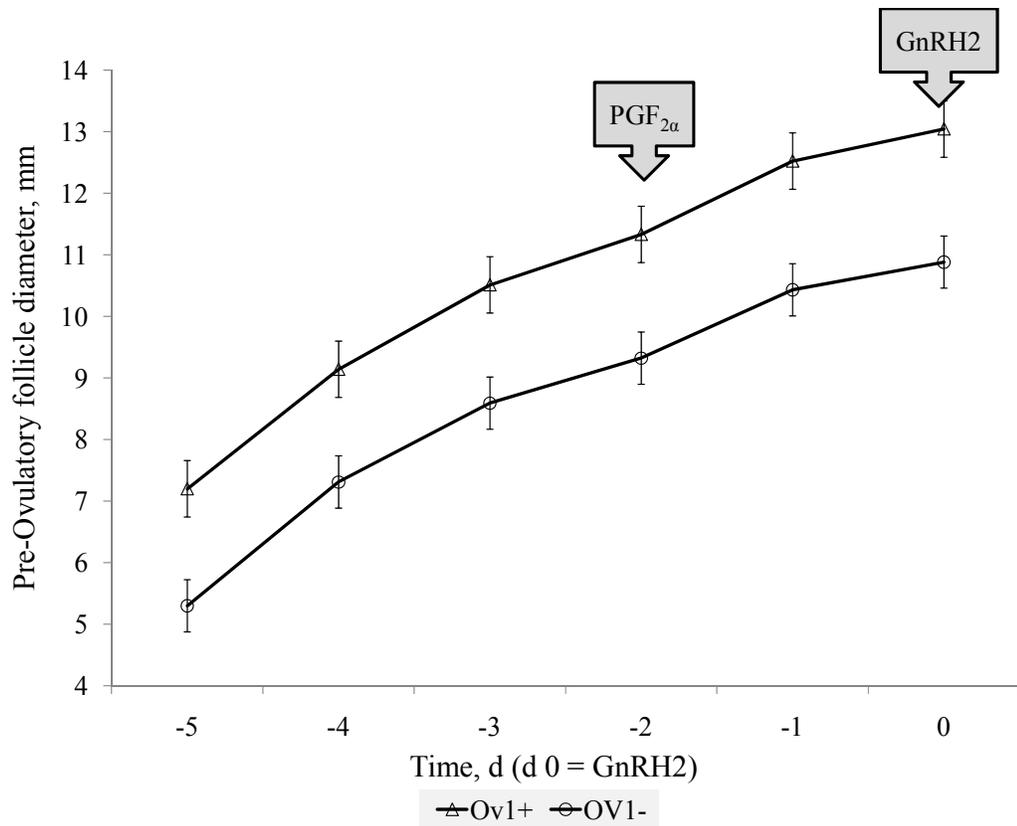
**Figure 4.1a** The percentage of cows with a class III follicle (> 9 mm) during the treatment period. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0. There was no difference in percentage of cows with a class III follicle between cows that did (Ov1+) or did not ovulate (Ov1-) after GnRH1 (a;  $P = 0.09$ ) during the treatment period.



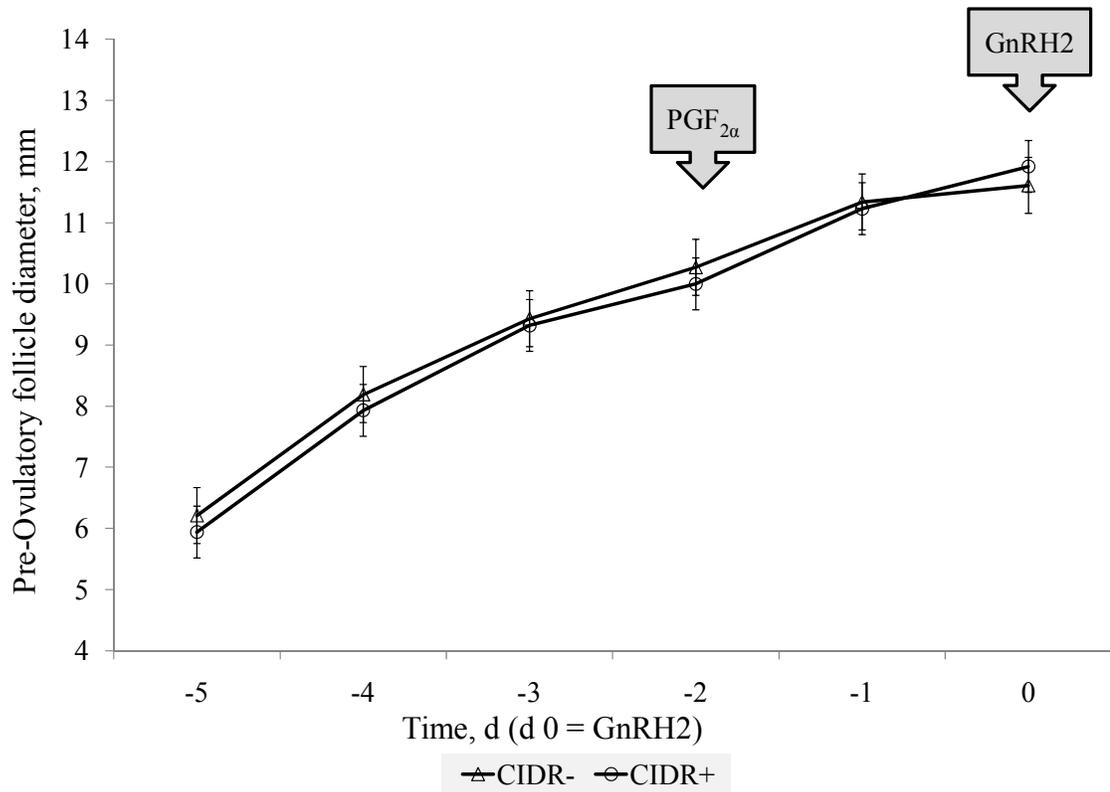
**Figure 4.1b** The percentage of cows with a class III follicle (> 9 mm) during the treatment period. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0. The percentage of cows with a class III follicle during the treatment period did not differ between those administered a CIDR (CIDR+) or not (CIDR-; b; *P* = 0.9).



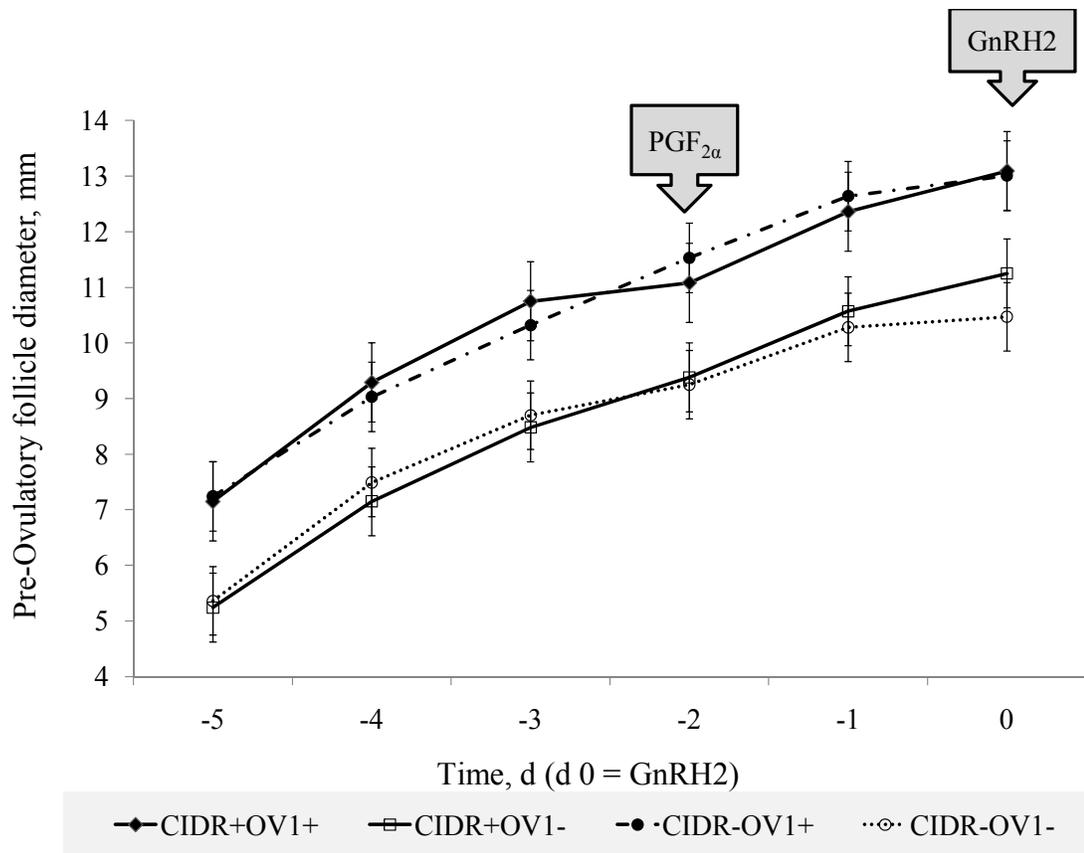
**Figure 4.1c** The percentage of cows with a class III follicle (> 9 mm) during the treatment period. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0. There was no interaction between ovulatory response and CIDR administration on the percentage of cows with a class III follicle over time (*c*;  $P = 0.21$ ).



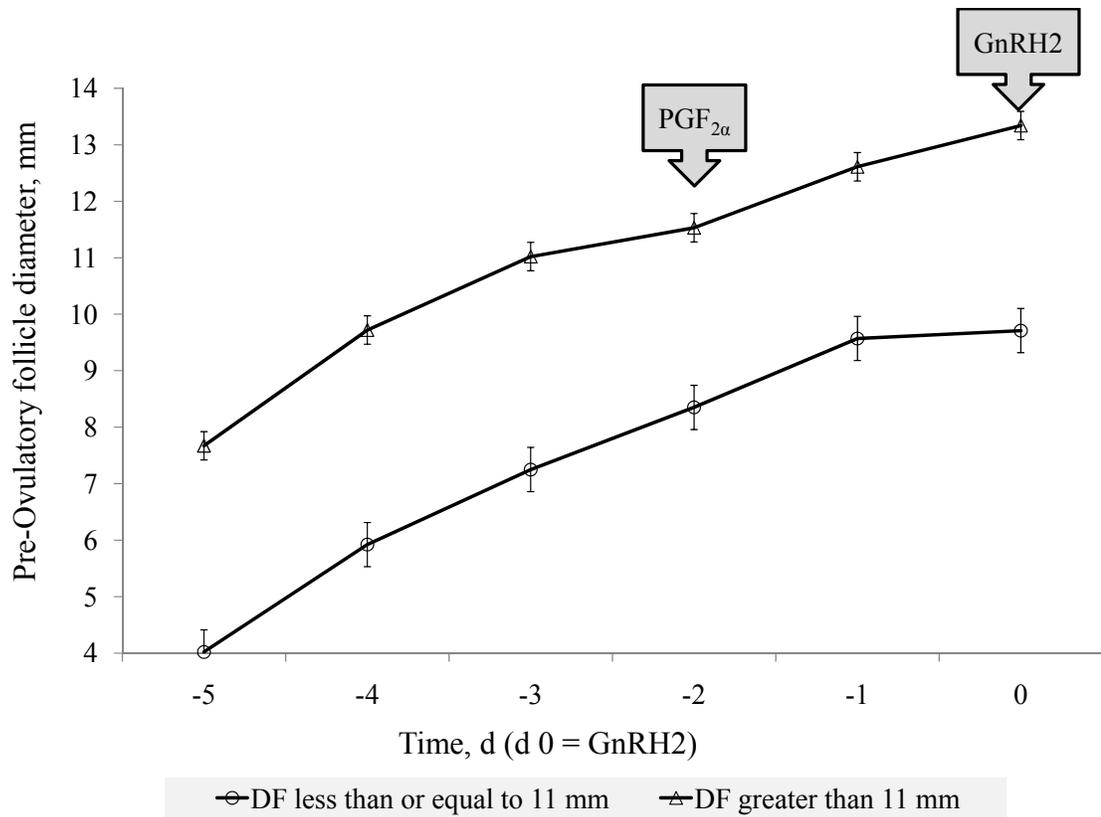
**Figure 4.2a** The pre-ovulatory follicle diameter leading up to GnRH2 by treatment. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0 and only cows that ovulated after GnRH2 were included. Cows that ovulated after GnRH1 (Ov1+; n = 16) had a similar follicular growth rate from d -5 to d 0 ( $P = 0.99$ ) and from d -2 to d 0 ( $P = 0.9$ ; a and c) compared to cows that did not ovulate after GnRH1 (Ov1-; n = 23). The ovulatory follicle was larger at d -5 in Ov1+ cows compared to Ov1- cows ( $P = 0.003$ ).



