EFFECTS OF BODY CONDITION SCORE AND POSTPARTUM INTERVAL ON OVARIAN FUNCTION OF ANESTROUS BEEF COWS

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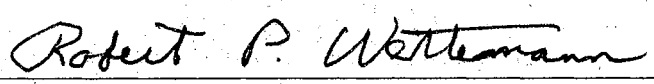

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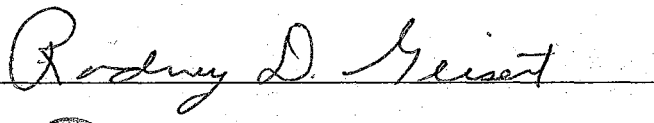

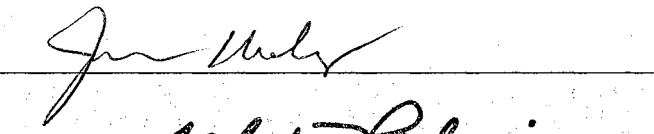

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I would like to dedicate this document to my wife, Stephanie White. She has made countless sacrifices, both personal and professional, so I could pursue my dreams and goals. The burdens of many responsibilities were shifted to her, so I could reach my professional goals. Stephanie is truly my best friend, and without her consistent support, this or any other accomplishment in my life would not have been possible. My wife is without question my soul mate, and I thank God for her every day. Stephanie, thank you. I will love you today, tomorrow, and always.

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thanks be to God! He gives us victory through our Lord Jesus Christ." 1 Corinthians 15:57

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CHAPTER I

INTRODUCTION

The beef industry is essential to the economies of the United States and Oklahoma. There were about 100 million beef and dairy cattle and calves in the United States in 2001, which resulted in a gross annual income of 40 billion dollars (USDA, 2003a). Oklahoma has the third largest beef herd in the United States, with about two million cows (USDA, 2003a). The number of beef operations in the United States has declined by 150 thousand since 1988, while the number of beef cows has remained consistent (USDA, 2003b). Because of the reduction in number of beef operations, producers must either increase their efficiency of production, or increase herd size, to maintain current production of beef and to sustain profitability. It is essential that beef cows produce a calf every 12 mo. Cows have a gestation length of about 385 days and must begin normal estrous cycles and become pregnant in 85 days after calving. Beef cows have a period of time after calving without normal estrous cycles and ovulation, termed the postpartum anovulatory interval.

Reproductive inefficiency due to a long postpartum anovulatory interval is a major source of economic loss for beef producers. Reproductive disease and suboptimal reproduction cost the beef industry between $441 and 502 million annually (Bellows et al., 2002), and the inability of a cow to rebreed during the breeding season is the largest factor affecting this cost. Non-pregnant and late-calving cows had an economic impact on 47 % of beef producers surveyed in 1997 (NAHMS, 1997). Cows are culled and sold if they do not become pregnant during the breeding season, and pregnancy status accounted for nearly one fourth of all cows sold (NAHMS, 1997). Wiltbank et al. (1961) concluded that non-pregnant cows at the end of a breeding season are the main factor affecting potential calf crops, and decreasing the postpartum anovulatory interval was recommended to increase calf crops. Non-pregnant beef cows cost the industry millions of dollars, but the mechanisms causing postpartum anovulation have not been completely revealed. Therefore, understanding factors controlling postpartum anovulation may lead to development of management decisions and treatments to increase reproductive performance and enhance profitability of beef producers.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Female gametes or ova must mature in the ovary before they are released for fertilization. Follicles contain cells that interact to provide the correct physiological environment for maturation of ova. Follicles are continuously changing, increasing in size and cell number as ova prepare to ovulate. Follicular cells secrete different hormones as the follicle grows to provide the correct endocrine milieu for maturation of ova and follicles. Once follicles reach maximum size, they ovulate, and ova, that are capable of fertilization, are released. Bovine follicles grow and mature in groups called follicular waves, which are cohorts of follicles that are recruited and grow together (Rajakoski, 1960; Ireland and Roche, 1983a; Pierson and Ginther, 1984). Two or three waves of follicular growth occur during an estrous cycle. Usually, only one of the bovine follicles per wave will become dominant and suppress growth of the other subordinate follicles. A dominant follicle will ovulate or regress, allowing a new wave of follicles to grow, and the fate of a dominant follicle is determined by an interaction of hormones from the hypothalamus, pituitary, and ovary.

In the early twentieth century, follicles were first described as endocrine tissues that regulate estrous cycles (Loeb and Hesselberg, 1917). Steroids secreted by the ovary influence gonadotropin secretion from the pituitary. A pituitary-ovarian axis was first suggested by Moore and Price (1932) and later determined that estradiol, from the dominant follicle, decreases follicle-stimulating hormone (FSH) release from the pituitary. The pituitary-ovarian axis was expanded to include the hypothalamus, when it was demonstrated that luteinizing hormone (LH) was released in pulses that are regulated by the hypothalamus (Diershke et al., 1970; Midgley and Jaffe, 1971; Zolman et al., 1973; Belchetz et al., 1978). This led to the hypothesis of a hypothalamic-pituitary-ovarian axis. Gonadotropins have receptors in the ovary, and exogenous gonadotropins increased RNA and protein synthesis of ovarian tissue (Reel and Gorski, 1968a,b; Rajaniemi and Vanha-Perttula, 1972). The relationship between the ovary and gonadotropins was expanded with the observation that estradiol has positive feedback on LH concentration causing the ovulatory LH surge (Labhsetwar, 1970; Short et al., 1972, 1973; Karsch et al., 1983). The ovary secretes steroids that regulate estrous cycles by actions at the hypothalamus and pituitary that alter peripheral LH and FSH concentrations. Ovarian capacity is interconnected to the hypothalamus and pituitary. Changes in hypothalamic, pituitary, or ovarian hormones may influence reproductive function including postpartum ovulation in cows.

A dominant follicle can be identified on the ovary an average of 10 d after calving in the beef cow, but only 11 % of first dominant follicles ovulate (Murphy et al., 1990). Beef cows averaged 3 to 8 follicular waves before the first ovulation (Murphy et al., 1990; Stagg et al., 1995); therefore, dominant follicles are present on the ovary during

postpartum anovulation, but final maturation and ovulation is prevented. Altered hypothalamic, pituitary, or ovarian function may prevent dominant follicles from maturing and ovulating during postpartum anovulation. The mechanisms regulating normal growth and ovulation of follicles during estrous cycles of cows and during postpartum anovulation must be revealed.

Bovine Estrous Cycle

Hormones from the hypothalamic-pituitary-ovarian axis interact throughout the bovine estrous cycle to control follicle growth and ovulation. The normal bovine estrous cycle ranges from 18-22 d with a mean of 20 d for heifers and 21 d for cows (Moeller and VanDemark, 1951; Olds and Seath, 1951; Trimberger, 1956; Sirois and Fortune, 1988; Ginther et al., 1989). Heape (1900) first classified and defined the 4 stages of the estrous cycle: estrus, metestrus, diestrus, and proestrus. These stages of the cycle are characterized by different endocrine, physiological, and behavioral changes.

Estrus occurs just prior to ovulation and is characterized by maximal concentrations of estradiol in systemic blood (Shemesh et al., 1972; Wettemann et al., 1972; Echternkamp and Hansel, 1973). During estrus, numerous physiological and behavioral changes occur; most significantly, the cow is receptive and will stand to be mounted by either males or females. Estrous behavior is the best external sign of the time of ovulation and has been used to estimate when ovulation occurs. Early studies determined that dairy cows ovulate 10.5 h after the end of estrus (Trimberger 1948) and beef cows ovulated 13 h after estrus (Brewster and Cole, 1941). Beef and dairy cows ovulated 31 (White et al., 2002) and 28 h (Walker et al., 1996) after the onset of estrus, respectively. Metestrus is the period of time from the end of estrous behavior until

concentrations of progesterone in plasma increases above 1 ng/mL, about 3 d after estrus (Swanson and Hafs, 1971; Wettemann et al., 1972). During diestrus, progesterone secretion increases and is maximal until approximately 15 d after estrus, at which time function of the corpus luteum decreases (Garverick et al., 1971; Wettemann et al., 1972; Christensen et al., 1974; Spicer et al., 1981; White et al., 2002). The time from the start of minimal concentrations of progesterone in plasma until the onset of estrous behavior is termed proestrus.

Follicular Growth

The bovine ovary develops prior to birth. Oocytes begin meiotic divisions about 75 to 80 d of gestation, and maximal numbers of ova (170,000) are found in the ovary at 150 to 170 d of fetal development in the bovine (Erickson, 1966a). After 170 d of prenatal development, oocytes are arrested in meiotic prophase and remain inactive until initiation of ovulation (Erickson, 1966a, reviewed by Richards, 1980). These oocytes are incapable of fertilization and must undergo a maturation process before ovulation. Most oocytes degenerate, fail to complete maturation, and will not ovulate. Oocytes mature in follicles, and follicles synthesize and release hormones that establish the correct endocrine environment for oocytes and follicles to grow and develop for ovulation. All growing follicles are being prepared for ovulation, but very few finish maturation. Ninety-nine percent of growing follicles fail to ovulate and become atretic, regress in size, and degenerate (reviewed by Ireland, 1987).

Folliculogenesis

Folliculogenesis is the growth and maturation of follicular cells and oocytes (Reviewed by Spicer and Echternkamp, 1986; Fortune, 1994). During folliculogenesis, a primordial follicle with a single layer of follicular cells grows and matures into a graafian follicle ready for ovulation. A graafian follicle consists of the oocyte, granulosa cells, theca cells, and an antrum or fluid filled center. The majority of follicles in the ovary are primordial follicles (Erickson, 1966b). Oocytes are arrested in a resting state and serve as a pool from which follicles will begin growing and preparing for ovulation throughout the life of the cow. At 170 d of prenatal development, primordial follicles are formed and consist of an oocyte arrested in meiotic prophase I surrounded by one layer of follicular cells (Erickson, 1966a). Approximately 133,000 primordial follicles are located in the bovine ovary at birth (Erickson, 1966b). An antrum is formed when bovine follicles reach 0.24 (Lussier et al., 1987) to 0.5 mm (Marion et al., 1968) in diameter. Follicles begin to grow prior to birth in the bovine, and follicles with an antrum develop in the fetal ovary by 250 d of gestation (Erickson, 1966a). Follicles 1 to 2 mm in size have the characteristics of a graafian follicle (Marion et al., 1968), and their oocytes can be fertilized in vitro (Pavlok et al., 1992, 1993). The number of primordial follicles declines to only 3,000 by 15 to 20 years of age in cows (Erickson, 1966b).