**Figure 4.2b** The pre-ovulatory follicle diameter leading up to GnRH2 by treatment. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0 and only cows that ovulated after GnRH2 were included. Cows that received a CIDR (CIDR+; n = 19) had a similar follicular growth rate from d -5 to d 0 ( $P = 0.76$ ) compared to cows that did not receive a CIDR (CIDR-; n = 20) but faster growth rate from d -2 to d 0 ( $P = 0.03$ ; b and c).



**Figure 4.2c** The pre-ovulatory follicle diameter leading up to GnRH2 by treatment. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0 and only cows that ovulated after GnRH2 were included. Cows that ovulated after GnRH1 (Ov1+; n = 16) had a similar follicular growth rate from d -5 to d 0 ( $P = 0.99$ ) and from d -2 to d 0 ( $P = 0.9$ ; a and c) compared to cows that did not ovulate after GnRH1 (Ov1-; n = 23). The ovulatory follicle was larger at d -5 in Ov1+ cows compared to Ov1- cows ( $P = 0.003$ ). Cows that received a CIDR (CIDR+; n = 19) had a similar follicular growth rate from d -5 to d 0 ( $P = 0.76$ ) compared to cows that did not receive a CIDR (CIDR-; n = 20) but faster growth rate from d -2 to d 0 ( $P = 0.03$ ; b and c).



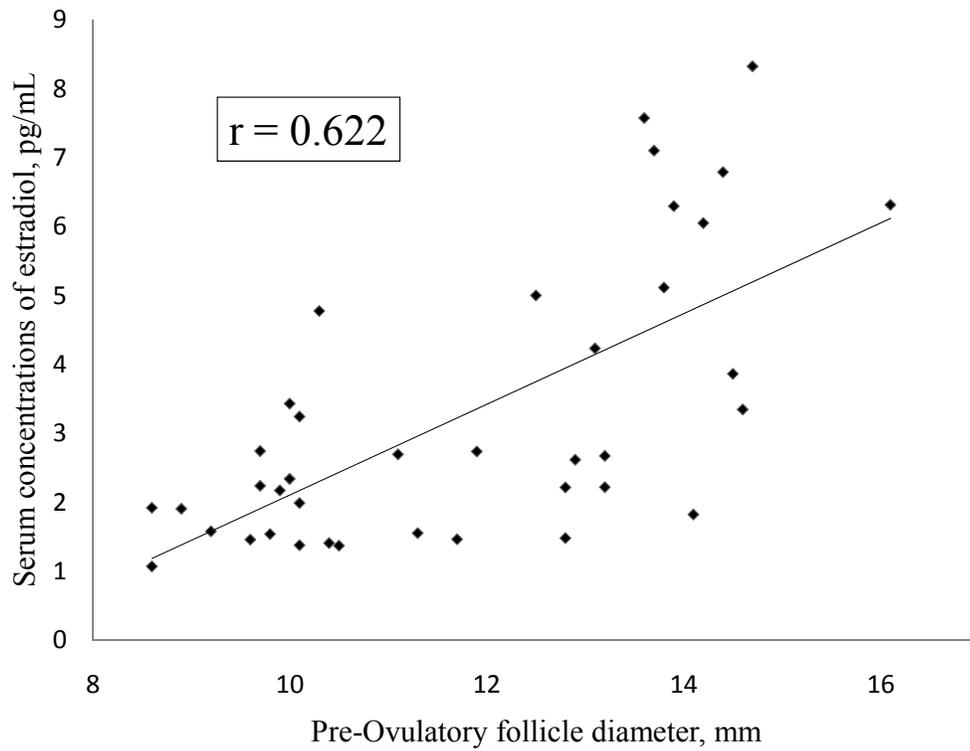
**Figure 4.3** The pre-ovulatory follicle diameter leading up to GnRH2 by ovulatory follicle size. All cows were administered GnRH1 on d -9, PGF<sub>2α</sub> on d -2 and GnRH2 on d 0 and only cows that ovulated after GnRH2 were included. Cows with a large ovulatory follicle (> 11 mm; n = 22) had a similar follicular growth rate from d -5 to d 0 compared to cows that ovulated a small follicle (≤ 11 mm;  $P = 0.01$ ; n = 17) but the follicle was already larger by d -5 in cows that ovulated a large follicle compared to cows that ovulated a small follicle ( $P < 0.001$ ).

positively correlated with size of the dominant follicle at GnRH2 ( $r = 0.62$ ;  $P < 0.0001$ ; Figure 4.4). Only 7 cows were in estrus at the time of GnRH2 and both estrus and follicle diameter had a significant positive relationship with serum concentrations of estradiol the day of GnRH2 ( $P < 0.0001$  and  $P = 0.003$ , respectively).

**Resumption of cyclicity.** The proportion of cows that resumed cycling following GnRH2 was similar between Ov1+ and Ov1- cows (16/20 and 20/35, respectively;  $P = 0.11$ ). Similarly, there was no effect of CIDR administration on the proportion of cows that resumed cycling ( $P = 0.33$ ) nor was there an interaction among treatments ( $P > 0.10$ ; Table 4.4). Of the cows that resumed cycling, there was a treatment interaction in the proportion of cows with a short cycle. Cows in the Ov1-CIDR- group had a lower proportion of normal estrous cycle lengths (more cows with a short cycle) than OV1+ and CIDR+ cows ( $P < 0.05$ ; Table 4.4).

## DISCUSSION

In the current study, there was a large variation in ovulatory follicle size at GnRH2. Perry et al. (2005) reported a range in ovulatory follicle diameter of 9 to 20 mm at the time of GnRH2 and AI and Atkins et al. (unpublished) reported a range of 7.7 to 18.2 mm in cycling beef cows with 43% of the follicles that ovulated less than or equal to 11 mm. Ovulatory follicle size and physiological maturity of the follicle are implicated in contributing to establishment and maintenance of pregnancy in beef (Lamb et al., 2001; Perry et al., 2005; Mussard et al., 2007) and dairy cattle (Vasconcelos et al., 2001). Lamb et al. (2001) reported that beef cows that ovulated a follicle smaller than 12 mm in diameter had reduced pregnancy rates compared to cows that ovulated follicles greater



**Figure 4.4** The ovulatory follicle diameter and serum concentrations of estradiol at GnRH2 were positively correlated ( $r = 0.622$  and  $P < 0.0001$ ). Only cows that ovulated after GnRH2 were included in the analysis ( $n = 39$ ).

**Table 4.4** The proportion (%) of cows that were cycling following GnRH2 and of those cycling, the proportion (%) having a normal length estrous cycle per treatment group.

Treatment <sup>1</sup>	n	Cycling <sup>2</sup>	Normal cycle <sup>3</sup>
Ov1+/CIDR+	9	7/9 (78)	7/7 (100) <sup>a</sup>
Ov1+/CIDR-	11	9/11 (82)	8/9 (89) <sup>a</sup>
Ov1-/CIDR+	17	12/17 (71)	10/12 (89) <sup>a</sup>
Ov1-/CIDR-	18	8/18 (47)	1/8 (12) <sup>b</sup>

<sup>1</sup> Ovulation after GnRH1 (Ov1+) or failure to ovulate (Ov1-) and presence (CIDR+) or absence (CIDR-) of a controlled internal drug releasing (CIDR) insert.

<sup>2</sup>Resumption of cyclicity was based on the pattern of serum concentrations of progesterone after GnRH2. All cows that either had a short luteal cycle followed by a second rise in progesterone or a regular length estrous cycle were consider to be cycling. Cows that continued to have serum concentrations of progesterone below 1.0 ng/mL for the duration of the experiment were considered anestrus.

<sup>3</sup>Of the cows that resumed estrous cycling, cows that had a short rise in serum concentrations of progesterone (< 16 d) were considered to have a short cycle. Cows that had elevated concentrations of progesterone consistent with a regular estrous cycle (18-24 d) were considered normal cycling.

Differing superscripts were different among treatments (<sup>ab</sup> $P < 0.05$ ).

than or equal to 12 mm. Perry and colleagues (2005) also reported that cows induced to ovulate small dominant follicles had reduced serum concentrations of progesterone following ovulation, reduced initial pregnancy rates (d 28 after AI), and more late embryonic/fetal loss by d 60 to 68 of gestation. Interestingly, follicle diameter did not affect pregnancy rates when cows displayed estrus indicating that the physiological status of the follicle is a key factor in establishment and maintenance of pregnancy (Perry et al., 2005). Mussard et al. (2007) also reported reduced serum concentrations of progesterone and pregnancy rates following GnRH-induced ovulation of immature follicles compared to spontaneous ovulation and pregnancy rates improved by 20% (Busch et al., 2008) to 62% (Perry et al., 2005) when cows displayed estrus compared to cows that did not exhibit estrus in a timed AI protocol (Busch et al., 2008).

In the current study, circulating concentrations of estradiol increased as ovulatory follicle diameter increased which has been reported previously in dairy cows (Vasconcelos et al., 2001), beef heifers (Atkins et al., 2008a), and cyclic beef cows (Atkins et al., unpublished). Similarly, Ireland and colleagues (1979) reported increased follicular fluid concentrations of estradiol in larger follicles compared to small follicles. The increase in serum concentrations of estradiol may be indicative of a more physiologically mature follicle. Concentrations of estradiol around the time of ovulation play a significant role in a number of events leading to establishment of pregnancy including sperm transport (Hawk, 1983), follicular cell maturation (McNatty, 1979), and oviductal (Buhi, 2002) and uterine environment (Miller and Moore, 1976; Ing and Tornesi, 1997). Hawk (1983) stated that estrogens are necessary for effective sperm transport through the female reproductive tract and estradiol decreased uterine pH levels

(Perry and Perry, 2008a; Perry and Perry 2008b) which in turn could affect sperm transport and viability. Follicular concentrations of estradiol were correlated with luteal cell secretion of progesterone (McNatty, 1979) suggesting that increased estradiol in the follicular cells may be important to luteal cell development and function. Extending the proestrus period from 1.5 to 2.5 d and thus increasing the duration of elevated preovulatory concentrations of estradiol resulted in increased subsequent luteal progesterone and increased pregnancy rates regardless of the size of the follicle that ovulated (Bridges et al., 2006). Estradiol also affects glycoprotein secretion in the oviduct which was proposed to facilitate sperm motility, fertilization, and possibly embryo cleavage (review by Buhi, 2002). Estradiol increased the expression of endometrial progesterone receptors (Ing and Tornesi, 1997) which may increase progesterone binding and secretions of uterine histotrophe required for early embryo development. Cows exposed to a short proestrus period had increased oxytocin receptor and cyclooxygenase-2 mRNA in the uterus 5 d after GnRH induced ovulation compared to cows ovulating after a long proestrus (Bridges et al., 2006), which could affect timing of uterine prostaglandin release. Finally, estradiol supplementation improved embryo survival in ewes (Miller and Moore, 1976). Taken together, this evidence suggests that adequate serum concentrations of estradiol around ovulation can improve pregnancy through optimizing gamete transport, luteal function, and oviductal and uterine environment. Premature ovulation of small follicles may limit concentrations of estradiol and affect subsequent downstream events associated with fertilization and pregnancy success.

Cows that ovulated after GnRH1 did have larger follicles at GnRH2 compared to cows that did not ovulate after GnRH1. Atkins et al. (unpublished) reported no difference in follicle size at GnRH2 based on ovulatory response to GnRH1 in cyclic beef cows; however, when only cows capable of ovulating at GnRH1 were included in the analysis, then cows that ovulated after GnRH1 had a larger follicle diameter that ovulated after GnRH2 than cows that did not ovulate after GnRH1. In both cycling (Atkins et al., unpublished) and anestrous cows (current study), cows that ovulated after GnRH1 had a larger follicle by d -5 than cows that did not ovulate after GnRH1 and these cows continued to have a larger follicle up to GnRH2. The presence of the larger follicle at d -5 through d 0 may indicate an improved control of the follicle leading to GnRH2 in cows that ovulated after GnRH1 compared to cows that did not ovulate following GnRH1. Cows that failed to ovulate at GnRH1 likely had a follicle either too young (small) to ovulate in response to the GnRH-induced LH surge (Sartori et al., 2001) or an atretic follicle. Considering the former scenario, these cows would have a follicle that is more advanced in age and growth at GnRH2 compared to cows that ovulated to GnRH1 and would likely either become atretic or undergo follicular turnover before the GnRH2 injection. Cows with an atretic follicle at GnRH1 would likely begin a new follicular wave around the same time as cows that ovulated to GnRH1 and may have a similarly synchronized follicular wave.

In the current study, the follicle growth rate for the 5 d leading up to GnRH2 was 1.1 mm/d in cows that ovulated at GnRH2. Dominant follicle growth is dependent on gonadotropins and their receptors as well as local growth factors (TGF $\beta$  super family [bone morphogenic proteins and growth differentiation factor 9 and their receptors],

fibroblast growth factor, and epidermal growth factors), insulin, insulin like growth factors and their binding proteins, controlled extracellular remodeling, and angiogenic factors (reviewed by Webb et al., 2004, Lucy, 2007, and Webb and Campbell, 2007). The average follicular growth rate was 1.6, 1.1, and 1.7 mm/d for the first, second, and third wave follicle in cycling Holstein heifers (n = 10; Fortune et al., 1988). Murphy and colleagues (1990) reported dominant follicle growth rates ranging from 1.4 to 2.1 mm/d in anestrous beef cows (Murphy et al., 1990). The growth rate in the current study was similar to the expected 1 to 2 mm/d (Fortune et al., 1988; Murphy et al., 1990) and to growth rates reported from the same herd in cyclic beef cows (0.89 mm/d; Atkins et al., unpublished).

There was no difference in long term follicle growth (5 d leading up to GnRH2) between cows with a CIDR insert and cows without the CIDR insert. Garcia-Winder et al. (1986) reported that anestrous cows administered a progestogen supplement (norgestomet) had an increase in LH pulse frequency 5 d after the start of treatment compared to cows without the supplement. It is possible that CIDR administration did not affect the long term growth of the ovulatory follicle due to timing of the increase in LH frequency. Progestogen supplementation increased follicle weight, concentrations of estradiol in follicular fluid, and LH receptors in the thecal and granulosa cells in postpartum beef cows compared to cows without supplementation (Inskeep et al., 1988) which may explain the increased short term follicle growth rate in CIDR+ compared to CIDR- cows.

Many reports indicate that progesterone supplementation can induce cyclicity in anestrous cows (Smith et al., 1987; Lucy et al., 2001; Twagiramungu et al., 1995). In the

current study, neither CIDR administration nor ovulation to GnRH1 increased the number of cycling cows. This was unexpected since both CIDR administration (Lucy et al., 2001) and GnRH- induced ovulation (Twagiramungu et al., 1995) were able to induce non-cycling heifers and cows to cycle. The CL that forms after the first ovulation has a shortened lifespan compared to subsequent CL (Berardinelli et al., 1979; La Voie et al., 1981) in suckled beef cows. Among cows that did return to estrous cycling, those that either received a CIDR or ovulated following GnRH1 (and therefore had a CL secreting progesterone) had reduced incidence of short luteal phases compared to OV1-CIDR-cows. Our results agree with the concept that previous progesterone exposure affects the timing of PGF<sub>2α</sub> release by the uterus and thus length of the subsequent luteal phase in lactating beef cows (reviewed by Inskoop, 2004 and Yavas and Walton, 2000). This shortened luteal phase is thought to occur due to an advanced release of PGF<sub>2α</sub> from the uterus (Copelin et al., 1989). Exposure of progesterone followed by estrogen is needed to correct the timing of PGF<sub>2α</sub> release from the uterus (Cooper et al., 1991; Kieborz-Loos et al., 2003). Administration of exogenous progesterone to anestrous cows primes the uterus for appropriate timing of PGF<sub>2α</sub> release resulting in normal CL lifespan and estrous cycle length.

In summary, anestrous cows that ovulated after GnRH1 had larger follicles and fewer of those cows ovulated a small follicle compared to cows that did not ovulate after GnRH1. Administration of a CIDR did not affect ovulatory follicle diameter. The long term follicular growth rate was independent of ovulatory response following GnRH1, CIDR administration, or size of the ovulatory follicle at GnRH2 in anestrous cows. Administration of a CIDR did increase the short term follicle growth rate. Ovulatory

follicle diameter was positively correlated with serum concentrations of estradiol on the day of GnRH2 in anestrous cows. These findings suggest that in anestrous beef cows, ovulation to GnRH1 increased the size of the follicle at GnRH2 and decreased the proportion of cows ovulating a small follicle at GnRH2. Thus, increasing the proportion of cows ovulating to GnRH1 would likely increase the fertility of anestrous cows ovulating at GnRH2.

## CHAPTER V

### CONTRIBUTIONS OF OOCYTE QUALITY AND UTERINE ENVIRONMENT TO ESTABLISHMENT AND MAINTANENCE OF PREGNANCY: USING RECIPROCAL EMBRYO TRANSFER

**ABSTRACT.** GnRH-induced ovulation of a small dominant follicle reduced pregnancy success in cattle. A reciprocal embryo transfer approach was used to differentiate between the effect of oocyte quality and uterine environment on pregnancy. Suckled beef cows (n = 1,166) were administered GnRH on d -9 (GnRH1), PGF<sub>2α</sub> on d -2, and GnRH (GnRH2) either with (donor cows; n = 810) or without (recipient cows; n = 354) artificial insemination on d 0. Single embryos (n = 394) or oocytes (n = 45) were recovered from the donor cows (d 7) and all live embryos were transferred into recipients the same day. Embryos from cows that ovulated a small follicle (< 12.5 mm) were transferred into cows that ovulated a large follicle (≥ 12.5 mm) and vice versa resulting in the following treatment groups; small to small (S-S; negative control; n = 71), small to large (S-L; primary effects of oocyte quality; n = 111), large to small (L-S; primary effects of uterine environment; n = 122) and large to large (L-L; positive control; n = 50). The probability of a live fertilized embryo increased with increasing diameter of the ovulatory follicle and serum concentrations of progesterone on d 7 ( $P = 0.01$  and  $0.02$ ,

respectively). The stage of embryo development was positively associated with serum concentrations of progesterone at PGF<sub>2α</sub> ( $P = 0.006$ ) but not associated with ovulatory follicle diameter at GnRH2 or d7 serum concentrations of progesterone ( $P = 0.6$  and  $0.4$ , respectively). There was no main effect of recipient or donor follicle size group on initial (d 27;  $P = 0.9$  and  $0.5$ , respectively) or final (d 72;  $P = 0.6$  and  $0.4$ , respectively) pregnancy rates nor was there an interaction between donor and recipient follicle size group (proportion pregnant at d 27 [35/71, 60/111, 67/122, and 28/50 pregnant in the S-S, S-L, L-S, and L-L treatments, respectively] and d 72 [33/71, 51/111, 64/122, and 25/50, in the S-S, S-L, L-S, and L-L treatments, respectively];  $P = 0.9$  and  $0.7$ , respectively). However when follicle diameter was analyzed as a continuous variable, the probability of pregnancy increased with increasing ovulatory diameter in the recipients cows ( $P = 0.05$ ) independent of donor follicle diameter ( $P = 0.6$ ). Similarly, recipient concentrations of progesterone at ET was a positive predictor of pregnancy but donor progesterone at ET did not influence pregnancy ( $P < 0.001$  and  $0.3$ , respectively). In summary, ovulatory follicle diameter of the donor cows influenced the probability of a fertilized and live embryo seven days after breeding but did not influence pregnancy rates subsequent to embryo transfer. Conversely, the probability of pregnancy increased as the diameter of the recipient ovulatory follicle and serum progesterone at ET increased suggesting that uterine environment (rather than oocyte competence) seven days after breeding is important to pregnancy success.

## INTRODUCTION

Ovulatory follicle size can affect pregnancy rates following fixed timed insemination in cattle (beef heifers: Perry et al., 2007; beef cows: Lamb et al., 2001,

Perry et al., 2005, Meneghetti et al., 2009; and dairy cows: Vasconcelos et al., 2001, Bello et al., 2006; Lopes et al., 2007). Cows induced to ovulate a small dominant follicle ( $\leq 11.3$  mm) had reduced pregnancy rates and more embryonic/early fetal loss compared to cows induced to ovulate a large follicle (optimum follicle diameter was 14.8 mm; Perry et al., 2005). However, ovulatory follicle diameter did not affect pregnancy outcome in cows that displayed estrus (Perry et al., 2005), suggesting that physiological maturity of the follicle rather than simply size is important to pregnancy success.

The mechanisms by which GnRH-induced ovulation of a small dominant follicle affect the establishment and maintenance of pregnancy are unknown but could involve ovulation of an incompetent oocyte (Arlotto et al., 1996; Brevini-Gandolfi and Gandolfi, 2001), inadequate corpus luteum (CL) function (Vasconcelos et al., 2001; Perry et al., 2005; Busch et al., 2008), altered oviductal environment, compromised uterine environment (Murdoch and van Kirk, 1998; Bridges et al., 2006; Moore, 1985), or a combination thereof. The oocyte continues cytoplasmic, nuclear, and molecular maturation within the developing follicle (reviewed by Sirard et al., 2006). Therefore, the following experiment used a reciprocal embryo transfer approach (embryos collected from donor cows that ovulated a large follicle were transferred into recipient cows that ovulated a small follicle and vice versa) to ascertain the contribution of oocyte competence vs. maternal environment to pregnancy success in suckled beef cows.

Our hypothesis was that the maternal environment established following ovulation of a large follicle would result in greater pregnancy rates compared to cows that ovulated a small follicle regardless of donor ovulatory follicle size. The primary objective of this experiment was to differentiate between the contribution of follicular

determinants of oocyte competence compared to maternal environment affects on the establishment and maintenance of pregnancy. The following endpoints were used to examine follicular determinants of pregnancy: CL function (by progesterone concentration), fertilization rate, embryo survival and grade (7 d after insemination), and prediction of pregnancy success based on ovulatory follicle size of the donor and recipient cows following single embryo recovery and transfer.

## MATERIALS AND METHODS

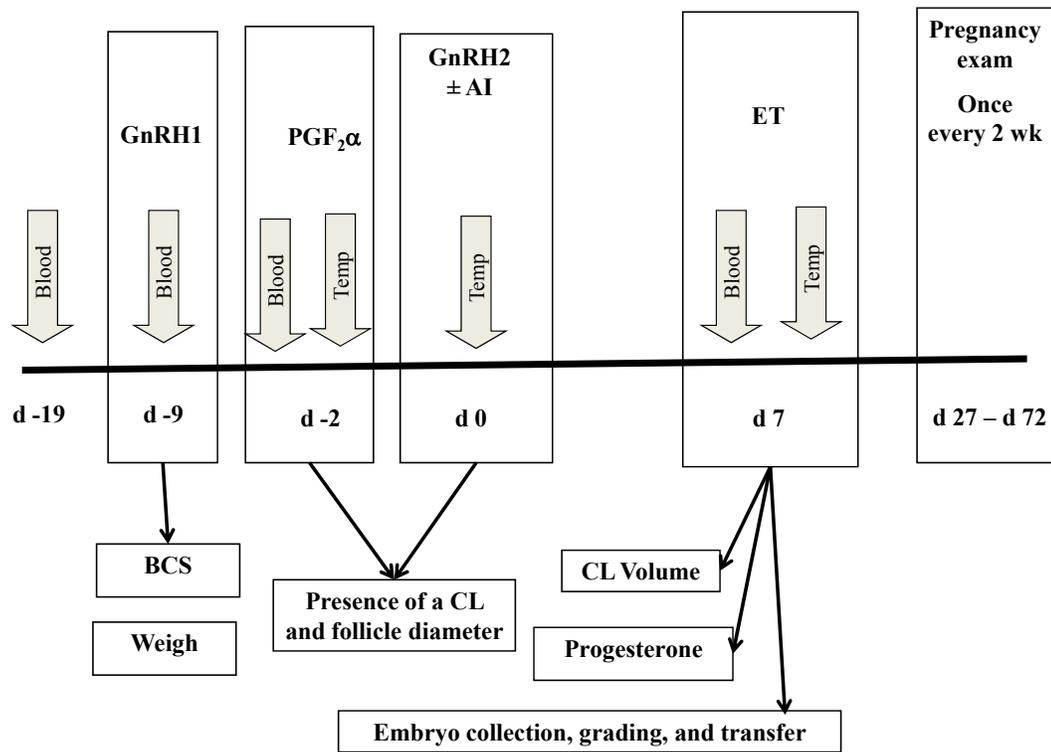
***Animal handling.*** All protocols and procedures were approved by the Fort Keogh Livestock and Range Research Laboratory (LARRL) Animal Care and Use Committee (IACUC approval no. 101106-3). This trial occurred over three years with 9, 11, and 5 groups of suckled postpartum beef cows in year 1, 2, and 3, respectively. This study includes data on 1,164 single ovulations in suckled beef cows that were synchronized with the CO-Synch protocol (GnRH [**GnRH1**] on d -9 followed by PGF<sub>2α</sub> [d -2] and GnRH [**GnRH2**] on d 0 with fixed-timed artificial insemination [**AI**] of which 810 were donor cows (inseminated on d 0) and 354 were recipient cows (not inseminated). Cows were inseminated by one of three artificial insemination technicians with semen from one sire (three different collections). Single embryos were recovered by uterine horn lavage 7 d after GnRH2 and all live embryos were transferred fresh into recipients on the same day as recovery. Body weights and body condition assessment (**BCS**; scale of 1 to 9 where 1 = emaciated and 9 = obese) were obtained on the day of GnRH1. Rectal temperature was collected on the day of GnRH1, GnRH2, and embryo

transfer (ET) for donor and recipient cows. See Figure 5.1 for experimental sample collection timeline.