Growth of a primordial follicle to a graafian follicle takes approximately the length of two estrous cycles (Lussier et al., 1987). Marion et al. (1968) concluded that follicles grow at a constant rate that is independent of stage of follicular development, but the current hypothesis is that follicular growth rate increases with the size of the follicle (reviewed by Fortune, 1994). During early follicular growth, granulosa cells proliferate although the initial rate of growth is slow; a follicle increases from 0.13 to 0.67 mm in

diameter in 27 d (Lussier et al., 1987). Antral follicles increase from 3.7 to 8.6 mm in only 8 d (Lussier et al., 1987); large follicles grow at a much faster rate and increase in diameter occurs mainly through antrum expansion rather than cell growth.

Rajakoski (1960) hypothesized that graafian follicles grow in groups or waves, but other authors concluded that follicles mature and grow continuously in the bovine ovary independent of stage of the estrous cycle (Choudary et al., 1968; Dufour et al., 1972). The development of ultrasound technology confirmed that follicles grow in waves (Pierson and Ginther, 1984, 1986, 1987). In the bovine, a cohort or group of follicles 4 to 5 mm in diameter simultaneously begins to grow and mature.

A follicular wave is the growth of a cohort of follicles and consists of 3 phases: recruitment, selection, and dominance. A group of follicles are initially recruited to grow and mature, but usually only one follicle (dominant) is selected to continue growth while the rest of the cohort undergoes atresia. The single healthy follicle establishes dominance over the rest of the cohort (subordinate) and prevents other follicles from maturing. Dominance is maintained until either the follicle ovulates or undergoes atresia, allowing a new wave of follicles to grow.

Follicular waves occur in pregnant cows (Pierson and Ginther, 1986; Ginther et al., 1996) and during normal estrous cycles. Cows have either two or three waves during an estrous cycle (Pierson and Ginther, 1984, 1987; Ginther et al., 1989), with 2 waves occurring most frequently in heifers (Ginther et al., 1989). Other authors have concluded that 3 instead of 2 follicular waves occur most frequently during estrous cycles of heifers (Savio et al. 1988; Sirois and Fortune, 1988).

Cohorts of follicles were recruited and began to grow on d 2, 9, and 16 of the estrous cycle in dairy heifers with 3 follicular waves, (d 0 = estrus; Sirois and Fortune, 1988), and a dominant follicle reached maximum size on d 6, 16, and 21 of the cycle, respectively. The ovulatory follicle is the dominant follicle of the second or third follicular wave and may be larger than the dominant follicles of the previous non-ovulatory waves (Savio et al., 1988).

Growth of follicular waves is similar for estrous cycles with 2 or 3 waves until d 16 of the cycle (Savio et al., 1988; Ginther et al., 1989). The dominant follicle continues to grow after d 16 in heifers with two follicular waves and becomes the ovulatory follicle, but if three follicular waves occur, the dominant follicle regresses and a new follicular wave begins. Dominant follicles of heifers with only two follicular waves may be larger than for heifers with three waves, because of a longer period of growth (Ginther et al., 1989). The number of follicular waves may be related to length of the luteal phase because regression of the corpus luteum occurs later in cows with three follicular waves (Sirois and Fortune, 1988; Ginther et al., 1989).

After a new wave of follicles begins to grow, one follicle establishes dominance over the rest of the cohort, and the other follicles become atretic. The rate of atresia in the rest of the follicles in the cohort increases once the dominant follicle is selected (Lussier et al., 1987). Dominant follicles can be identified on the ovary by both their diameter and capacity to synthesize estradiol. Dominant follicles are estradiol active while subordinate follicles are estradiol inactive (Sunderland et al., 1994; Austin et al., 2001; Rivera et al., 2001). The future dominant follicle is the first follicle to grow to 4 mm and maintains its size advantage over the next largest follicle until it establishes

dominance over the rest of the cohort (Kulick et al., 1999). Dominant follicles maintain a constant growth rate, while the rest of the cohort have a decrease in growth rate.

Deviation is the first time when growth rates of the dominant and subordinate follicles differ during a follicular wave. Growth rates are similar for dominant and the largest subordinate follicle prior to deviation; however, at deviation, a dominant follicle continues to grow while subordinate follicles grow at a decreasing rate, eventually ceasing to grow. Deviation in cattle occurs an average of 60 to 61 h after a new follicular wave emerges, at which time the follicle is approximately 8 to 8.5 mm in diameter (Ginther et al., 1998; Kulick et al., 1999). Dominant follicles were significantly larger than subordinate follicles when dominant follicles were 9 to 9.9 mm in diameter (Beg et al., 2002) and at day 5 post-estrus (Stewart et al., 1996). The growth rates of the third and fourth largest follicles of the first follicular wave of an estrous cycle began decreasing 16 h before the largest follicle was 8.5 mm in diameter (Ginther et al., 2001a). The growth rate of the largest subordinate follicle began decreasing between 0 and 16 h after the largest follicle reached 8.5 mm in diameter (Ginther et al., 2001a) or when the largest subordinate follicle was 8 to 8.9 mm in diameter (Beg et al., 2002).

Deviation marks the beginning of the dominance phase of a follicular wave because the dominant follicle has acquired the ability to suppress growth of the other follicles in the cohort by secreting factors that act either locally or systemically, and the endocrine milieu of dominant and subordinate follicles differ (summarized in Table 1). Subordinate follicles grew faster and were larger when the dominant follicle was removed from the ovary at the time of expected deviation (8.5 mm; Beg et al., 2002). Dominant follicles inhibit growth of other follicles in a cohort. If one to three of the

Table 1. Concentrations of hormones in follicular fluid (FF) and receptor in granulosa cells (G.C.) of dominant and subordinate follicles after deviation

Hormone in FF/receptor in G.C.	Dominant Follicles	Subordinate Follicles	References
Estradiol	+++	+	Sunderland et al., 1994; Stewart et al., 1996
Androstenedione	+++	+	Stewart et al., 1996
Progesterone	++	++	Stewart et al., 1996
LH receptor	+++	+	Xu et al., 1995a, Boa et al., 1997; Beg et al., 2001; Stewart et al., 1996
Total IGF-I	++	++	de la Sota et al., 1996; Stewart et al., 1996
IGF-I receptor	++	++	Stewart et al., 1996
IGFBP-2	+	+++	de la Sota et al., 1996; Rhodes et al., 2001; Spicer et al., 2001
IGFBP-3	++	++	de la Sota et al., 1996; Rhodes et al., 2001; Spicer et al., 2001
IGFBP-4	+	+++	de la Sota et al., 1996; Rhodes et al., 2001; Spicer et al., 2001
IGFBP-5	+	+++	de la Sota et al., 1996; Rhodes et al., 2001; Spicer et al., 2001
Proteolysis of IGFBP-2	+	+	Spicer et al., 2001
Proteolysis of IGFBP-3	+	+	Spicer et al., 2001
Proteolysis of IGFBP-4	+++	+	Spicer et al., 2001; Rivera and Fortune, 2003a
Proteolysis of IGFBP-5	+++	+	Spicer et al., 2001; Rivera and Fortune, 2003a
Free IGF	+++	+	Beg et al., 2001,2002; Ginther et al., 2003; Rivera and Fortune, 2003a

+ = low concentration/ activity; ++ = moderate concentration/activity; +++ = high concentration/activity.

largest follicles are removed from the ovary, the next largest follicle greater than 7 mm in diameter will become the dominant follicle; however, follicles less than 7 mm in diameter at removal of the dominant follicle cannot become dominant (Ginther et al., 2001a). Subordinate follicles greater than 7 mm in diameter have the ability to become dominant after deviation but dominant follicles inhibit their growth.

Future dominant follicles are larger than subordinate follicles at recruitment (4.2 vs. 3.6 mm; Kulick et al., 1999), and because a cohort of follicles grows at a similar rate until deviation, the dominant follicle maintains its size advantage. Selection of the dominant follicle is a race between a cohort of follicles to reach a level of maturity and size (> 7 mm) that allows the selected follicle to continue growing while preventing growth of other subordinate follicles.

The ovulatory follicle can be identified on the ovary by d 16 of the estrous cycle (Savio et al., 1988). It is the largest follicle on the ovary 3 d prior to estrus, and continues to increase in size until estrus (Dufour et al., 1972; Pierson and Ginther, 1986; Savio et al., 1988). During final growth and maturation of the ovulatory follicle, estradiol secretion is maximal and induces estrous behavior and the ovulatory surge of LH.

Ovulation was originally thought to be due to increasing pressure from fluid in the antrum (reviewed by Espey, 1994). While this pressure may be important for final rupture of the follicle, other mechanisms are involved. Tissue breakdown has a critical role in ovulation of the mammalian follicle. Two hypotheses have been developed to explain this tissue breakdown, and evidence of both necrosis (cell death; reviewed by Espey, 1994) and apoptosis (programmed cell death; reviewed by Murdoch, 1999) can

occur in the ovary near ovulation. Necrosis with inflammation and vascular injury occurs at ovulation, and anti-inflammatory drugs inhibit ovulation (Espey, 1994, Murdoch et al., 1999a,b).

Prior to ovulation, a stigma forms at the apex of the follicle and becomes the site of ovulation. Once a stigma has formed in heifers, ovulation is completed quickly (1.2 h), and evacuation of follicular fluid occurs between 6 s and 14.5 min (Kot and Ginther, 1999). Before ova can be released, five layers of tissue at the stigma must be removed (reviewed by Espey, 1994). The outermost layer is the surface epithelium of the ovary, which surrounds the tunica albuginea or connective tissue of the ovary. Inside the tunica albuginea, the theca externa is the first layer of the follicle. The theca interna, basement membrane, and granulosa cells are the final follicular barriers to ovulation. During ovulation, these layers interact to allow apoptosis.

During apoptosis, the cytoplasm shrinks, and DNA fragments at the apex of the follicle, while the rest of follicle or ovary is not damaged (Murdoch, 1995; Murdoch et al., 1999a,b). Near ovulation, signs of apoptosis are observed in both the surface epithelium of the ovary and theca and granulosa cells of the follicle (Murdoch, 1995). Tumor necrosis factor-α induces ovarian apoptosis in vitro, and antibodies against tumor necrosis factor-α inhibit ovulation in the ewe (Murdoch et al., 1997).

Gonadotropins activate plasmin, which is a protease that regulates the formation of a follicular stigma at ovulation (Colgin and Murdoch, 1997; Murdoch, 1999; Dow et al., 2002). Plasmin stimulates collagenases that degrade connective tissue in the ovary and follicle and increase tumor necrosis factor-α activity (Murdoch et al., 1999a). The

interaction of gonadotropin-stimulated plasmin and collagenases allows the stigma to form and stimulates tumor necrosis factor-α to cause the release of ova.

Hypothalamic and Pituitary Control

The hypothalamus and pituitary control growth, maturation, and ovulation of follicles by regulating gonadotropin secretion. Luteinizing hormone and FSH are synthesized and stored in the anterior pituitary, and they modulate follicular waves and estrous cycles of cows. Luteinizing hormone and FSH are regulated by the hypothalamus through gonadotropin releasing hormone (GnRH) synthesis and secretion. The interaction of GnRH and gonadotropins provide a neuroendocrine mechanism that is the driving force of folliculogenesis, and factors that alter secretions of GnRH, LH, and FSH influence follicular growth.