***Estrous Detection.*** Visual estrous detection occurred once daily from GnRH1 to PGF<sub>2α</sub> and twice daily for 1 hr from PGF<sub>2α</sub> until the day of GnRH2 in all groups and continued after GnRH2 in a subset of the groups (2 groups in year 1 and all groups in years 2 and 3). Estrus Alert (Western Point Inc., Apple Valley, MN) estrous detection patches were used on all cows to aid in estrous detection. Data from cows that were in estrus before GnRH2 or 2 to 7 d after GnRH2 were not included in the study.

***Ovarian structures.*** Ovaries were examined by using an Aloka 500V ultrasound with a 7.5 MHz transducer (Aloka, Wallingford, CT). The diameter of the largest follicle on each ovary was measured on d -2 (PGF<sub>2α</sub>) and d 0 (GnRH2) and the number of CL present on d -2 and d 0. The diameter and lumen of the CL was measured in both donor and recipient cows on the day of ET (d 7). The diameters of the follicles and CL were calculated as the average of the width at the widest point and the width perpendicular to the widest point. The volume of both the CL and lumen were calculated by using the equation for volume of a sphere ( $4/3\pi r^3$ ) and the functional CL volume was determined by subtracting the luminal volume from the CL volume (Utt et al., 2009). Follicles  $\leq$  12.4 mm were considered small follicles and  $\geq$  12.5 mm considered large follicles based on previous reported follicle diameter in beef cows (Lamb et al., 2001 and Perry et al., 2005).

***Ovulatory Response.*** Ovulation following GnRH1 was estimated using cyclicity status and number of CL present at PGF<sub>2α</sub>. Cows with 0 CL or cycling cows with 1 CL



**Figure 5.1** Experimental design. See table 5.1 for a description of the treatment groups. Includes data on 1,164 single ovulations in suckled beef cows that were treated with the CO-Synch protocol (GnRH1 on d -9, PGF<sub>2α</sub> on d -2, and GnRH2 with [donor cows; n = 810] or without [recipient cows; n = 354] fixed timed artificial insemination [AI] at d 0). Embryo collection, grading, and transfer (ET) occurred 7 d after GnRH2 administration. Pregnancy diagnosis began on d 27 to 29 (d 0 = GnRH2) and continued every other week until d 72 to 74 (d 0 = GnRH2). Blood = blood collection for quantification of serum concentrations of progesterone, Temp = rectal temperature, BCS = body condition score.

were classified as not ovulating after GnRH1. Non-cycling cows with one CL at PGF<sub>2α</sub> and cycling cows with two CL were considered to have ovulated in response to GnRH1. There was a subset of cows that were unable to be classified due to inconsistencies in the number of CL or cycling cows that had low progesterone at GnRH1 and a CL at PGF<sub>2α</sub> (n = 217). Cows with a CL at ET on the same side as the dominant follicle at GnRH2 that had not been in estrus the previous 5 d were considered to have ovulated following GnRH2.

***Embryo recovery and transfer.*** Embryos were recovered from donor cows by using a non-surgical transcervical uterine catheterization technique on d 7 (d 0 = GnRH2). Since these were all single embryo recoveries, the catheters were placed in the uterine horn ipsilateral with the CL and a single horn was lavaged with ViGro Complete Flush Solution (Bioniche Animal Health, Canada Inc., Bellville, Ontario). Embryos were transferred fresh into the uterine horn ipsilateral to the CL on the same day as collection by one of two technicians. Embryos collected from cows that ovulated a small follicle were transferred into cows that ovulated a large (n = 111) or small (n = 71) follicle. Similarly, embryos collected from cows that ovulated a large follicle were transferred into cows that ovulated a large (n = 50) or small (n = 122) follicle resulting in four treatment groups: 1) small into small, 2.) small into large, 3) large into small, and 4.) large into large (Table 5.1). The small to small treatment group was used as a negative control since these recipients should have the lowest pregnancy rate. Similarly, the large to large treatment group was a positive control as they should have the highest pregnancy rate. The small to large treatment group was designed to test if an embryo derived from an oocyte from a small follicle transferred into the uterine environment established by

**Table 5.1** Description of treatment group (trt), the primary effect tested, and number of embryos transferred in each treatment.

Trt <sup>1</sup>	GnRH-induced follicle size			n
	Donor	Recipient	Test	
S-S	Small	Small	Negative control	71
S-L	Small	Large	Effect of oocyte	111
L-S	Large	Small	Effect of uterine environment	122
L-L	Large	Large	Positive control	50
			Total	354

<sup>1</sup> Treatments based on ovulatory follicle size at GnRH2 of the donor and recipient cows. Embryos collected from cows that ovulated a small follicle (<12.5 mm) or large follicle ( $\geq 12.5$  mm) were transferred into recipients that ovulated a small or large follicle resulting in the following treatment groups: small to small (S-S), small to large (S-L), large to small (L-S), and large to large (L-L).

ovulation of a large follicle would have normal pregnancy rates. The large to small transfer group was designed to test if an embryo derived from an oocyte from a large follicle transferred into a uterine environment established by ovulation of a small follicle would have normal pregnancy rates.

***Embryo handling.*** Embryos and oocytes were washed 3 times in holding media (Biolife Holding Media, AgTech Inc., Anderson Avenue, Manhattan, KS) and stored at 26°C until grading and transfer. The time of grading was recorded and used as an estimation of embryo age (time from GnRH2 administration until grading). Embryo development (scale of 1 to 7 where 1 = unfertilized oocyte [**UFO**] and 7 = expanded blastocyst) and quality (scale of 1 to 4 where 1 = excellent to good [85% of the cellular mass was intact and healthy in appearance], 2 = fair [between 50 to 85% of the cellular mass was intact and healthy in appearance and no abnormalities in embryo shape], 3 = poor [over 50% of the cellular mass is extruded or degenerating or gross abnormalities in the structure of the embryo], and 4 = degenerate or dead; based on the classifications set by the International Embryo Transfer Society [Savoy, IL]) were determined. All live embryos (quality grades 1 to 3) were transferred into recipients except embryos that were lost or damaged prior to transfer (n = 8) or did not have a recipient (n = 2). Additionally, there were some structures recovered that appeared to be empty zona pellucida (n = 6). The empty zona pellucida were not included in the analysis.

***Detection of spermatozoa within the zona pellucida.*** Spermatozoa within the zona pellucida of a portion of the degenerating embryos (n = 8), UFOs (n = 21), an empty zona (n = 1) and a normal embryo (n = 1) were detected by using previously described procedures (Machaty et. al., 1998). Briefly, embryos and UFOs were fixed at the time of

collection in individual tubes containing 1 mL of 10% neutral buffered formalin. At the time of staining, the preceding structures were recovered and placed in 2 microliter drops of media (KSOM) containing a DNA stain (bisBenzimide; Sigma Chemical Co, St Louis, MO) that had been diluted 1:1000 (stock concentration = 2 mg/ml; 3.8 mM). Spermatozoa within the zona pellucida were detected and counted with a fluorescent microscope.

***Pregnancy diagnosis.*** Pregnancy diagnosis began on d 27 to 29 after GnRH2 (or 20 to 22 d after transfer) and continued once every 2 wk until d 70 to 72 after GnRH2 for a total of four pregnancy exams per recipient. An Aloka 500V ultrasound was used with a 7.5 MHz and 5.0 MHz transducer (depending on the stage of pregnancy; Aloka, Wallingford, CT) to determine presence of a fetus and fetal heartbeat.

***Blood collection and radioimmunoassay (RIA).*** Blood was collected via tail venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA) on d -19, -9 (GnRH1), -2 (PGF<sub>2α</sub>), and 7 (ET). Blood was incubated at 4°C for 24 hr and centrifuged at 1,200 g for 25 min. Serum was harvested and stored at -20°C until use in RIA. Serum concentrations of progesterone were quantified by RIA with a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA) as described previously (Bellows et al., 1991). Intra and inter-assay coefficients of variation were 1.8 and 13%, respectively and the assay sensitivity was 0.08 ng/mL for the progesterone RIAs. Serum concentrations of progesterone at d -19 and d -9 were used to determine cycling status of the cows (if both samples < 1.0 ng/mL then the cow was considered to be anestrus).

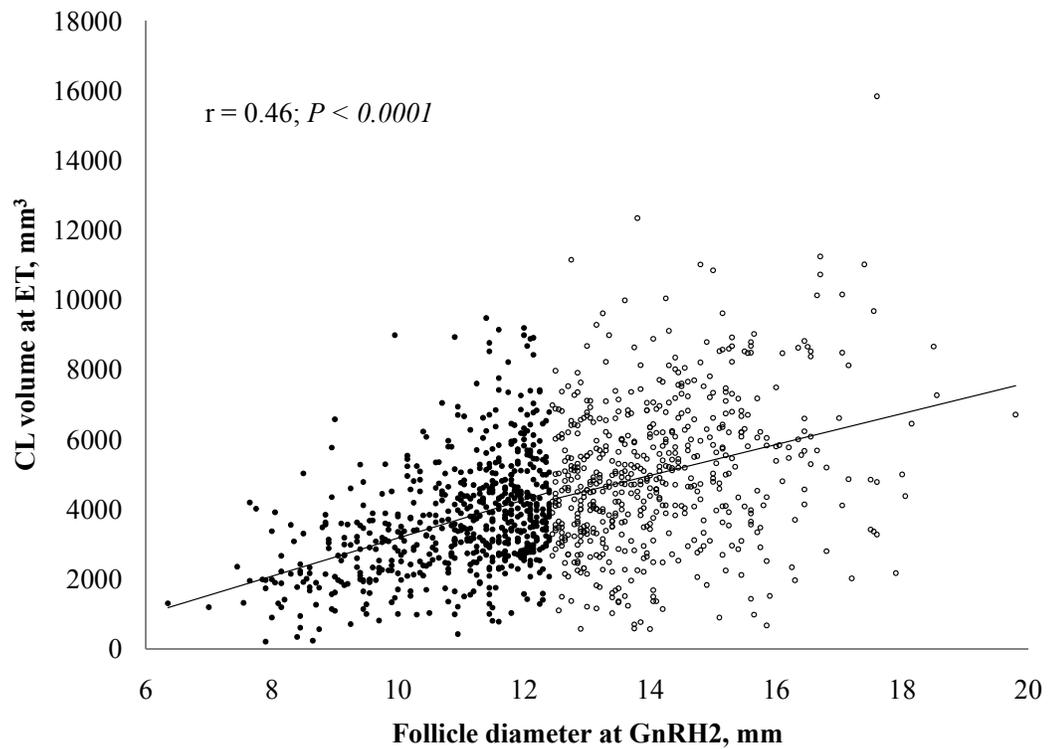
***Statistical analysis.*** All statistical analyses were conducted by using SAS (SAS Inst. Inc., Cary, NC). Serum concentrations of progesterone at PGF<sub>2α</sub> and follicle size at

GnRH2 between cows that did or did not ovulate in response to GnRH1 were analyzed with the two-sample T test. The correlation between follicle diameter at GnRH2 and serum concentrations of progesterone and CL volume were analyzed with PROC CORR. Binomial response variables (fertilization, embryo survival, transferrable embryos, and pregnancy [initial and final]) were analyzed with PROC LOGISTIC for multi-variate logistic regression. Initially all variables that could potentially affect the response were included in the analysis and non-significant variables were removed from the model, beginning with the variable with the highest P value, until all variables had a P value of  $\leq 0.2$ . Variables included in the model for fertilization, embryo survival, and embryo grades were days post partum at the start of treatment, cycling status, age, weight, BCS, year (orthogonal contrast between year 1 and 2 and comparing year 1 and 2 to year 3), group, AI technician (orthogonal contrast between AI technician 1 and 2 and comparing 1 and 2 to 3), semen collection (orthogonal contrast between collection 1 and 2 and comparing 1 and 2 to 3) follicle size, serum concentrations of progesterone (at PGF<sub>2 $\alpha$</sub>  and ET), rectal temperature (at GnRH2 and ET), estrus, ovulation to GnRH1, and follicle size. The above variables and approach of removing none significant variables were also included in the PROC GLM procedure to analyze differences in embryo stage and quality. The same variables were included in the model for pregnancy plus the addition of embryo quality, stage, and transfer technician. Predictions of success (fertilization, embryo survival, transferrable embryo, and pregnancy) were calculated from the multiple regression model generated above.