Follicle-stimulating hormone has a major role in early folliculogenesis and initiates growth of a cohort of follicles during recruitment (reviewed by Fortune 1994). Concentrations of FSH in serum increase prior to the onset of each follicular wave (Sunderland et al., 1994; Ginther et al., 1998), and cows with two or three waves have two and three surges of FSH, respectively (Adams et al., 1992). Concentrations of FSH in serum increase 2 to 4 d prior to the growth of a new cohort of follicles (Adams et al., 1992) and continue to increase until 8 h after wave emergence (Ginther et al., 1998). Similarly, follicular waves during pregnancy are also initiated by increased concentrations of FSH in serum (Ginther et al., 1996).

Changes in FSH concentrations alter follicular growth, and several models have been developed to either augment or diminish concentrations of FSH to study its role in folliculogenesis. Removal of the dominant follicle increases FSH in serum, which

precedes the recruitment of a new follicular wave (Adams et al., 1992; Ginther et al., 2001a). Exogenous FSH after removal of dominant follicles resulted in four to seven times more follicles recruited compared with control animals (Gibbons et al., 1997). Decreasing FSH concentrations by immunizing heifers against GnRH induces anovulation (Prendiville et al., 1995, 1996), and immunized heifers will begin to grow follicles 5 to 9 mm in diameter after treatment with exogenous FSH (Crowe et al., 2001). Follicle-stimulating hormone also regulates follicular cell maturation and growth in vitro. Follicle-stimulating hormone increases bovine granulosa cell proliferation and estradiol production in cell culture systems (Gutierrez et al., 1997; Glister et al., 2001; Spicer et al., 2002a). Follicle-stimulating hormone increased steroidogenesis and proliferation of cultured granulosa cells from small follicles, so that steroidogenesis of cells from small and large follicle was similar (Gutierrez et al., 1997). These data provide evidence that FSH regulates the emergence of a follicular wave and growth of follicles until selection of a dominant follicle (approximately 9 mm), but other factors must regulate follicular growth after the time of deviation of the dominant follicle.

The dominant follicle may continue growing after deviation because it requires less FSH than the rest of the cohort of follicles. At follicle deviation, concentrations of FSH decrease and concentrations of LH increase in serum (Evans et al., 1997; Ginther et al., 1998; Kulick et al., 1999). Concentrations of FSH began to decrease 24 h before expected deviation of the first follicular wave of an estrous cycle, but concentrations of LH started to increase 32 h before deviation (Kulick et al., 1999). These data have led to the hypothesis that final growth of the dominant follicle is dependent on concentrations of LH. Inhibiting LH concentrations by exogenous progesterone decreased growth of

dominant follicles after deviation without influencing timing of deviation (Ginther et al., 2001b). Progesterone treatment (every 8 h beginning when the dominant follicle was 7 mm or 9 mm in diameter) prior to deviation did not influence growth or maturity of dominant follicles; however, progesterone treatment after deviation inhibited growth and estradiol synthesis by dominant follicles (Ginther et al., 2001b). The dominant follicle is dependent on concentrations of LH and FSH. Treating GnRH immunized heifers with FSH or LH had no effect on the number of large follicles (> 9 mm; Crowe et al., 2001), but giving both FSH and LH together increased the number of large dominant follicles (> 9 mm; Crowe et al., 2001). Decreasing concentrations of FSH in serum at deviation inhibited follicular growth and estradiol synthesis although concentrations of LH were not influenced (Bergfelt et al., 2000). A basal concentration of FSH is required for follicular growth even after deviation when LH is the main hormone regulating growth and maturation of dominant follicles.

Steroids alter secretion of LH and FSH and consequently folliculogenesis. Peripheral progesterone concentrations determine the fate of dominant follicles. Dominant follicles will become atretic when progesterone is secreted by corpus luteum, and a new follicular wave will occur. Progesterone concentrations decline during proestrous, allowing concentrations of LH in serum to increase. Concentrations of LH in plasma increase after progesterone concentrations decrease, and LH continues increasing until the ovulatory surge of gonadotropins (Swanson and Hafs, 1971; Wettemann et al., 1972; Walters and Schallenberger, 1984). If LH concentrations are maintained but ovulation is prevented, the dominant follicles continue to grow and synthesize estradiol, which results in a larger ovulatory follicle (Sirois and Fortune, 1990; Stock and Fortune,

1993). The preovulatory LH surge occurs 28 to 32 h prior to ovulation (Swanson and Hafs, 1971; Christenson et al., 1975), and stimulates the cellular mechanisms responsible for the rupture of the ovulatory follicle.

Ovarian Mechanisms

Gonadotropins are essential for folliculogenesis, and factors from the ovary modulate secretion of gonadotropins and their effects at the ovary. Dominant follicles inhibit growth of other follicles through paracrine, autocrine, and/or endocrine mechanisms. The dominant follicle causes atresia of other follicles in its cohort as well as prevents the emergence of another follicular wave by inhibiting FSH secretion. Follicles $\geq$ 5 mm may decrease FSH concentrations in serum (Gibbons et al., 1997), and large dominant follicles have increased ability to suppress FSH concentrations (Ginther et al., 2000b). Exogenous follicular fluid prohibits secretion of FSH and delays the recruitment of a new cohort of follicles (Turzillo and Fortune, 1990; Adams et al., 1992). Removal of the dominant follicle increases secretion of FSH, followed by a new follicular wave (Adams et al., 1992). Injecting FSH increases the number of follicles recruited (Gibbons et al., 1997) and can result in more than one dominant follicle (codominance) on the ovary (Rivera and Fortune, 2001). Therefore subordinate follicles have the ability to become dominant follicles, but dominant follicles suppress their growth and maturation by decreasing FSH to concentrations less than those required for continued growth. Dominant follicles suppress FSH and growth of other follicles through secretion of estradiol (Ginther et al., 2000a,b).

Peripheral concentrations of FSH decrease as concentrations of estradiol increase during follicular maturation (Evans et al., 1997). Dominant follicles have greater

concentrations of estradiol in follicular fluid than subordinate follicles (Merz et al., 1981; Ireland and Roche, 1983b; Sunderland et al., 1994; Stewart et al., 1996). Concentrations of follicular fluid estradiol increased in dominant follicles when they are approximately 0.4 mm in diameter less than the size at which deviation occurs (8.4 mm; Ginther et al., 2003). Removal of the dominant follicle at deviation decreases the amount of estradiol released into the caudal vena cava by 4 h after ablation, and peripheral concentrations of FSH increased between 4 and 12 h after follicle ablation. Removal of the dominant follicle also increased concentrations of estradiol in the largest subordinate follicle, and concentrations were similar to those in dominant follicles at 12 h post deviation (Beg et al., 2002; Ginther et al., 2002). Concentrations of estradiol modulate peripheral FSH concentrations, and exogenous estradiol decreased FSH concentrations (Ginther et al., 2000a). These data support the hypothesis that dominant follicles have increased estradiol secretion at follicular deviation, which attenuates FSH concentrations. Growth of subordinate follicles is inhibited because of decreased peripheral FSH concentrations. Dominant follicles maintain growth when FSH concentrations are minimal, so they must have a mechanism allowing continued growth and development with minimal FSH. Dominant follicles continue to grow and mature when FSH concentrations are minimal because they have greater LH receptors. Luteinizing hormone receptors may have a critical role in deviation of the dominant follicle (Stewart et al., 1996; Bao and Garverick, 1998; Webb et al., 1999). Late in follicular growth, the dominant follicle becomes less dependent on FSH, and acquires the ability to respond to LH. Luteinizing hormone receptor mRNA increased in granulosa cells of dominant follicles from day 2 to 4 of a follicular wave (Xu et al., 1995a; Bao et al., 1997), and LH receptor mRNA increased

when the follicle was 0.5 mm in diameter less than the size when deviation occurs (8.5-8.9 mm; Beg et al., 2001). Subordinate and atretic follicles have fewer LH receptors than dominant follicles (Stewart et al., 1996), and LH receptor mRNA decreased at the onset of atresia (Xu et al., 1995a). The importance of LH concentrations in post deviation growth of the dominant follicle is demonstrated by inhibiting LH concentrations. Reducing LH concentrations after deviation by treating cows with exogenous progesterone inhibited synthesis of estradiol and growth of the dominant follicle (Ginther et al., 2001b). Treating GnRH immunized heifers with FSH and LH increased growth of large estradiol active follicles, which augmented peripheral concentrations of estradiol (Crowe et al., 2001). The preovulatory increase in peripheral estradiol concentrations stimulates the ovulatory LH surge. Treating with estradiol increases serum concentrations of LH similar to the preovulatory surge in both ovariectomized heifers and cows (Short et al., 1973; Hausler and Malven, 1976), and immunization against estradiol inhibited the LH surge in ewes (Martin et al., 1978).

Other molecules influence folliculogenesis through mechanisms that are in synergy or independent of estradiol and gonadotropins. Concentrations of FSH begin to decrease before deviation when concentrations of estradiol are still minimal (Kulick et al., 1999). Factors other than estradiol must also alter concentrations of FSH. Dominant follicles may secrete factors other than estradiol to inhibit secretion of FSH, because a steroid free fraction of follicular fluid, void of estradiol, decreased concentrations of FSH and estradiol and size of the dominant follicle at deviation (Bergfelt et al., 2000). Other molecules modulate selection and dominance of follicles by either autocrine and/or paracrine pathways (at the ovary) or endocrine mechanisms (effecting gonadotropin

secretion). Inhibin, activin, insulin like growth factor I, insulin like growth factor II, and insulin like growth factor binding proteins have been identified as possible modulators of folliculogenesis (reviewed by Roche, 1996; Bao and Garverick, 1998; Webb et al., 1999). Establishment of dominance is dependent on modulation of growth and steroid synthesis by a complex interaction of many different factors and mechanisms that determine the fate of dominant follicles (Table 1).