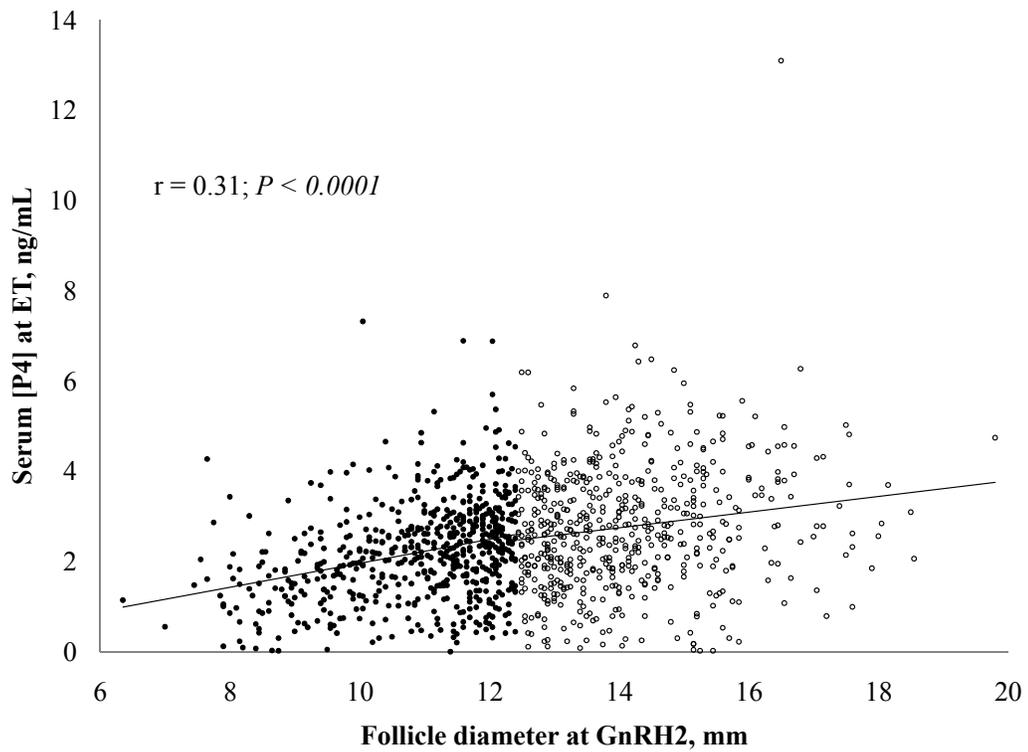
## RESULTS

The mean follicle diameter of all cows at GnRH2 was  $12.5 \pm 0.06$  mm. Mean follicle diameter was likely skewed to a smaller size as more of the treatment groups required a small rather than a large follicle. From the estimates of ovulation to GnRH1, 59% of the cows responded to GnRH1 (564/949) and cows that ovulated after GnRH1 had an increased follicle diameter at GnRH2 ( $12.4 \pm 0.7$  mm compared to  $12.1 \pm 0.1$  mm, respectively;  $P = 0.02$ ). Cows that ovulated after GnRH1 also had increased serum progesterone at PGF<sub>2 $\alpha$</sub> , but estrous response was not different between cows that did or did not ovulate to GnRH1. Serum concentrations of progesterone at PGF<sub>2 $\alpha$</sub>  were negatively correlated with ovulatory follicle diameter at GnRH2 ( $r = -0.18$ ;  $P < 0.0001$ ) but positively correlated with serum concentrations of progesterone at ET ( $r = 0.214$ ;  $P < 0.0001$ ). Follicle diameter at GnRH2 was positively associated with CL volume and serum concentrations of progesterone at ET ( $r = 0.46$  and  $0.31$ , respectively;  $P < 0.0001$ ; Figure 5.2a and b).

*Fertilization.* The total recovery rate of both oocytes and embryos was 54% (439/810). Of these, 10.3% were unfertilized (45 UFOs), 2 were lost before grading, and 6 empty zona pellucidas were found which were not included in the analysis. Among donor cows from which a structure was recovered, as follicle diameter increased, the probability of fertilization increased ( $P = 0.005$ ). Time of year (group), AI technician, age of the donor, and rectal temperature at GnRH2 were all significant in the prediction equation for fertilization ( $P = 0.04$ ,  $0.02$ ,  $0.01$ , and  $0.04$ , respectively). There was a decrease in fertilization rates earlier in the season, in older cows, and in cows with lower rectal temperature at AI (Table 5.2). Some of the ‘unfertilized oocytes’ had 1 or more



**Figure 5.2a** Scatter plot displaying correlation between diameter of the dominant follicle at GnRH2 and volume of the corpus luteum (CL) at embryo transfer (ET). Follicle diameter and CL volume were positively correlated ( $r = 0.46$ ;  $P < 0.0001$ ;  $n = 1164$ ).



**Figure 5.2b** Scatter plot displaying correlation between dominant follicle diameter at GnRH2 and serum concentrations of progesterone [P4] at reciprocal embryo transfer (ET). Follicle diameter and concentrations of P4 were positively correlated ( $r = 0.31$ ;  $P < 0.0001$ ;  $n = 1164$ ).

**Table 5.2** Mean ( $\pm$  SEM) ovulatory follicle diameter (mm), age of the dam (yr), and rectal temperature (F) in cows from which an embryo (fertilized) or unfertilized oocyte was recovered.

Variable	Fertilized (n = 394)	Unfertilized (n = 45)	<i>P</i> value
Follicle size	12.6 $\pm$ 0.1	11.7 $\pm$ 0.4	0.005
Age of the donor:	4.2 $\pm$ 0.1	5.0 $\pm$ 0.4	0.01
Donor rectal temp at GnRH2	101.5 $\pm$ 0.03	101.3 $\pm$ 0.1	0.04

\*Additionally time of year of embryo recovery (two week increments starting at the end of May), year, and artificial insemination technician were significant and included as covariates ( $P = 0.04$ ,  $0.09$ , and  $0.02$ , respectively).

sperm bound to the zona pellucida (5/21, four of which were from donors that ovulated a small follicle and one was from a donor that ovulated a large follicle).

*Embryonic development stage.* The majority of embryos collected were morulas followed by early blastocysts, early morulas, 2 to 12 cell embryos, blastocysts and expanded blastocysts (n = 221, 79, 54, 19, 9, and 1). Neither ovulatory follicle diameter nor serum concentrations of progesterone affected embryonic stage of development ( $P = 0.6$  and  $0.4$ , respectively; Table 5.3). As time from GnRH2 to embryo grading increased there was an increase in developmental stage ( $P = 0.01$ ). There was a negative relationship between donor age and embryo stage of development ( $P = 0.02$ ) and a positive relationship between serum concentration of progesterone at PGF<sub>2α</sub> and embryo stage ( $P = 0.006$ ; Table 5.3). Other factors that were significant and included in the model were year and AI technician ( $P = 0.004$  and  $P = 0.02$ , respectively).

*Embryo quality.* The majority of the embryos collected had a quality grade of excellent to good followed by fair, poor, and dead embryos (n = 242, 84, 34, and 26). Follicle diameter at GnRH2 was positively related to embryo quality ( $P = 0.04$ ; Table 5.4) and survival ( $P = 0.05$ ). Other factors that were significant in predicting embryo quality were rectal temperature of the donor cow at ET (positively related to quality;  $P = 0.04$ ) and AI technician ( $P = 0.002$ ). The proportion of embryos that were alive at ET was increased in cows that had elevated concentrations of progesterone at PGF<sub>2α</sub> but serum concentrations of progesterone at ET was not predictive of embryo survival ( $P = 0.03$  and  $0.2$ , respectively; data not shown). Collectively, the probability of having a transferable embryo (not a UFO or a dead embryo) at ET was positively associated with

**Table 5.3** Mean  $\pm$  SEM embryo age<sup>1</sup> (hours), donor age (yr), serum concentrations of progesterone (P4; ng/mL) on the day of PGF<sub>2 $\alpha$</sub>  administration, and on the day of embryo transfer (ET; 7 days after GnRH2), and size of the GnRH- induced ovulatory follicle (mm) in cows from which embryos of various stages<sup>2</sup> were recovered.

	2 to 12 cell	Early Morula	Morula	Early Blastocyst and beyond <sup>3</sup>	<i>P</i>
n	19	54	224	89	-
Embryo age	176 $\pm$ 0.6	175.3 $\pm$ 0.3	176.1 $\pm$ 0.1	176.7 $\pm$ 0.2	0.01
Donor age	4.3 $\pm$ 0.4	4.6 $\pm$ 0.32	4.4 $\pm$ 0.16	3.4 $\pm$ 0.16	0.02
P4 at PGF <sub>2<math>\alpha</math></sub>	3.4 $\pm$ 0.5	3.9 $\pm$ 0.3	4.6 $\pm$ 0.2	5.1 $\pm$ 0.3	0.006
P4 at ET	2.03 $\pm$ 0.2	2.3 $\pm$ 0.14	2.5 $\pm$ 0.07	2.5 $\pm$ 0.1	0.4
Follicle size	12.0 $\pm$ 0.5	12.8 $\pm$ 0.24	12.7 $\pm$ 0.1	12.3 $\pm$ 0.15	0.6

<sup>1</sup>Embryo age determined as the amount of time from GnRH2 administration to embryo grading.

<sup>2</sup>Also year and artificial insemination technician were significant and included as covariates in the model (*P* = 0.004 and 0.02, respectively). This analysis did not include unfertilized oocytes, empty zona pellucidas, or non-graded embryos (n = 45, 6, and 2, respectively).

<sup>3</sup>Includes nine blastocysts and one expanded blastocyst.

**Table 5.4** Mean  $\pm$  SEM ovulatory follicle diameter (mm; at GnRH -induced ovulation), serum concentrations of progesterone (P4, ng/mL; at embryo transfer [ET]) and rectal temperature ( $^{\circ}$ F) at ET in cows from which excellent, fair, poor, or dead embryos<sup>1</sup> were recovered.

	Excellent	Fair	Poor	Dead	<i>P</i>
n	242	84	34	26	--
Follicle size	12.6 $\pm$ 0.1	12.7 $\pm$ 0.2	12.2 $\pm$ 0.3	12.2 $\pm$ 0.5	0.05
P4 at ET	2.45 $\pm$ 0.1	2.47 $\pm$ 0.1	2.4 $\pm$ 0.2	2.0 $\pm$ 0.2	0.3
Temperature ET	101.4 $\pm$ 0.04	101.3 $\pm$ 0.1	101.2 $\pm$ 0.1	101.3 $\pm$ 0.1	0.04

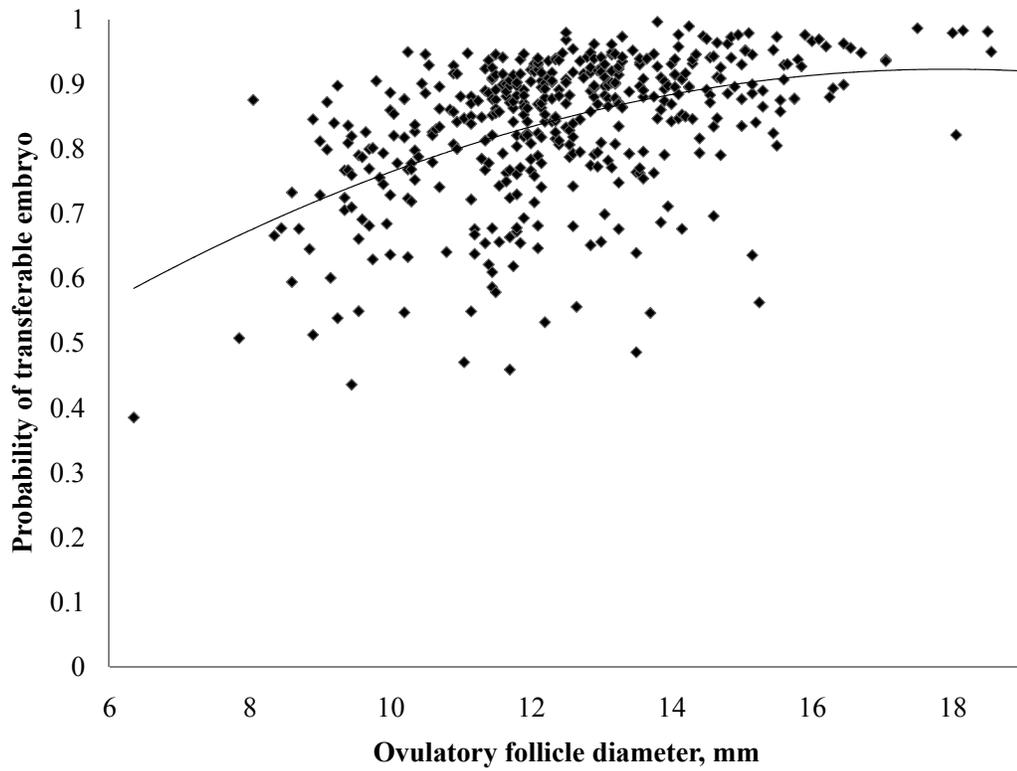
Artificial insemination technician ( $P = 0.002$ ) and days post partum ( $P = 0.1$ ) were included in the model as a covariates. This analysis did not include unfertilized oocytes, empty zona pellucidas, or non-graded embryos (n = 45, 6, and 2, respectively).