Inhibin and activin are glycoproteins that are produced by follicles and influence folliculogenesis by autocrine/paracrine or endocrine mechanisms. Inhibins are heterodimers that contain α and β subunits, while activin is a homodimer containing two inhibin β subunits (Robertson et al., 1985). Activin is an inhibin antagonist, and inhibin and activin bind to the same receptor (reviewed by Mihm et al., 2002). Bovine follicular fluid contains eight different forms of dimeric inhibin with different molecular weights (Good et al., 1995). Estrogen active dominant follicles have greater concentrations of high molecular weight inhibins than estrogen inactive follicles, and the onset of atresia is associated with increased concentrations of low molecular weight forms of inhibins but decreased concentrations of high molecular weight forms of inhibin (Ireland et al., 1994; Mihm et al., 1997; Austin et al., 2001). Concentrations of inhibin A and estradiol increase concurrently and together they may decrease concentrations of peripheral FSH, which is associated with selection of the dominant follicle (Bleach et al., 2001; Kaneko et al., 2002). Dimeric forms of inhibin decreased in vitro synthesis of FSH from ovine pituitary cells (Good et al., 1995). Concentrations of inhibin and activin in follicular fluid increased between 5 and 33 h after maximum concentrations of peripheral FSH (Austin et al., 2001). Heifers immunized against bovine inhibin α had greater

concentrations of peripheral FSH than non-immunized heifers (Scanlon et al., 1993). However, inhibin may have a negative effect on granulosa cell estradiol synthesis, and treating cultured granulosa cells with antibodies to bovine inhibin increased estradiol synthesis (Jimenez-krassel et al., 2003). Inhibin and activin are important regulators of antral follicle growth and maturation, and may influence folliculogenesis through hypothalamic/pituitary and ovarian mechanism.

Insulin-Like Growth Factor System. Insulin-like growth factor-I (IGF-I) is a potent stimulator of cell proliferation and DNA and protein synthesis in a variety of tissues including the ovary. The hormone was first named somatomedin-C because it influences many different physiological actions and mimics the actions of somatotropin (reviewed by Jones and Clemmons, 1995). Insulin-like growth factor activity is associated with two separate polypeptides with 62 % amino acid homology (Rinderknecht and Humbel, 1976a,b), and the two polypeptides are designated as IGF-I and IGF-II. The somatomedin hypothesis implies that biological actions of growth hormone are facilitated by IGF-I, which is produced by the liver (reviewed by Isaksson et al., 2001). Growth hormone increases IGF-I from the liver, while IGF-I decreases GH secretion by the pituitary (Daughaday and Reeder, 1966; Daughaday et al., 1972; Jones and Clemmons, 1995).

Insulin-like growth factors have similar actions to insulin, but their physiological actions occur independent of the insulin receptor (Marshall et al., 1974; Megyesi et al., 1975). Insulin-like growth factor-I and IGF-II influence a cell by binding to two types of receptors (Type 1 and 2). The type 1 IGF receptor mediates the signal of IGF-I and IGF-II for most physiological mechanisms, and this receptor binds IGF-I with greater affinity

than IGF-II, while the type 2 IGF receptor has a greater capacity to bind IGF-II than IGF-I (Jones and Clemmons, 1995).

The physiological actions of IGF-I are controlled by a complex system of IGF binding proteins (IGFBP) and IGFBP proteases (reviewed by Spicer and Echternkamp, 1995; Fortune et. al., 2001; Mihm and Austin, 2002) allowing a similar concentration of IGF-I to differ in its ability to stimulate the follicle depending on the presence of receptors, IGFBP, and IGFBP proteases. Granulosa and theca cells have IGF type 1 receptors and can respond to changes in concentrations of IGF-I. Bovine granulosa and thecal cells bind IGF-I (Spicer et al., 1994; Stewart et al., 1996), and the amount of receptors are greater in granulosa cells from large (> 8 mm) vs. small (1-5 mm) follicles (Spicer et al., 1994). Amount of granulosa cell IGF-I binding sites increased after the formation of an antrum in bovine fetal ovaries (Wandji et al., 1992). However, expression of IGF type 1 receptor (Perks et al., 1999) and IGF-I binding (Stewart et al., 1996) in granulosa cells were not influenced by follicular size. Armstrong and coworkers (2000) found IGF type 1 receptor mRNA in granulosa and theca cells, and granulosa cells have more IGF-I receptors than theca cells (Stewart et al., 1996; Armstrong et al., 2000). Others concluded that the IGF type 1 receptor mRNA was located only in granulosa cells (Perks et al., 1999). The discrepancies between studies could be due to differences in experimental model, size and types of follicles used in the experiment, or methods to detect receptors.

Insulin-like growth factor-I has a major function in follicular growth and maturation, and effects of IGF-I on steroidogenesis and cell proliferation in vitro may be influenced by gonadotropin concentrations. Insulin-like growth factor-I increases

numbers of granulosa cells and progesterone and estradiol secretion from large bovine follicles in vitro (Spicer et al., 1993; Spicer et al., 2002a). Furthermore, IGF-I extends the half-life of FSH receptor transcripts (Minegishi et al., 2000), and FSH and IGF-I act in synergy to increase estradiol synthesis by bovine granulosa cells by increasing FSH induced aromatase activity (Spicer et al., 2002a). The amount of IGF-I required to stimulate 50% of maximal estradiol production (ED_{50}) was reduced by 4-fold when cells were concurrently cultured with FSH (Spicer et al., 2002a). Gene expression for aromatase was observed only in murine granulosa cells that had concurrent expression of FSH and IGF receptors (Zhou et al., 1997). Similarly, IGF-I increased bovine thecal cell numbers, LH binding, and androstenedione and progesterone synthesis in vitro (Stewart et al., 1995; Spicer and Chamberlain, 1998).

The importance of IGF-I in reproductive processes has been demonstrated by creating mice without a functional IGF-I gene. Insulin-like growth factor-I null female mice are dwarfs, infertile, and cannot ovulate after gonadotropin treatment (Baker et al., 1996). Insulin-like growth factor-I null mice have reduced ovarian FSH receptor and aromatase mRNA (Zhou et al., 1997). Treating IGF-I null mice with exogenous IGF-I restored FSH receptors to normal levels (Zhou et al., 1997). Insulin-like growth factor-I is essential for normal steroid synthesis of follicles, and removal of IGF-I has detrimental consequences on follicle growth and maturation in mice, possibly through its effects on FSH receptors.

Altering peripheral and ovarian concentrations of IGF-I in the bovine also influences follicular growth. Daily growth hormone injections increased concentrations of IGF-I and the number of follicles 1-5 mm in diameter (Gong et al., 1991). Similarly,

heifers immunized against growth hormone releasing factor had reduced IGF-I concentrations in serum and follicular fluid, delayed puberty, as well as fewer follicles > 7 mm in diameter (Cohick et al., 1996). Cattle that have a natural deficiency in growth hormone receptor have reduced plasma IGF-I concentrations, which is associated with a decrease in the number of follicles 2-5 mm in diameter and size of the dominant follicle during the luteal phase of the estrous cycle (Chase et al., 1998). *Bos Indicus* and twinning cattle have increased numbers of large follicles as well as increased concentrations of peripheral IGF-I (Echternkamp et al., 1990; Alvarez et al., 2000). Spicer et al. (2000a) increased intraovarian IGF-I for 7 d, which increased estradiol concentrations of small follicles and diameter of large follicles. In the ovine, IGF-I infusion into the ovarian artery increased estradiol concentrations (Scaramuzzi et al., 1999). These data provide in vivo evidence that IGF-I regulates folliculogenesis.

According to the somatomedin hypothesis, the actions of growth hormone are mediated by liver IGF-I; however, many tissues synthesize IGF-I, and the validity of the original somatomedin hypothesis is questioned (reviewed by Isaksson et al., 2001). Liu et al. (2000) created a mouse that was void of liver IGF-I, but capable of synthesizing IGF-I in other tissues. These mice had decreased IGF-I in serum without any effect on growth. Exogenous growth hormone increased tissue IGF-I and growth (Liu et al., 2000). Tissues were capable of responding to growth hormone even though their livers could not synthesize IGF-I, so local production of IGF-I may have physiological importance.

Granulosa cells produced IGF-I mRNA and protein in vitro (Spicer et al., 1993; Spicer and Chamberlain, 2000) and mRNA in vivo (Schams et al., 2002; Yuan et al.,

1998). Synthesis of IGF-I is hormonally controlled (reviewed by Spicer and Echternkamp, 1995; Spicer and Chamberlain, 2000). However, other authors could not detect IGF-I mRNA in antral follicles (Perks et al., 1995; Armstrong et al., 2000). This discrepancy between studies could be due to differences between type and sensitivity of methods used to access IGF-I mRNA or experimental models.

Changes in concentrations of IGF-I during folliculogenesis could influence follicle maturation. Insulin-like growth factor-I was positively correlated with size of the dominant follicle and concentrations of estradiol and progesterone in follicular fluid (Spicer et al., 1988; Spicer and Enright, 1991; Echternkamp et al., 1994). Concentrations of IGF-I were greater in large (> 8 mm in diameter) than medium or small follicles (< 8 mm in diameter; Spicer and Enright et al., 1991), and estrogen active follicles have grater amounts of IGF-I than estrogen inactive follicles (Echternkamp et al., 1994). Gene expression for IGF-I was greater in dominant than subordinate follicles with increased expression as size and maturity of the dominant follicle increased (Yuan et al., 1998). However, concentrations of total IGF-I in follicular fluid were similar for dominant and subordinate follicles (de la Sota et al., 1996; Stewart et al., 1996) and did not change during follicular growth (de la Sota et al., 1996; Funston et al., 1996). These data fail to support the hypothesis that follicular fluid IGF-I regulates follicular growth, but the studies evaluated concentrations of total IGF-I in follicular fluid. Insulin-like growth factor binding proteins sequester IGF-I and alter the biological activity of IGF. Therefore, concentrations of IGF binding proteins (IGFBP) must be considered when evaluating the amount of IGF-I that can bind to receptors and have biological activity (free IGF-I). Insulin-like growth factor-I that is not bound to IGFBP (free IGF-I) is

biologically active and can bind to its receptor. Subordinate follicles have decreasing free IGF-I prior to follicle deviation; however, the dominant follicle maintains concentrations of free IGF-I during follicular growth (Beg et al., 2001, 2002; Ginther et al., 2003). Furthermore, removal of the dominant follicle at expected deviation increased free IGF-I in the largest subordinate follicle (Beg et al., 2002). Exogenous progesterone decreased free IGF-I in follicular fluid and size of dominant follicles (Ginther et al., 2001b). Free IGF-I in follicular fluid may be an important regulator of follicular growth, and minimal concentrations in subordinate follicles probably inhibit growth.

Insulin-like growth factor binding proteins are IGF transporter proteins; six IGFBP have been identified (Jones and Clemmons, 1995; Spicer and Echternkamp, 1995). Insulin-like growth factor binding proteins transport IGFs throughout the body, increase the half-life of IGFs, sequester IGFs in certain tissue, and alter the interaction of IGFs with receptors. They may also have IGF independent actions (Jones and Clemmons, 1995). Six IGFBP bands were described in the bovine ovary, and they were identified as one of four IGFBP: IGFBP-2 (34-35 kDa), IGFBP-3 (40-49 kDa), IGFBP-4 (22-28 kDa), and IGFBP-5 (30 kDa; Echternkamp et al., 1994; de la Sota et al., 1996). Granulosa cells of small follicles (1-5 mm) produce IGFBP-2, -4, and -5, and theca cells of large follicles ($\geq$ 8 mm) synthesize IGFBP-2, -3, -4, and -5 in vitro (Chamberlain and Spicer, 2001). Synthesis and production of IGFBP by granulosa and theca cells are hormonally regulated (Spicer and Echternkamp, 1995; Chamberlain and Spicer, 2001; Spicer and Chamberlain, 2002; Voge et al., 2004).