<sup>1</sup>Embryo classifications based on the guidelines set forth by the International Embryo Transfer Society.

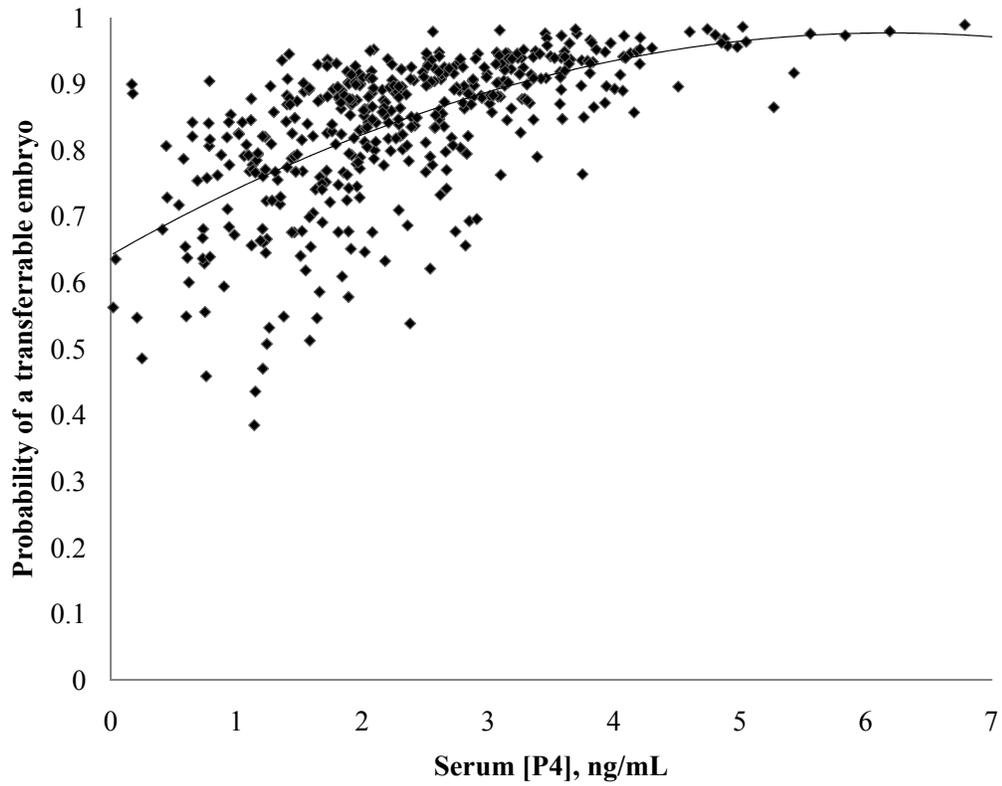
follicle diameter at GnRH2 and serum concentrations of progesterone at ET ( $P = 0.01$  and  $0.02$ , respectively; Figure 5.3 a and b).

*Pregnancy rate and follicle size group.* All live embryos were transferred except for six embryos that were either lost before transfer, damaged due to embryo handling, or there were not enough recipients available for transfer. There was an initial (d 27; d 0 = GnRH2) and final (d 72; d 0 = GnRH2) pregnancy rate of 54% and 49%, respectively (Table 5.5 and 5.6). Neither donor follicle size group nor recipient follicle size group affected initial (d 27 to 29 after GnRH2;  $P > 0.1$ ; Table 5.5) or final (d 70 to 72 after AI;  $P > 0.1$ ; Table 5.6) pregnancy rates. There was a year by donor follicle size group interaction in both the initial and final pregnancy rates (Table 5.5 and 5.6). There was also no interaction between donor and recipient follicle size group at initial or final pregnancy exam ( $P = 0.7$ ; Table 5.7 and 5.8). As recipient concentrations of progesterone at ET increased, the probability of pregnancy also increased ( $P < 0.0001$ ; Figure 5.4) but donor concentrations of progesterone at ET was not significant ( $P > 0.4$ ). Recipient rectal temperature at ET was also a positive predictor of pregnancy ( $P = 0.003$ ; data not shown). The probability of pregnancy tended to increase with increasing stage of embryo development at ET ( $P = 0.06$ ). Other significant factors that were included in the model for the probability of pregnancy were year, recipient cycling status, and transfer technician ( $P = 0.07$ ,  $0.06$ , and  $0.0003$ , respectively, data not shown).

*Pregnancy rates and follicle size as a continuous variable.* Since optimal follicle diameter varies among different populations of females (see Perry et al., 2005), a second analysis for pregnancy prediction by follicle size was conducted with follicle size



**Figure 5.3a** Probability of the structure recovered being a transferable embryo (not an unfertilized oocyte or dead embryo) from cows based on the size of the ovulatory follicle (mm) at GnRH2 ( $P = 0.01$ ;  $n = 365$  cows from which an embryo or unfertilized oocyte was recovered).



**Figure 5.3b** Probability of recovering a transferrable embryo (not an unfertilized oocyte or dead embryo) from cows based on serum concentrations of progesterone (P4, ng/mL) on the day of embryo transfer ( $P = 0.02$ ;  $n = 365$  cows from which an embryo was recovered).

**Table 5.5** Proportion (%) of embryo transfers resulting in a pregnancy (27 to 29 d after GnRH2) per year and across all years by main effects of recipient or donor follicle size group.

Trt <sup>1</sup>	Year 1	Year 2	Year 3	Total
R-S	30/59 (51)	49/93 (53)	23/41 (56)	102/193 (53)
R-L	14/36 (39)	60/98 (61)	14/27 (52)	88/161 (55)
<sup>2</sup> <i>P</i>	0.2	0.9	0.4	0.6
D-S	11/33 (33)	55/101 (54)	29/48 (60)	95/182 (52)
D-L	33/62 (53)	54/90 (60)	8/20 (40)	95/172 (55)
<sup>*2</sup> <i>P</i>	0.07	0.4	0.04	0.8
<sup>3</sup> D-S	11/45 (24)	55/120 (46)	29/60 (48)	95/225 (42)
<sup>3</sup> D-L	33/77 (43)	54/101 (53)	8/22 (36)	95/200 (48)
<sup>4</sup> <i>P</i>	0.05	0.4	0.2	0.7

<sup>1</sup>Treatments based on ovulatory follicle size at GnRH- induced ovulation of the donor (D) and recipient (R) cows. Follicles <12.5 mm were considered small (S) and follicles ≥ 12.5 mm were considered large (L).

<sup>2</sup>*P* value compares main affects of recipient or donor follicle size group. The model for the main effects of recipient and donor follicle size group (only considering donor cows from which a transferred embryo was recovered) included year, embryo stage, transfer technician, recipient rectal temperature at ET, and recipient concentrations of progesterone at ET (*P* = 0.04, 0.08, 0.0003, 0.001, and 0.0001).

<sup>\*</sup>There was a donor follicle size group by year interaction (year 1 and 2 were different than year 3; *P* = 0.004).

<sup>3</sup>Considering dead embryos or unfertilized oocytes from donor cows as non pregnant cows in the total.

<sup>4</sup>*P* value comparing main affects of donor follicles size group when all donors from which an embryo or unfertilized oocyte were recovered. The model included year and embryo stage (*P* = 0.03 and 0.009, respectively). There was a year by follicle size group interaction (year 1 and 2 were different than year 3; *P* = 0.02).

**Table 5.6** Proportion (%) of embryo transfers resulting in a pregnancy (72 to 74 d after GnRH2) per year and across all years by main effects of recipient or donor follicle size group.

Trt <sup>1</sup>	Year 1	Year 2	Year 3	Total
R-S	28/59 (47)	49/93 (53)	21/41 (51)	97/193 (50)
R-L	12/36 (33)	51/98 (52)	13/27 (48)	76/161 (47)
<sup>2</sup> <i>P</i>	0.1	0.4	0.3	0.3
D-S	10/33 (30)	48/101 (48)	26/48 (54)	84/182 (46)
D-L	30/62 (48)	51/90 (57)	8/20 (40)	89/172 (52)
<sup>*2</sup> <i>P</i>	0.06	0.1	0.4	0.2
<sup>3</sup> D-S	10/45 (22)	48/120 (40)	26/60 (43)	84/225 (37)
<sup>3</sup> D-L	30/77 (39)	51/101 (50)	8/22 (36)	89/200 (45)
<sup>4</sup> <i>P</i>	0.07	0.2	0.5	0.5

<sup>1</sup>Treatments based on ovulatory follicle size at GnRH- induced ovulation of the donor (D) and recipient (R) cows. Follicles < 12.5 mm were considered small (S) and follicles ≥ 12.5 mm were considered large (L).

<sup>2</sup>*P* value compares main affects of recipient or donor follicle size group. The model for the main effects of recipient and donor follicle size group (only considering donor cows from which a transferred embryo was recovered) included year, embryo stage, donor cyclicity, transfer technician, recipient rectal temperature at ET, and recipient concentrations of progesterone at ET ( $P = 0.05, 0.09, 0.05, 0.0004, 0.006, \text{ and } 0.002$ ).

\*There was a tendency for a donor follicle size group by year interaction (year 1 and 2 were different than year 3;  $P = 0.07$ ).

<sup>3</sup>Considering dead embryos or unfertilized oocytes from donor cows as non pregnant cows in the total.

<sup>4</sup>*P* value comparing main affects of donor follicles size group when all donors from which an embryo or unfertilized oocyte were recovered. The model included year and embryo stage as covariates ( $P = 0.04 \text{ and } 0.01$ , respectively). There was a year by donor follicle size group interaction (year1 and 2 were different compared to year 3;  $P = 0.02$ ).

**Table 5.7** Proportion (%) of pregnant cows by year and across all years for each treatment group (trt) 27 to 29 d after GnRH2.