Concentrations of IGFBP change with follicular maturation. Dominant follicles have less IGFBP-2, -4, and -5 than subordinate follicles (de la Sota, 1996; Rhodes et al.,

2001; Spicer et al., 2001), and these binding proteins increase with atresia (de la Sota et al, 1996). In contrast, the relative concentration of IGFBP-3 did not change with atresia and was similar between dominant and subordinate follicles (de la Sota et al., 1996; Spicer et al., 2001). The dominant follicle of the first follicular wave was the largest follicle with the least amount of IGFBP-4 and -2 by 84 h after the FSH surge (Austin et al., 2001), and codominant follicles induced with exogenous FSH had IGFBP-4 concentrations similar to single dominant follicles (Rivera and Fortune, 2001).

Proteases specific for IGFBP are present in follicular fluid and could influence concentrations of IGFBP. Proteolysis of IGFBP-4 and -5 were greater in dominant than subordinate follicles (Rivera et al., 2001; Spicer et al., 2001). Codominant follicles that were induced by exogenous FSH had greater IGFBP-4 proteolysis than subordinate follicles (Rivera and Fortune, 2001). Dominant and subordinate follicular fluid of cattle had little or no proteolytic activity for IGFBP-3 in vitro (Spicer et al., 2001). Proteolysis of IGFBP-4 and -5 by bovine follicular fluid has been reported (Spicer et al., 2001; Rivera and Fortune, 2003a; Monget et al., 2003), and appears to be greater in dominant than subordinate follicles (Spicer et al., 2001; Rivera and Fortune, 2003a).

Proteolysis of IGFBP-2, -4, and -5 is a result of a zinc dependent metalloproteinase (Mazerbourg et al., 2001; Rivera et al., 2001; Monget et al., 2003; Rivera and Fortune, 2003b). Insulin-like growth factor binding protein-4 degradation is regulated by positive feedback of IGF-I (Mazerbourg et al., 2000). Increasing IGF-I concentrations augment IGFBP-4 proteolysis and results in more free IGF-I.

Pregnancy-associated plasma protein-A (PAPP-A), a zinc dependent metalloproteinase, may be responsible for IGFBP proteolysis. Antibodies to PAPP-A

inhibited IGFBP-2, -4, and -5 degradation in bovine preovulatory follicular fluid (Mazerbourg et al., 2001; Monget et al., 2003), and actions of PAPP-A is IGF dependent (Mazerbourg et al., 2001). Pregnancy-associated plasma protein-A mRNA was expressed in bovine granulosa cells and positively correlated with LH receptor and aromatase mRNA (Mazerbourg et al., 2001). Pregnancy-associated plasma protein-A mRNA was expressed in granulosa cells of human and murine ovaries (Hourvitz et al., 2000, 2002), and PAPP-A expression may be induced by gonadotropins (Hourvitz et al., 2002).

Postpartum Anovulation

Beef cows have postpartum anovulatory intervals in which ovulation and estrous behavior does not occur for a period of time after calving. This phenomenon in bovine has been well documented (Wettemann et al., 1980; Yavas and Walton, 2000b; Wiltbank et al., 2002), and Hancock (1948) classified bovine ovaries as ovulatory, anovulatory, and inactive depending on the physiological activity of the ovaries. Anovulatory ovaries were defined as having follicular growth without the formation of corpora lutea, while both follicular growth and ovulation were inhibited in inactive ovaries, and ovulatory ovaries were described as having normal follicular growth that resulted in the formation of functional corpora lutea (Hancock, 1948). Acute and chronic nutrient restriction can also induce anovulation in beef cows (Richards et al., 1989a; Bossis et al., 1999; Mackey et al., 1999; White et al., 2001). Anovulation in cows has been classified according to follicular development and is due to either a wave of follicles that: 1. emerge but stop growing at deviation, 2. deviate but do not mature and ovulate, or 3. continue to grow past ovulatory size without ovulation (follicular cyst; Wiltbank et al., 2002).

Postpartum anovulatory cows have follicular waves with dominant follicles similar to normal estrous cycles; however, final maturation and ovulation of dominant follicles do not occur. A dominant follicle can be identified on the ovary of beef cows about 10 d after calving (Murphy et al., 1990; Crowe et al., 1993; Crowe et al., 1998), but only 11 % of first dominant follicles ovulate (Murphy et al., 1990). Beef cows averaged 3 to 8 follicular waves before the first ovulation (Murphy et al., 1990; Stagg et al., 1995).

Dairy cows also have follicular waves with dominant follicles by 10 d after calving (Wagner and Hansel, 1969; Savio et al., 1990; Beam and Butler, 1997), however, more dairy than beef cows ovulate the first wave dominant follicle (reviewed by Yavas and Walton, 2000b). The average postpartum interval to the first ovulation for dairy cows is 13-17 d with an interval of 14-34 d to the first estrus (Marion and Gier, 1968; Wagner and Hansel, 1969; Callahan et al., 1971; Savio et al., 1990). Differences in postpartum anovulatory intervals for beef and dairy cows could be due to genetic or management differences. Dairy cows that were suckled had a postpartum anovulatory interval of 28 d, without any of the cows exhibiting estrus by 30 d postpartum, compared with a postpartum anovulatory interval of 14 d in milked cows (Wagner and Hansel, 1969). Anovulatory dominant follicles during the early postpartum period occur more frequently on ovaries of beef cows than dairy cows. During postpartum anovulation, maturation and ovulation of dominant follicles may be inhibited by altered hypothalamus, pituitary, or ovarian function.

Hypothalamus and Pituitary

During early gestation, cows have follicular waves that are related to fluctuating gonadotropin concentrations during early gestation (Pierson and Ginther, 1986; Ginther

et al., 1996), but during late stages of gestation, follicular growth is inhibited (Choudary et al., 1968; Ginther et al., 1996) and concentrations of LH and FSH are minimal (Crowe et al., 1998). Concentrations of FSH in plasma increase from d 1 to 5 after calving, and surges of FSH are associated with the recruitment of follicular waves during postpartum anovulation (Beam and Butler, 1997). Concentrations of GnRH in the hypothalamus do not change during the postpartum anovulatory interval (Nett et al., 1988). Concentrations of FSH in plasma and GnRH in the hypothalamus do not limit follicular growth and ovulation in postpartum cows.

Pulsatility of LH is minimal and insufficient during postpartum anovulation for final maturation and ovulation of dominant follicles (reviewed by Wettemann 1980; Short et al., 1990; Yavas and Walton et al., 2000a; Wiltbank et al., 2002). Luteinizing hormone pulsatility resumes around 25 d postpartum and increases with time during postpartum anovulation in beef cows (Riley et al., 1981, Leung et al., 1986), but concentrations of peripheral LH are variable and influenced by body energy stores of cows (Rutter and Randel, 1984; Bishop et al., 1994). Dairy cows may resume LH pulses earlier after calving than beef cows (Peters et al., 1981). Basal LH and the number of LH pulses increase during the postpartum anovulatory interval of beef cows (Rawlings et al., 1980; Garcia-Winder et al., 1984; Stagg et al., 1998). Pituitary concentrations of LH are at their nadir after calving and increase until 30 d postpartum (Nett et al., 1988). Pituitary stores of LH are minimal during the early postpartum period, but after 30 d postpartum, pituitary stores are replenished and do not repress follicular growth. Gonadotropin-releasing hormone receptors increase in the pituitary by 15 d postpartum (Nett et al., 1988), and exogenous GnRH will increase LH concentrations and the number of LH

pulses in anestrous beef cows (Echternkamp et al., 1978; Walters 1982b; Spicer et al., 1986b). The pituitary responds to exogenous GnRH during postpartum anestrus; therefore, inadequate release of GnRH from the hypothalamus inhibits LH secretion from the pituitary after pituitary stores of LH are replenished. The importance of gonadotropins in the regulation of postpartum follicular growth was demonstrated by treatment of postpartum anovulatory cows with exogenous GnRH or LH. A single injection of exogenous GnRH induced a gonadotropin surge and caused 100 % of treated cows to ovulate the first postpartum dominant follicle (Crowe et al., 1993). Similarly, hourly pulses of exogenous LH caused 50 % of cows to ovulate their first postpartum dominant follicle (Duffy et al., 2000).

The hypothalamus may be more sensitive to estradiol during postpartum anovulation, and small increases in estradiol may decrease GnRH release from the hypothalamus. However, studies evaluating the effects of the ovary and estradiol on gonadotropin secretion are inconclusive. Removing ovaries of cows by 5 d after calving increased the number of LH pulses (Schallenberger and Peterson, 1982) and mean LH (Hinshelwood et al., 1985), implying that a factor from the ovary was inhibiting gonadotropin release during anestrus. These studies did not use ovary intact cows as controls and had a small numbers of cows per treatment. Postpartum ovariectomized cows had less GnRH in the median eminence and greater concentrations of LH in plasma than intact cows at 31 – 37 d postpartum when cows were not nursing a calf (Zalesky et al., 1990). However, intact cows could have resumed normal estrous cycles by 37 d postpartum, and concentrations of GnRH and LH change with the estrous cycle of the cow. Ovariectomy of cows with normal estrous cycles also increases the number of

pulses and concentrations of LH (Schallenberger and Peterson, 1982). The ovary inhibited LH concentrations if cows were anestrous or had normal estrous cycles, so the ovary may not prevent gonadotropin release during postpartum anovulation. Treatment of postpartum beef cows with estradiol decreased LH pulse frequency (Walters et al., 1982b). Estradiol treatment of ovariectomized beef cows decreased concentrations of LH in serum after 21 d postpartum (Acosta et al., 1983), but there was no effect earlier in the postpartum interval. An implant of an anti-estrogen (enclomiphene) decreased the postpartum interval and increased mean LH concentrations, but injections of another anti-estrogen (clomiphene citrate) increased the postpartum anestrous interval (Chang and Reeves, 1987). Estradiol treatments can also increase LH secretion. Estradiol implants induced an LH surge at 10 and 17 d postpartum in beef cows (Peters, 1984). These conflicting results may be due to differences in the concentrations of estradiol released from an implant compared to concentrations of estradiol found in cows. The constant release of estradiol from an implant may stimulate an LH surge, but concentrations of peripheral estradiol continuously change in cows and remain constant only for a short period of time. Negative feedback of estradiol may inhibit LH secretion from the pituitary and ovulation of postpartum anovulatory beef cows, but future research is needed to confirm the role of the ovary and estradiol in controlling postpartum anovulation.