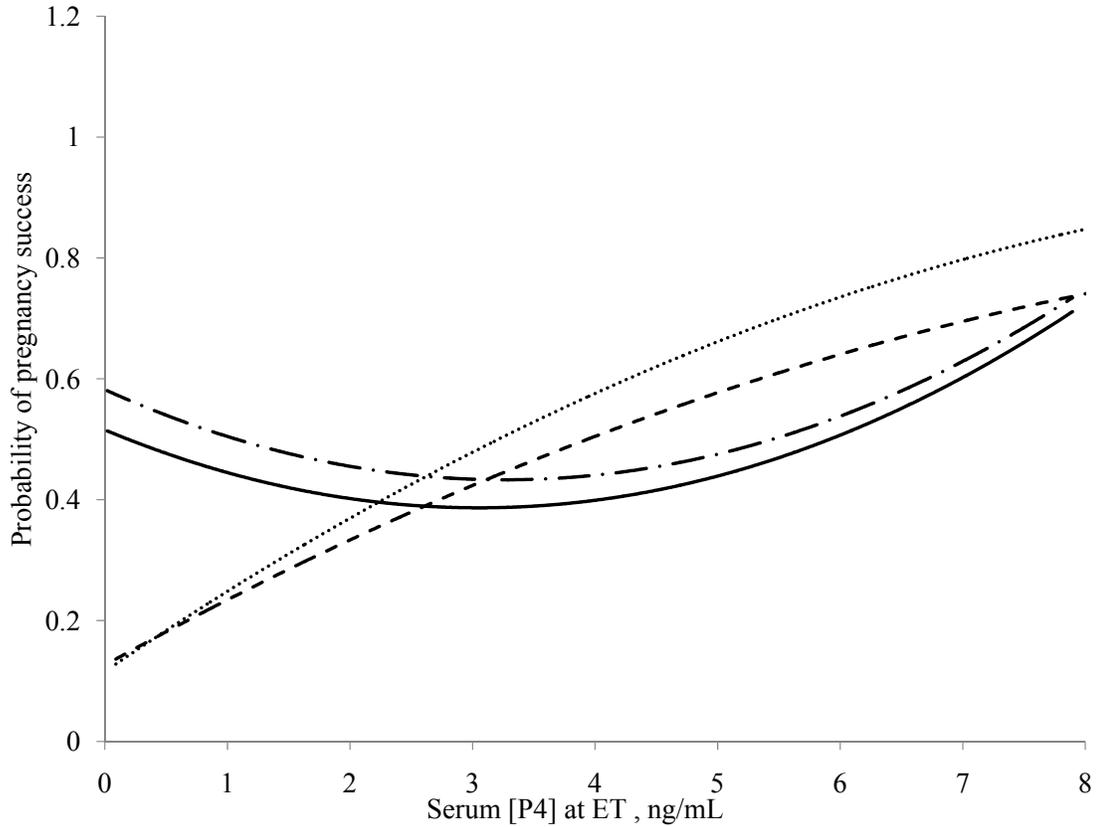
Trt <sup>1</sup>	Year 1	Year 2	Year 3	Total
S-S	5/11 (45)	11/27 (41)	19/33 (58)	35/71 (49)
S-L	6/22 (27)	44/74 (59)	10/15 (67)	60/111 (54)
L-S	25/48 (52)	38/66 (58)	4/8 (50)	67/122 (55)
L-L	8/14 (57)	16/24 (67)	4/12 (33)	28/50 (56)
Total	44/95 (46)	109/191 (57)	37/68 (54)	190/354 (54)
<i>P</i>	<i>0.4</i>	<i>0.7</i>	<i>0.3</i>	<i>0.9</i>

<sup>1</sup>Treatments based on ovulatory follicle size at GnRH2 of the donor and recipient cows. Embryos collected from cows that ovulated a small follicle (<12.5 mm) or large follicles (≥ 12.5 mm) were transferred into recipient cows that ovulated a small or large follicle resulting in the following treatment groups: small to small (S-S), small to large (S-L), large to small (L-S), and large to large (L-L).

**Table 5.8** Proportion (%) of pregnant cows by year and total for each treatment group (trt) 72 to 74 d after GnRH2.

Trt <sup>1</sup>	Year 1	Year 2	Year 3	Total
S-S	5/11 (45)	11/27 (41)	17/33 (52)	33/71 (46)
S-L	5/22 (23)	37/74 (50)	9/15 (60)	51/111 (46)
L-S	23/48 (48)	37/66 (56)	4/8 (50)	64/122 (52)
L-L	7/14 (50)	14/24 (58)	4/12 (33)	25/50 (50)
Total	40/95 (42)	99/191 (52)	34/68 (50)	173/354 (48)
<sup>2</sup> P	0.4	0.9	0.9	0.8

<sup>1</sup>Treatments based on ovulatory follicle size at GnRH2 of the donor and recipient cows. Embryos collected from cows that ovulated a small (< 12.5 mm) or large follicle (≥ 12.5 mm) were transferred into recipient cows that ovulated a small or large follicle resulting in the following treatment groups: small to small (S-S), small to large (S-L), large to small (L-S), and large to large (L-L).



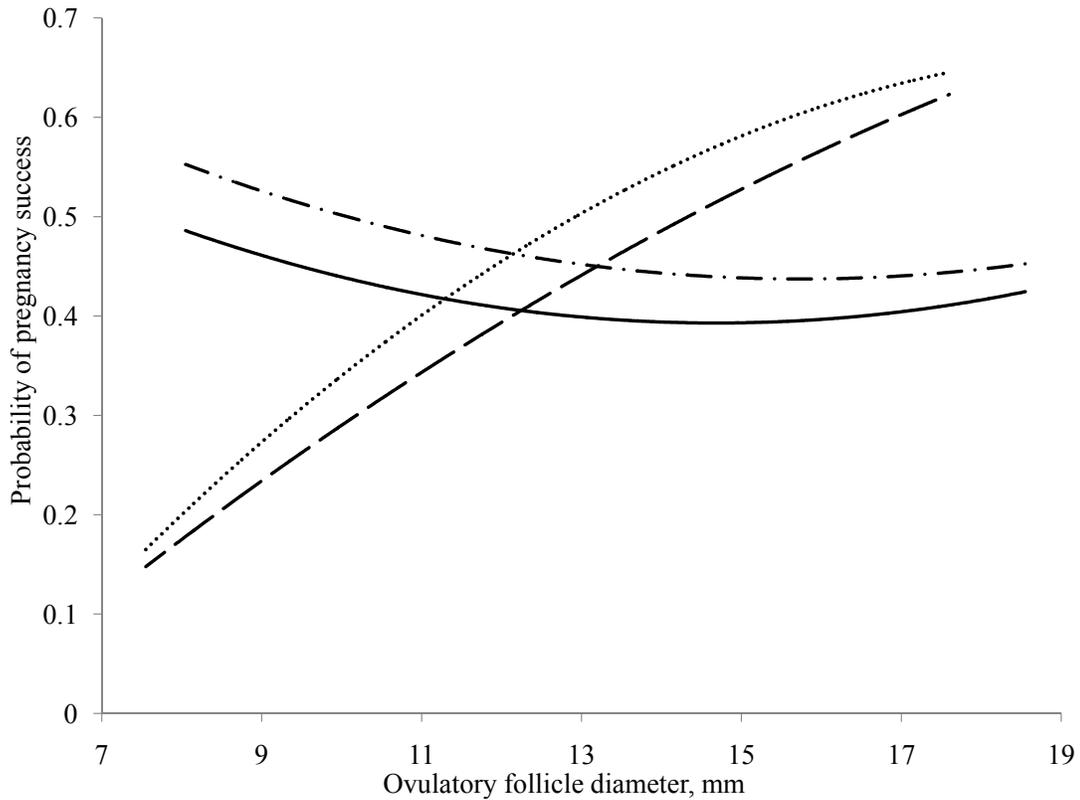
**Figure 5.4** Probability of pregnancy success 27 to 29 d GnRH2 (embryo transfer [ET] 7 d after GnRH2; dotted line in recipients and long/dot/dash line in donors) and 72 to 74 d after GnRH2 (dashes in recipients and solid line in donors) based on serum concentrations of progesterone (P4, ng/mL) at ET. Recipient concentrations of progesterone at ET was a significant predictor of pregnancy but donor concentrations of progesterone were not significant ( $P < 0.01$  and  $> 0.4$ , respectively;  $n = 354$  embryo transfers).

included as a continuous variable rather than follicle size group (large or small). As recipient follicle diameter increased, the likelihood of d 27 and 72 pregnancy increased ( $P = 0.05$  and  $0.05$ ; Figure 5.5). Donor follicle size was not predictive of pregnancy at either d27 or d72 ( $P = 0.6$  and  $0.9$ , respectively; Figure 5.5). The same covariates that significantly predicted the model for pregnancy success in the follicle size group analysis were also predictive of pregnancy in the continuous follicle size analysis.

*Embryonic loss.* There were a total of 17/175 pregnancies (9.7%) that experienced embryonic/early fetal loss from d 27 to 72. The majority of the loss occurred by d 40 to 44, followed by d 56 to 58, and d 70 to 72 (8, 7, and 2, respectively). Of the failed pregnancies, nine were from the small to large treatment group, two in the small to small treatment group, and three each in the large to small and large to large treatment groups. Embryonic loss did not differ among recipient follicle size group, donor follicle size group, or the interaction of the donor and recipient follicle size groups ( $P = 0.15$ ,  $0.2$ , and  $0.18$ , respectively).

## DISCUSSION

The likelihood of pregnancy establishment or maintenance was reduced when cows were induced to ovulate small compared to large follicles (Lamb et al., 2000; Vasconcelos et al., 2001; Perry et al., 2005; Bello et al., 2006; Waldmann et al., 2006; Stevenson et al., 2008; Dias et al., 2009; Meneghetti et al., 2009). While the underlying cause of a reduction in pregnancy following induced ovulation of a small follicle is unknown, inadequate oocyte competence and (or) uterine environment are likely responsible. The oviductal and uterine environments are responsive to changes

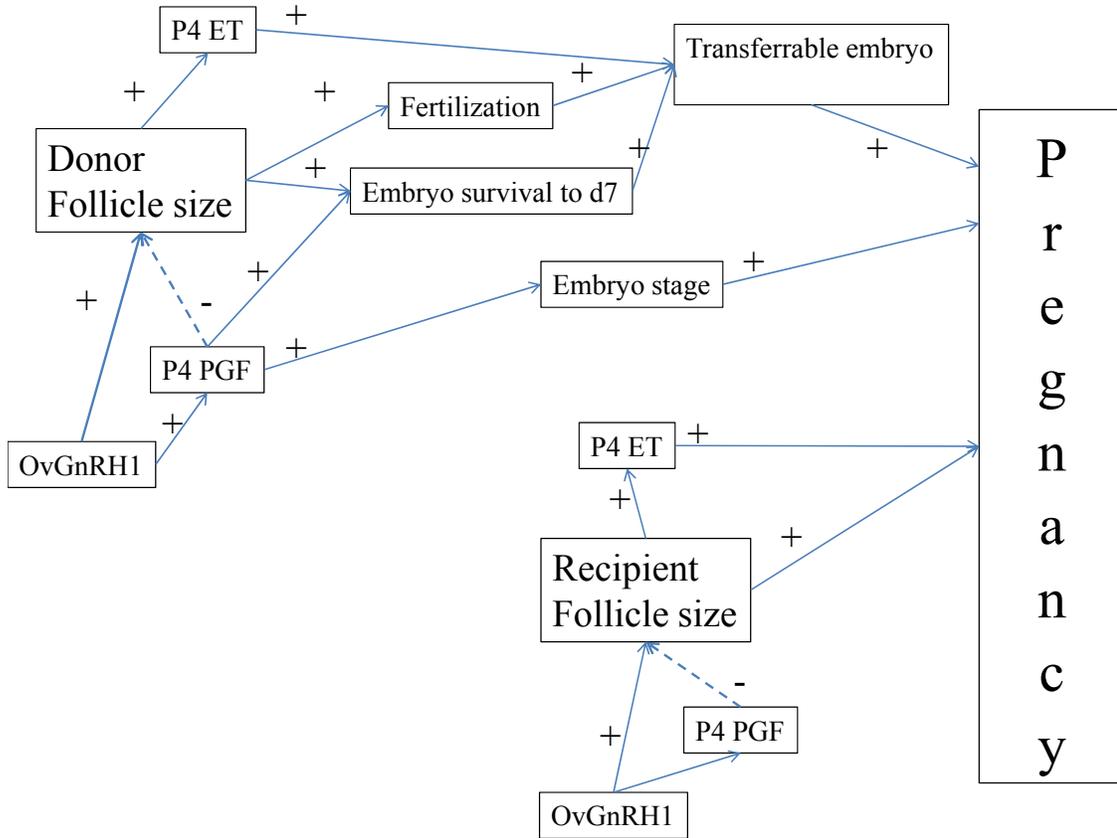


**Figure 5.5** Probability of pregnancy success 20 to 22 and 63 to 65 days after embryo transfer days (ET; n = 354 embryo transfers). Recipient ovulatory follicle diameter significantly increased the probability of pregnancy success at both the initial (dotted line) and final (long dashed line) exams ( $P = 0.05$ ). Donor ovulatory follicle diameter was not predictive of pregnancy success at either the initial (long dash and dot) or final (solid line) pregnancy exam ( $P = 0.6$  and  $0.9$ , respectively).

in the ovarian steroids and altered endocrine profiles associated with premature ovulation of a follicle could lead to impaired viability/transport of gametes, fertilization, and embryo/fetal development. Induced ovulation of a small follicle was associated with reduced serum concentrations of estradiol at ovulation (Vasconcelos et al., 2001; Bello et al., 2006; Perry et al., 2005; 2007; Busch et al., 2008) and subsequent serum concentrations of progesterone (Vasconcelos et al., 2001; Perry et al., 2005; Busch et al., 2008; Stevenson et al., 2008). Either or both of the preceding alterations in ovarian steroid secretion could alter uterine environment.