Ovary

Follicular growth changes as the postpartum anovulatory interval progresses. Maximum size and growth rate of dominant follicles progressively increase with time after parturition (Murphy et al., 1990; Stagg et al., 1995). Follicular steroidogenesis may

also change during the postpartum anovulatory interval. Concentrations of progesterone in follicular fluid of large follicles (> 8 mm) increased three fold from 7 to 14 d postpartum but then decreased to 28 d postpartum (Spicer et al., 1986a). Large follicles had increased estradiol synthesis from 14 to 28 d postpartum (Spicer et al., 1986a), but gonadotropin binding of large follicles did not change from calving to 56 d postpartum (Spicer et al., 1986a). Estradiol active follicles collected from beef cows after calving or during the luteal phase of an estrous cycle had similar concentrations of estradiol, progesterone, androstenedione, LH receptors, and FSH receptors; however preovulatory follicles had greater estradiol and androstenedione than follicles collected either during the luteal phase or postpartum (Braden et al., 1986). Cows injected with GnRH every 2 h for 2 to 4 d had a four-fold increase in concentrations of progesterone and a two-fold increase of estradiol in large follicles (Spicer et al., 1986c). Follicles of postpartum anovulatory beef cows may synthesize less estradiol than follicles of ovulatory cows, and estradiol synthesis of follicles increases as the first postpartum ovulation approaches.

All dairy cows do not ovulate the first dominant follicles after calving, and follicles that failed to ovulate were smaller and contained less estradiol and IGF-I in follicular fluid than follicles that ovulated (Beam and Butler, 1997). Concentrations of IGF-I in follicular fluid may be an important regulator of postpartum ovarian function; however, concentrations of IGF-I in large follicles of beef cows did not change with time postpartum (Spicer et al., 1988). The previous study only evaluated concentrations of total IGF-I in follicular fluid and did not measure changes in free IGF-I, IGFBP, or IGFBP proteases. Insulin-like growth factor-I binding proteins and IGFBP proteases determine the amount of IGF-I that is biologically active. Changes in concentrations of

IGFBP and their proteases during the postpartum anovulatory interval must be determined before the role of IGF-I in regulating postpartum reproductive function can be determined.

The ovary of postpartum cows may not respond to gonadotropins. Treating postpartum anovulatory cows with LH (Duffy et al., 2000) or GnRH (reviewed by Wettemann et al., 1980; Yavas and Walton, 2000a) will not induce ovulation in all cows implying all follicles of anestrous cows are not responsive to a LH surge. Dominant follicles that did not ovulate after hourly pulses of LH had less estradiol than follicles that did ovulate even though treatments were given for 3 – 5 d commencing on the second day of dominance (Duffy et al., 2000). Therefore, some dominant follicles of postpartum anovulatory cows may not ovulate even when adequate gonadotropins are secreted.

An ovarian mechanism may control ovarian function in seasonally anestrous ewes. Steroid concentrations in dominant follicles differed for anestrous ewes and ewes with normal estrous cycles even though size of the follicles and peripheral FSH concentrations were similar (Bister et al., 1999). Dominant follicles collected from ewes with normal estrous cycles had greater in vitro estradiol synthesis than dominant follicles from seasonally anestrous ewes, even after treatment with gonadotropins (Noel et al., 1999). Follicles from anestrous ewes had lower concentrations of estradiol after treatment with gonadotropins, so an ovarian mechanism in addition to inadequate concentrations of LH may inhibit estradiol synthesis of ovine dominant follicles. Inadequate gonadotropin receptors, steroid precursors, or free IGF-I in seasonally anestrous ewes may act in synergy with the hypothalamus and pituitary to prevent ovulation of dominant follicles. A similar mechanism in the postpartum anovulatory cow

has not been determined, and ovarian factors could influence the postpartum anovulatory interval of beef cows.

Factors Regulating the Postpartum Anovulatory Interval

The duration of the postpartum anovulatory interval is regulated by suckling, nutrition, age, genetics of the dam, and retained placentas (reviewed by Yavas and Walton, 2000b; Wiltbank et al., 2002; Wettemann et al., 2003). Suckling and nutrition are the major factors influencing the length of time from calving until the first ovulation and estrus, with the other factors having minor roles. The negative effects of suckling on the postpartum anovulatory interval are well documented (reviewed by Williams, 1990; Wettemann et al., 2003). Cows with a nursing calf have a prolonged period until the first estrus (Short et al., 1972; Smith and Vincent, 1972; Bellows et al., 1974). Early weaning of calves decreases the interval to the first postpartum estrus (Smith and Vincent, 1972; Bellows et al., 1974; Acosta et al., 1983). Cows with calves that suckled one time per day had a shorter postpartum anovulatory interval than when calves suckled ad libitum (Randel, 1981; Reeves and Gaskins, 1981; Stagg et al., 1998), but cows with calves suckling two times per day or ad libitum had a similar interval to first postpartum ovulation (Lamb et al., 1999).

The mechanism by which suckling regulates postpartum reproduction includes the suckling stimulus, bond between cow and calf, and gonadotropin secretion. Cows suckled once a day and housed near their calves had a longer postpartum anovulatory interval than cows suckled once a day but isolated from calves (Stagg et al., 1998). Cows that had contact with their calves but were not suckling had a longer interval to first postpartum ovulation than cows with weaned calves (Hoffman et al., 1996). Milking

beef cows (2 to 5 times per day) with weaned calves did not influence the postpartum anovulatory interval when compared to non-milked beef cows with weaned calves (Lamb et al, 1999). These results suggest that the maternal bond between a cow and calf in addition to the physical stimulus of suckling may increase the postpartum anovulatory interval.

Suckling prolongs intervals to the first estrus and ovulation through a neuroendocrine mechanism. Suckling inhibits LH secretion from the pituitary (Walters et al., 1982a; Williams et al., 1983; Shively and Williams, 1989), but pituitaries from suckled cows will secrete LH after treatment with GnRH (Walters et al., 1982b; Myers et al., 1989). Weaning at 21 d postpartum increased basal LH, number of LH pulses, responsiveness of pituitaries to GnRH, and the number of follicular LH receptors (Walters et al., 1982a, b). Cows with weaned calves had increased concentrations of GnRH in cerebrospinal fluid (Gazal et al., 1998). These studies provide evidence that suckling may prolong the postpartum anovulatory interval by decreasing GnRH release from the hypothalamus and as a result LH secretion from the pituitary.

Suckling may alter gonadotropin secretion by modulating concentrations of endogenous opioid peptides in the brain. Treatment of cows with an opioid receptor antagonist (Naloxone) increased peripheral concentrations of LH in suckled cows (Whisnant et al., 1986b; Myers et al., 1989). Naloxone increased concentrations of LH in serum of suckled beef cows at 14, 28, and 42 days postpartum, but a larger dose was required to increase LH concentrations at 14 d postpartum (Whisnnant et al., 1986a). Suckling probably inhibits gonadotropin secretion through an opioid mechanism, but beef cows suckling a calf and treated with naloxone at 28 d postpartum had concentrations of

LH that were less than in cows with weaned calves (Myers et al., 1989). Suckling may inhibit gonadotropin secretion through neuroendocrine mechanisms in addition to opioid peptides.

A model to explain suckling effects on gonadotropin concentrations was proposed by Williams (1990). Suckling or maternal bond between cow and calf may activate neurons that signal the brain and increase neurotransmitters like opioid peptides. These neurotransmitters may hinder GnRH release by acting on the hypothalamus or indirectly through other interneurons. Decreased GnRH release results in insufficient LH secretion for final follicular growth and ovulation.

Nutrition

Nutrition also regulates reproductive efficiency of cattle (reviewed by Wettemann 1980; Randel, 1990; Short et. al., 1990; Wettemann et al., 2003). Energy intake influences the onset of puberty in heifers (Short and Bellows, 1971; Yelich et al., 1995) and duration of the postpartum anestrous interval in cows (Wiltbank et al., 1963; Spitzer et al., 1995). Body condition score at calving is the most important factor that determines the length of the postpartum anovulatory interval (Richards et al., 1986) and if a cow will become pregnant in the breeding season (Selk et al., 1988; Spitzer et al., 1995). Cows calving with a good BCS (5 or greater) have shorter postpartum anovulatory intervals (Richards et al., 1986; Lents et al., 2000; Looper et al., 2000), and primiparous cows with a greater BCS at calving have greater pregnancy rates and a greater percentage in estrus by the end of the breeding season (Spitzer et al., 1995). While nutrition is a major factor regulating reproductive efficiency, the mechanism by which nutrition controls reproductive function has not been elucidated.

Models to Study Nutrition and Reproduction

Several models have been developed to study the effects of nutrition on reproduction (Table 2). Reduced nutrient intake can inhibit reproductive function of cows, and different types of nutrient restriction have been used to identify possible mechanisms linking nutritional status of a cow with reproductive function. Beef heifers fed to lose 1 % of BW per week (chronic nutrient restriction) ceased to ovulate, and became anestrus after 26 (Richards et al., 1991) and 32 wk (Bossis et al., 1999). Similarly, restricting heifers early after puberty to 0.4 of maintenance requirements for 14 d (acute nutrient restriction) prevented ovulation in 60 to 70 % of heifers (Mackey et al., 1999; White et al., 2001).

Nutrition alters follicular growth through its effects on the hypothalamic-pituitary-ovarian axis. Restriction of dietary energy reduced size of follicles in prepubertal (Bergfeld et al., 1994) and postpubertal (Murphy et al., 1991) heifers. Chronic nutrient restriction decreased size (Rhodes et al., 1995; Rhodes et al, 1996; Bossis et al., 1999) and growth of dominant follicles (Bossis et al., 1999). Heifers fed 0.4 M had smaller dominant follicles that grew slower when compared with heifers fed 1.2 M (Mackey et al., 1999). Fasting heifers for 48 h reduced size of large dominant follicles (Spicer et al., 1992).

Concentration, pulse frequency, and pulse amplitude of LH in serum prior to anovulation were less in chronically restricted than maintenance fed heifers (Rhodes et al., 1996; Bossis et al., 1999). The negative effects of nutrient restriction on growth of dominant preovulatory follicles could be due to a decrease in LH secretion. However, acute nutrient restriction of heifers did not alter the frequency of LH pulses (Mackey et

Table 2. Effects of type of nutrient restriction on follicular growth and concentrations of hormones

Characteristic/ hormone	Model		
	Chronic restricted	Acute restricted	Fasted
Growth of DF	Decreased (Bossis et al., 1999)	Decreased (Mackey et al., 1999)	-
Size of DF	Decreased (Rhodes et al., 1995, 1996; Bossis et al., 1999)	Decreased (Mackey et al., 1999)	Decreased (Spicer et al., 1992)
LH pulses	Decreased (Rhodes et al., 1996; Bossis et al., 1999)	No effect (Mackey et al., 2000, 2001)	-
Estradiol	Inhibited (Bossis et al., 1999)	Inhibited (Lents et al., 2001)	Inhibited (Spicer et al., 1992)
LH surge	Inhibited (Bossis et al., 1999)	Inhibited (Mackey et al., 1999, Lents et al., 2001)	-
Glucose	Decreased (Richards et al., 1989; Richards et al., 1995; Bossis et al., 1999)	No effect (White et al., 2000)	Decreased (McCann and Hansel, 1986; Frohil and Blum, 1988; Lents et al., 1996)
Insulin	Decreased (Richards et al., 1989; Bossis et al., 1999)	No effect (White et al., 2000)	Decreased (McCann and Hansel, 1986; Frohil and Blum, 1988; Lents et al., 1996)
IGF-I	Decreased (Richards et al., 1995; Bossis et al., 1999)	Decreased (Mackey et al., 2000; White et al., 2001)	Decreased (Spicer et al., 1991; Amstalden et al., 2000)
NEFA	Increased (Bossis et al., 1999)	Increased (Mackey et al., 2000; White et al., 2000)	Increased (Murphy et al., 1991, Lents et al., 1996; Jorritsma et al., 2003)
Leptin	-	Tended to decrease (White et al., 2000)	Decreased (Amstalden et al., 2000)
Thyroxine	Decreased (Richards et al., 1995)	Decreased (White et al., 2000)	

al., 2000; Lents et al., 2001). The effects of acute nutritional restriction on follicular growth may be independent of secretion of gonadotropins and due to intraovarian factors. Acute nutritionally restricted heifers that did not ovulate did not have an ovulatory surge of LH, but all heifers that ovulated had an LH surge (Mackey et al., 1999; Lents et al., 2001). Chronic (Bosssis et al., 1999) and acute (Lents, 2001) nutritionally restricted heifers that did not ovulate also did not have an estradiol surge. Heifers fasted for 48 h had decreased estradiol in follicular fluid of follicles greater than 6 mm in diameter (Spicer et al., 1992). These results suggest that dominant follicles of heifers with reduced nutrient intake are unable to induce an LH surge because they fail to secrete sufficient estradiol. Because nutrition of cows affects the hypothalamic-pituitary-ovarian axis, metabolic hormones that change with nutrient restriction may be signals to the brain and ovary influencing reproductive function.

Metabolic Hormones

Metabolic hormones and energy metabolites are possible signals that indicate the energy status of animal to the hypothalamic-pituitary-ovarian axis (Table 2). Nutrient availability alters both glucose and insulin in plasma of cows and heifers (McCann and Hansel, 1986; Richards et al., 1989b; Bossis et al., 1999; Ciccioli et al., 2003). However, the effects of glucose and insulin on LH concentrations are inconclusive. Chronic infusion of insulin or insulin and glucose into the brain of feed restricted ovariectomized ewes increased LH secretion, but infusion of just glucose had no effect on LH secretion (Daniel et al., 2000). Acute infusion of insulin in the brain did not alter LH secretion in growth restricted ovariectomized ewes (Hileman et al., 1993). Infusion of glucose and

treatment with insulin did not change LH secretion in postpartum beef cows (Garmendia, 1984).

Concentrations of insulin in plasma are sensitive to diets of animals, and increasing nutrient intake augmented plasma insulin in heifers (Mackey et al., 2000). Chronic nutrient restriction of heifers (Richards et al., 1989b; Bossis et al., 1999) and fasting prepubertal calves (Frohli and Blum, 1988; Lents et al., 1996) decreased concentrations of insulin and glucose in plasma. Acute nutrient restriction did not alter concentrations of insulin and glucose in plasma (White et al., 2000), and concentrations of insulin and glucose probably are not limiting reproductive function of acute nutritionally restricted heifers.

Chronic and acute nutritional restriction of beef heifers increased plasma NEFA (Bossis et al., 1999; Mackey et al., 2000; White et al., 2000). Heifers that were fasted also had increased concentrations of NEFA in plasma (Lents et al., 1996; Jorritsma et al., 2003). Concentrations of plasma NEFA are inversely related to the frequency of LH pulses in primiparous suckled beef cows (Grimard et al., 1995). Lambs infused with free fatty acids had increased NEFA, but concentrations of LH secretion were not effected (Estienne et al., 1990). The role of NEFA in regulating the hypothalamus-pituitary-ovarian axis needs further research.

Acute and chronic nutrient restriction decreased plasma IGF-I in heifers (Bossis et al., 1999; White et al., 2001). Fasting heifers for 48 h decreased IGF-I in plasma but not in follicular fluid of follicles greater than 6mm in diameter (Spicer et al., 1992). Realimentation of acute and chronic restricted heifers increased concentrations of IGF-I in plasma and stimulated anovulatory heifers to resume normal estrous cycles (Bossis et

al., 2000; White et al., 2001). Concentrations of IGF-I change with reproductive function of beef heifers and may provide a mechanism whereby nutrition modulates reproduction (Wettemann and Bossis, 2000).

Concentrations of IGF-I increased with level of nutrition, and as heifers approach puberty (Yelich et al., 1996). Heifers immunized against growth hormone releasing hormone had decreased IGF-I secretion and delayed puberty (Armstrong et al., 1992). Ewes with low BCS had less LH and IGF in serum than those with high BCS (Snyder et al., 1999). Ovine pituitaries have receptors for IGF-I, and IGF-I stimulated LH secretion from ovine pituitary cells in vitro (Adam et al., 2000). Insulin-like growth factor knockout mice are infertile and will not ovulate after treatment with gonadotropins (Baker et al., 1996). These studies suggest that the nutritional status of an animal could control reproduction by decreasing IGF-I concentrations.

Leptin is secreted by adipose tissue, and concentrations of leptin change with diet and fat reserves of ruminants (Delavaud et al., 2000; Ehrdardt et al., 2000; Wettemann et al., 2003). Acute nutrient restriction tended to decrease concentrations of leptin (14 % less) in plasma compared with heifers fed a normal diet (White et al., 2001). Heifers fasted for 48 h had decreased leptin mRNA in fat and decreased peripheral leptin concentrations (34 % decrease; Amstalden et al., 2000). Ovine pituitaries have leptin receptor mRNA (Dyer et al., 1997) with leptin receptors identified specifically in gonadotropes (Iqbal et al., 2000). Leptin receptor mRNA is expressed in the hypothalamus of ewes (Dyer et al., 1997; Williams et al., 1999; Adam et al., 2002), and feed restriction (Dyer et al., 1997) and fasting (Adam et al., 2002) increased leptin receptor mRNA in the hypothalamus. Changes in peripheral concentrations of leptin may

influence the brain by crossing the blood-brain barrier (Kastin and Pan, 2000) or the blood-cerebrospinal fluid barrier (Thomas et al., 2001). Leptin may alter reproduction via an ovarian mechanism. Granulosa cells have receptors for leptin (Spicer and Francisco, 1997), and leptin had a negative effect on in vitro steroid production by theca and granulosa cells (Spicer and Francisco, 1997, 1998; Spicer et al., 2000b). These data provide indirect evidence that leptin may be a metabolic signal to the hypothalamic-pituitary-ovarian axis to influence reproduction, but future research is needed to support this hypothesis.

Conclusion

Before oocytes can be released for fertilization, follicles must progress through a series of changes providing the correct physiological and endocrine environment for oocytes. Most follicles will not complete development and become atretic failing to release their oocytes; however, a few follicles become dominant and prevent the growth of other follicles. Follicular growth occurs in waves in the bovine with one follicle establishing dominance per wave. Follicular growth is precisely timed and controlled by the interaction of hormones from the hypothalamic-pituitary-ovarian axis that have endocrine, paracrine, or autocrine effects. Factors that alter the hypothalamic-pituitary-ovarian axis can alter follicular growth and ovulation.

Beef cows are anovulatory for a period of time after calving, and many cows fail to resume normal estrous cycles by the end of the breeding season. A major goal for beef producers is to have each cow produce one calf per year, but many cows have postpartum anovulatory intervals that are longer than the breeding season. Cows that fail to become pregnant during the breeding season are culled and sold. This decreases efficiency and

profitability for producers. Therefore, management strategies to decrease the length of time that postpartum cows are anovulatory would benefit the beef industry.

Length of the postpartum anovulatory interval is controlled by body condition score and nutrient intake. Nutritional restriction alters reproductive function providing evidence that nutrient status of the animal must communicate with the hypothalamic-pituitary-ovarian axis, and metabolic hormones may be possible signals. Evidence from several models indicates that concentrations of IGF-I change with nutritional status of animals. These data suggest that fluctuations in concentrations of IGF-I may influence reproductive processes. Concentrations of IGF-I are important modulators of follicular growth, and the activity of IGF-I is controlled by a complex system of IGFBP and IGFBP proteases.

The mechanism preventing postpartum cows from ovulating dominant follicles is not completely understood. Postpartum anovulatory cows have decreased secretion of LH, which reduces follicular growth and prevents ovulation. Hypothalamic and pituitary function during postpartum anovulation has been extensively studied, but changes in ovarian function during the postpartum anovulatory interval are not well documented. Dominant follicles of postpartum beef cows have decreased estradiol concentrations, and decreased concentrations of steroid precursors and steroidogenic enzymes may prevent estradiol synthesis. Concentrations of IGF-I, IGFBP, and IGFBP proteases regulate normal follicular growth and steroid synthesis; therefore, changes in the IGF system may also influence dominant follicles of postpartum beef cows. Body condition score at calving affects the length of time from parturition until cows resume normal estrous cycles, but the effects of BCS on ovarian function have not been elucidated. Body

energy stores at calving may modulate steroid synthesis and/or the IGF system in dominant follicles. A better understanding of the mechanism causing postpartum anovulation may provide management practices and/or treatments to increase postpartum reproductive performance and profitability of beef producers. Therefore, the objectives of this research were: 1. to determine the effects of days after calving and interval before the first postpartum ovulation on concentrations of IGF-I, IGFBPs, progesterone, androstenedione, estradiol, and aromatase mRNA in dominant follicles, and 2. to evaluate effects of BCS at calving and length of the postpartum anovulatory interval on concentrations of IGF-I, IGFBPs, progesterone, androstenedione, estradiol, aromatase mRNA , leptin receptor mRNA, and pregnancy-associated plasma protein-A mRNA in dominant follicles.

CHAPTER III

Effects of days after calving and days before first estrus on insulin-like growth factor-I, insulin-like growth factor binding proteins, progesterone, androstenedione, estradiol, and aromatase mRNA in dominant follicles of postpartum beef cows.