The follicular microenvironment likely affects the oocyte as well. The oocyte continues cytoplasmic, nuclear, and molecular maturation within the growing follicle (reviewed by Sirard et al., 2006) and oocyte growth is intimately associated with follicular maturation (reviewed in cattle by Fair, 2003). Premature ovulation may result in the release of an oocyte that is less capable of being fertilized, less able to activate the embryonic genome, or possibly result in reduced development potential of the embryo/fetus/offspring.

The main objective of this experiment was to differentiate between oocyte and uterine environment effects in GnRH- induced ovulation of small and large follicles and our results suggest that both are indicators of pregnancy but may vary in importance temporally (Figure 5.6). Oocyte competence may affect pregnancy establishment since the likelihood of having a transferrable embryo increased with increasing ovulatory size of the donor cow. While the size of the ovulatory follicle in the donor cow may reflect primary effects due to oocyte competence, the role of the oviductal and (or) uterine



**Figure 5.6** Hypothesized role of the donor and recipient follicle size in establishing and maintaining pregnancy. The results of the recipricol embryo transfer experiment suggest the donor follicle size affects the ability of the oocyte to be fertilized and the resulting survival of the embryo to day 7. There was no direct affect of follicle diameter on the stage of the embryo development; however serum concentrations of progesterone at PGF induced luteolysis was a positive indicator of embryo stage. Given the presence of a transferrable embryo, there was no further affect of donor follicle size on the subsequent maintenance of pregnancy. Pregnancy after embryo transfer was positively associated with recipient ovulatory follicle size and serum concentrations of progesterone at embryo transfer. These results suggest that donor follicle diameter affects fertilization and early embryo survival but after day 7, the follicular determinants of pregnancy appear to be mediated through the uterine environment.

environment between ovulation and embryo/oocyte recovery cannot be ruled out as factors affecting embryo formation and survival.

There was no difference in the initial or final pregnancy rates due to follicle size treatment groups; however, the recipient ovulatory follicle diameter (treated as a continuous variable) was a positive predictor of pregnancy following embryo transfer independent of the ovulatory follicle size of the donor cow. This finding may indicate that our criteria for establishing the follicle size treatment groups may not be accurate in this population of cattle, as previous reports have indicated differences in optimal follicle diameters between herds (Perry et al., 2005).

In the present study, the probability of successful fertilization increased with increasing diameter of the ovulatory follicle, which could signal an inherent defect in the oocyte. Follicular environment may affect oocyte quality as administration of an aromatase inhibitor reduced the ability of oocytes to mature to metaphase II (Zelinski Wooten et al., 1993). Members of the transforming growth factor  $\beta$  family have also been implicated in the acquisition of oocyte competence (Silva and Knight, 1998; Patel et al., 2007). Interestingly, some of the ‘unfertilized’ oocytes (5 out of 21) had sperm bound to the zona pellucida suggesting the oocytes at least had contact with spermatozoa but failed to develop into a two cell embryo. The mechanism for such a loss is unknown but may relate to cytoplasmic or nuclear maturation of the oocyte as the orchestration of the cortical reaction, and syngamy of the pronuclei require remodeling of the organelles in the cytoplasm (Albertini et al., 2003).

The ovum, zygote, and the early embryo are dependent on mRNA and proteins synthesized prior to the LH surge. The oocyte continued protein and mRNA synthesis in

follicles up to 15 mm in cattle (Arlotto et al., 1996). In bovine oocytes, transcription increased until just prior to germinal vesicle breakdown (Rodriguez and Farin, 2004), after which the oocyte is unable to continue mRNA synthesis. The bovine embryo is unable to transcribe mRNA until the maternal to embryonic transition which occurs around the 8 to 16 cell stage (Brevini-Gandolfi and Gandolfi, 2001). Therefore, premature exposure of an oocyte to an LH surge could reduce the maternally derived mRNAs that might be important for development prior to activation of the embryonic genome.

Several maternal effect genes are known to be expressed in bovine oocytes and there is a gradual decrease in expression until embryonic genome activation (*dnlc1*, *zp2*, *gtl3*, *fankl*, *btg4*, *cullin1*, *mater*, *zar1*, *gdf9*, *bmp15*, and *nalp9* [Sendai et al., 2001; Brevini et al., 2004; Pennetier et al., 2005; Hwang et al., 2005]). This pattern of gene expression suggests that these genes are important maternal sources of mRNA prior to embryonic activation of transcription. Recently, several maternal effect genes have been described in mice that are required for the maternal to zygotic transition (Minami, 2007) and null mice do not survive past the 1 to 2 cell stage (as the embryonic genome is activated by the 2 cell stage in mice).

Another emerging area of research that is relevant to oocyte affects on the establishment and maintenance of pregnancy is the epigenetic control and imprinted gene contribution to embryo development. Several genes are reported to be imprinted for specific expression by either the paternal or maternal allele (reviewed by Tycko and Morison, 2002 and Miyoshi et al., 2006) and aberrant imprinting has been implicated in abnormalities during embryonic, fetal, and neonatal development (Moore and Reik, 1996;

Farin et al., 2006). It is possible that the follicular environment may alter imprinting of specific genes as oocytes from superstimulated mice and women had a differential imprinting pattern compared to controls (Sato et al., 2007) and concentrations of steroid and gonadotropins were reported to change the global methylation pattern of mouse oocytes in vivo (Murray et al., 2008).

Elevated serum concentrations of progesterone from the cycle before conception have also been associated with increased conception rates (Folman et al., 1973; Corah et al., 1974; Fonseca et al., 1983). Bello et al., (2006) also reported an increase in the probability of pregnancy with increasing concentrations of progesterone at PGF- induced luteolysis (during an OV-Synch protocol). Serum concentrations of progesterone at PGF were positively associated with embryo stage of development and neither ovulatory follicle size nor serum concentrations of progesterone at embryo transfer (7 d after GnRH administration) were predictive of embryo development. While it is not known how elevated circulating concentrations of progesterone from the previous cycle affect conception rate, it is possible that oocyte competence is affected by low levels of progesterone (Mihm et al., 1994).

Ovulation following GnRH1 initiates a new follicular wave resulting in the synchronization of a dominant follicle at the final GnRH- induced ovulation associated with fixed timed AI programs. It is interesting that ovulation following GnRH1 was positively associated with both serum concentrations of progesterone at PGF and follicle diameter at GnRH2 yet serum concentrations of progesterone were negatively correlated with follicle diameter at GnRH2 both of which were positive predictors of embryo survival. This suggests that ovulation at the first GnRH injection led to increased

concentrations of progesterone at PGF and increased follicle diameter at GnRH2 which may be ideal for embryonic survival. Previous studies from our lab reported an increase in follicle diameter in cows that ovulated to GnRH1 (Atkins et al., unpublished) but not in heifers (Atkins et al., 2008a). Bello et al. (2006) reported an increase in the variation of follicle diameter in cows that failed to ovulate following GnRH1 compared to cows that did ovulate after GnRH1.

The relationship between donor ovulatory follicle diameter and fertilization rate, embryo survival, and embryo quality may also be due to the oviductal and uterine environment during the 7 d after GnRH2 administration. Uterine environment 7 d after estrus differed between cows that had a normal embryo compared to cows with a poorly developed or dead embryo (Wiebold, 1988). As mentioned previously, cows that were induced to ovulate a small dominant follicle had reduced serum concentrations of estradiol at insemination and reduced progesterone during subsequent luteal formation (Perry et al., 2005). These ovarian steroids induce changes in secretion and gene expression in the oviduct and uterus which alter the environment to which gametes and embryos are exposed.

The duration of the proestrous period may also affect pregnancy outcome as cows with a shorter proestrus had decreased serum concentrations of progesterone after ovulation and less developed embryos compared to cows that ovulated following a long proestrus (Bridges et al., 2006). Estradiol may affect the oviductal or uterine environment by changing the pH of the uterus (Elrod and Butler, 1993; Perry and Perry, 2008a and 2008b), altering sperm transport and longevity (Allison and Robinson, 1972; Hawk, 1983), oviduct secretions (for example oviductal glycoprotein; reviewed by Buhi,

2002) and indirectly stimulate progesterone activity through induction of progesterone receptors in the uterus (Stone et al., 1978; Zelinski et al., 1982; Ing and Tornesi, 1997).

Clearly progesterone is also important to the establishment and maintenance of pregnancy. Progesterone affects uterine environment by inducing histotrophe secretions from the uterine glands and decreasing muscle contractions. Mauer and Echterkamp (1982) reported a benefit of progesterone as early as 2 to 3 d after estrus on embryo development. It is possible that progesterone has a direct effect on the embryo as bovine embryos were recently reported to have progesterone receptors (Clemente et al., 2009). However, addition of progesterone to cultured embryos did not change embryo quality or development; therefore, a direct progesterone action on the embryo is still unclear (Fukui and Ono, 1989).

When only considering the transferred embryos, subsequent pregnancy was not affected by ovulatory follicle size of the donor but recipient ovulatory follicle size and serum concentrations of progesterone were predictive of pregnancy. This suggests that if an embryo is present by d 7, the uterine environment is the main determinant of successful pregnancy. Mussard et al. (2003) similarly reported increased pregnancy rates following embryo transfer into recipients with larger follicles at ovulation. The uterine environment is likely the result of both serum concentrations of estradiol around the time of ovulation (see above section) and serum concentrations of progesterone from the developing CL.

Ovulatory follicle diameter was correlated with CL volume, similar to results described previously (Vasconcelos et al., 2001). Since luteal cells are derived from follicular cells, factors affecting follicular differentiation and maturation may also affect

subsequent luteinization. Follicular secretion of estradiol may affect corpus luteum production of progesterone as ewes treated with an aromatase inhibitor prior to induced ovulation had a delayed rise in serum concentrations of progesterone (Benoit et al., 1992). Progesterone production by human luteinized granulosa cells was affected by the follicular environment (McNatty and Sawers, 1975) or gonadotropin exposure prior to ovulation (Lobb and Younglai, 2001). It has also been suggested that the vascular development of the follicle could affect the blood supply to the subsequent corpus luteum (Tamanini and De Ambrogi, 2004).

Progesterone stimulates histotrophe production from the uterine glands which is critical for embryonic and fetal development (Geisert et al., 1992; Spencer et al., 2004). Progesterone supplementation is able to maintain pregnancy in cattle (Zimbelman and Smith, 1966) and ewes (Foote et al., 1957) that were ovariectomized at breeding. Several recent studies have attempted to understand the differential gene expression pattern in the uterus of cycling, pregnant, and progesterone supplemented animals (Spencer et al., 2008; Satterfield et al., 2009; Mitko et al., 2008; Bauersachs et al., 2005, 2006; Forde et al., 2009). Progesterone supplementation in cyclic and pregnant heifers tended to advance the gene expression such that the uterine expression profiles from supplemented heifers on d 5 after estrus were similar to expression profiles in non treated heifers 2 d later (d 7; Forde et al., 2009).

Progesterone supplementation following breeding has yielded conflicting results. A meta-analysis of 17 experiments with progesterone supplementation revealed a 5% improvement with supplementation (Mann and Lamming, 1999). While several report a benefit to progesterone supplementation (for example, Garret et al., 1988; Mann and

Lamming, 2001; Carter et al., 2008) others report no benefit of supplementation (for example Funston et al., 2005; Galvao et al., 2006; Stevenson et al., 2007). Part of the discrepancy may reflect differences in the fertility of the cow population. Starbuck et al. (2001) reported an improvement in pregnancy rates with progesterone supplementation in cows with reduced serum concentrations of progesterone but no added benefit in cows with elevated progesterone 5 d following AI.

In summary, the follicular environment of the donor cow affected fertilization rate, and embryonic survival prior to d 7 but maintenance of pregnancy subsequent to d 7 was dependent on the ovulatory follicle size and subsequent progesterone concentrations in the recipient cows (independent of the ovulatory size of the donor cow) following single embryo recovery and transfer. This suggests a potential role of oocyte competence in fertilization and early embryo survival (although the oviductal and uterine environment may play a role) 7 d following breeding. The uterine environment affected the maintenance of pregnancy following embryo transfer.

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## VITA

Jacqueline (Jackie) Atkins, nee Clement, was born and raised in Mandan, North Dakota. After graduating from Mandan High School, Jackie took a year off to “see the world”. She was an aupair in Switzerland for 6 mo. and then moved back to North Dakota for a few months before starting her undergraduate education at Montana State in Bozeman, MT. Jackie completed a B. S. in Veterinary Molecular Biotechnology in 2002 and started a Master’s of Science in January of 2004 with Dr. Michael F. Smith at the University of Missouri. While in Bozeman, Jackie met her future husband, Brandon Atkins and the two were married in the summer of 2003.