ABSTRACT: The effect of days after calving and interval before the first postpartum ovulation on IGF-I, IGFBP, progesterone, androstenedione, estradiol, and aromatase mRNA in dominant follicles (DF) was evaluated in Angus x Hereford cows. Growth of DF (> 9 mm) was monitored daily by ultrasonography and fluid from DF was collected in vivo at either 30 ± 2 d or 47 ± 2 d postpartum. Follicular fluid (FF) was also aspirated from DF of contemporary ovulatory cows at proestrus. Estrous behavior was monitored continuously with HeatWatch, and progesterone in plasma collected twice weekly was used to assess luteal activity. Anovulatory DF aspirated 30 and 47 d postpartum had similar concentrations of IGF-I, IGFBP, progesterone, estradiol and androstenedione in FF and IGF-I and IGFBP in plasma. The intervals from aspiration to estrus were similar for cows aspirated 30 and 47 d postpartum, so time of follicular aspiration of anovulatory cows was classified as < 35 d or >35 d before the first postpartum estrus and ovulation. The amounts of IGFBP-3 and -4b (20-kDa) in FF were greater ($P = 0.06$; $P < 0.05$, respectively) in DF aspirated < 35 d before the first estrus, or at proestrus, than in DF aspirated > 35 d before estrus. Concentrations of progesterone in FF were less ($P < 0.01$)

in DF > 35 d before estrus (29 ± 6 ng/mL) than in DF < 35 d (76 ± 15 ng/mL) before estrus and in proestrous follicles (94 ± 10 ng/mL). Concentrations of androstenedione in FF were greater ($P < 0.01$) in proestrous follicles (34 ± 10 ng/mL) than in DF aspirated at > 35 d (3 ± 5 ng/mL) and < 35 d (10 ± 2 ng/mL) before the first postpartum estrus, and tended ($P = 0.08$) to be greater in DF < 35 than in DF > 35 d before estrus. Proestrous follicles had greater ($P < 0.01$) estradiol (538 ± 148 ng/mL) than DF > 35 d (72 ± 39 ng/mL) or < 35 d (95 ± 32 ng/mL) before estrus. Concentrations of IGF-I in FF, IGF-I and IGFBP in plasma, and aromatase mRNA in granulosa cells were similar for anovulatory and proestrous cows. In conclusion, estradiol production by DF of postpartum anovulatory cows may be limited by inadequate production of androstenedione, and alterations in IGFBP in FF during the postpartum anovulatory interval may influence follicular maturation.

Key Words: Beef cows, Postpartum, IGF-I, IGFBP, Steroids, Aromatase

Introduction

Postpartum anovulatory beef cows have follicular waves and dominant follicles (DF) by 10 d after calving; however, only 11 % of beef cows ovulated the first DF (Murphy et al., 1990). Beef cows had an average of seven follicular waves before the first ovulation (Stagg et al., 1995), and postpartum follicular growth is influenced by prepartum and postpartum nutrient intake (Perry et al., 1991). Beef cows must resume normal estrous cycles and ovulation by about 80 d post partum to produce a calf every 12 mo. The inability of a cow to become pregnant during the breeding season is a major cost for beef production (NAHMS, 1997; Bellows et al., 2002). The mechanisms controlling postpartum anovulation have not been completely revealed and could be due to factors

influencing the hypothalamus, pituitary, and/or ovary. While the hypothalamus and pituitary have been extensively studied (reviewed by Wettemann 1980; Short et al., 1990; Wiltbank et al., 2002; Wettemann et al., 2003), research is limited on ovarian changes during the transition from anovulation to normal estrous cycles.

Insulin-like growth factor-I may be an important regulator of ovarian activity (Wettemann and Bossis, 2000. Concentrations of IGF-I regulate in vitro cell proliferation and estradiol production by granulosa cells from large bovine follicles (Spicer et al., 1993). The physiological actions of IGF-I are controlled by a complex system of IGFBPs, IGF-I receptors, and IGFBP proteases (reviewed by Spicer and Echternkamp, 1995; Fortune et. al., 2001; Mihm and Austin, 2002) allowing a concentration of total IGF-I in follicular fluid (FF) to differ in its ability to stimulate follicles depending on the entire IGF system. Insulin-like growth factor-I that is not bound to IGFBP (free IGF-I) is biologically active and can bind to its receptor. Peripheral IGF-I increased prior to first postpartum ovulation when cows were fed a nutrient restricted diet (Roberts et al., 1997; Stagg et al., 1998; Ciccioli et al., 2003). Concentrations of IGF-I in FF of beef cows did not change with time postpartum (Spicer et al., 1988); however, concentrations of IGFBP were not evaluated. Objectives of this study were to determine the effects of days after calving and interval before the first postpartum ovulation on concentrations of IGF-I, IGFBPs, progesterone, androstenedione, estradiol, and aromatase mRNA in DF of postpartum anovulatory cows, and to compare concentrations in DF of postpartum anovulatory cows to concentrations in DF of ovulatory proestrous cows.

Materials and Methods

Animals and Experimental Protocol

Cows weighed 551 ± 10 kg and had a body condition score of 4.5 ± 0.1 (1 = emaciated, 9 = obese; Wagner et al., 1988) pre calving. Cows were fed 1.5 kg/d of a 40% CP supplement prior to calving with ad libitum dormant grass hay. After calving, cows were fed 1.8 kg/d of a 40% CP supplement with ad libitum access to dormant native grass pasture and hay to maintain body condition.

Cows (n = 17) were blocked by calving date, and commencing at 22 or 42 d post partum, growth of DF (> 9 mm) was monitored daily by ultrasonography (7.5-MHz probe; Aloka, Corometrics Medical Systems, Wallingford, CT) until DF grew < 0.8 mm in 24 h. Ultrasound images of the ovary were recorded at each evaluation, and the diameter of the follicle was the average of the length and the width (Pierson and Ginther, 1988). When a follicle reached its maximum size, ultrasound-guided transvaginal follicle aspiration was used to collect fluid from DF either at 30 ± 2 d (n = 7) or 47 ± 2 d (n = 10) after calving. Local anesthesia was induced by caudal epidural treatment with 5 mL of 2% lidocaine. Ovaries were positioned against the vaginal wall, and a vaginal 5-MHz probe (Aloka, Corometrics Medical Systems, Wallingford, CT) was used to guide an 18 G, 55 cm needle (Cook Veterinary Products, Spencer, IN) to puncture the follicle and aspirate FF to a 3-mL syringe. Time of follicular aspiration of anovulatory cows was classified as < 35 d or >35 d before the first estrus and ovulation. Follicular fluid was also aspirated from DF of proestrous cows (n = 4). Cows exhibiting normal estrous cycles were given $PGF_{2\alpha}$ (Lutalyse, 25 mg; Pharmacia Animal Health, Kalamazoo, MI) during the luteal phase of an estrous cycle (8 d after onset of estrus), and follicles were

measured on the day of $PGF_{2\alpha}$ treatment and for two consecutive days. The DF increased at least 0.8 mm/d, and it was aspirated at 48 h after $PGF_{2\alpha}$ treatment.

Blood plasma was collected by tail venipuncture twice weekly (Monday and Thursday) from 22 d post partum until 23 d after the first postpartum estrus and ovulation. Progesterone in plasma was used to access luteal activity, and cows were classified as anovulatory at follicular aspiration if plasma progesterone was < 0.5 ng/mL in all previous samples. Commencing at 20 d postpartum, estrous behavior was monitored with HeatWatch® (DDx Inc., Denver, CO) as previously described (White et al., 2002), and onset of estrous behavior was defined as the first of two mounts within a 4 h period. The first postpartum estrus and ovulation was defined as the first estrus associated with plasma progesterone > 0.5 ng/mL within 7d after estrus and with 3 consecutive samples (≥ 8 d) > 0.5 ng/mL. Concentrations of IGF-I in plasma were measured weekly for 6 wk before the first ovulation and at follicular aspiration. Concentrations of IGFBP in plasma were determined for each anestrous cow (n = 17) at 30 d postpartum (27 ± 3 d), 47 d post partum (45 ± 3 d), and at proestrus.

Hormone and mRNA Analysis

Follicular fluid was placed in 5 mL cryogenic polypropylene conical vials on ice for 10 min and then centrifuged at 2000 x g for 7 min to separate fluid and granulosa cells. Granulosa cells and fluid were stored in separate 5 mL cryogenic polypropylene conical vials. TRIzol (500μL; Invitrogen Corp., Carlsbad CA) was added to vials containing granulosa cells to extract RNA. Granulosa cells and FF were immediately frozen in liquid nitrogen, and FF was stored at –20°C and granulosa cells were stored at –80°C until analyzed.

Vials containing granulosa cells and TRIzol were thawed at 20°C, and 100 μL of chloroform (molecular biology grade) was added to each vial. Samples were incubated for 2 min at 20°C and then were centrifuged at 3500 x g for 30 min at 4°C. The upper aqueous phase was transferred to a 1.5 mL micro centrifuge tube, 250 μL of isopropanol (molecular biology grade) was added, and the tube was incubated at 20°C for 10 min. Then samples were centrifuged at 3500 x g for 10 min at 4°C to precipitate RNA, and the supernatant was decanted. Pellets containing RNA were washed with 500 μL of 75 % ethanol and centrifuged at 3500 x g for 30 min at 4°C. The supernatant was decanted, and the RNA was stored in 30 μL of RNAase free water (Qiagen, Valencia, CA) at – 80°C until analyzed (< 3 months after extraction). The quantity and quality of RNA in granulosa cells were determined by spectrophotometry by evaluating the absorbance at 260 nm (total μg of RNA = OD_{260} x dilution factor x 40μg/ml x total sample volume) and 260 nm/280 nm (all samples had a ratio > 1.5).

Concentrations of aromatase mRNA in granulosa cells was determined by real-time RT-PCR in 25 μL reactions. Primers and probe were created from the sequence of bovine aromatase (GeneBank accession number = Z32741) using Primer Express™ software (Foster City, CA) as described by Voge et al. (2004). A BLAST search (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) for primers and probe was conducted to ensure that only target genes were evaluated, and RT-PCR product was electrophoresed on a 2% agarose gel to further validate that size of product was consistent with the predicted size (65 bp). A master mix was created and the following reagents were added for each reaction: 1 μL of probe from a 10 μ*M* solution (sequence: TGGTGACCATCTGTGCTGATTCCATCA; bp 661 to 687), 1 μL of both forward and

reverse primers from 5 μ*M* solutions (forward sequence: CCTGGCCTGGTGCGC, bp 645 to 659; reverse sequence: TCCAGCCTGTCCAGATGCTT, bp 690 to 709), 12.5 μL of RT-PCR master mix (Qiagen, Valencia, CA), 0.25 μL of reverse transcriptase mix (Qiagen, Valencia, CA), and 8.25 μL of RNase-free water (Qiagen, Valencia, CA). Reactions also included 1 μL (25 ng) of total RNA. The probe contained a 5' reporter dye (TET) and 3' quencher dye (TAMRA) that allowed amplification of RNA to be evaluated on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction conditions consisted of 1 cycle to reverse transcribe RNA (50 °C for 30 min then 95 °C for 15 min) proceeded by 45 cycles to amplify cDNA (94°C for15 s then 60 °C for 60 s). The relative concentration of aromatase mRNA was determined in duplicates (intraassay CV was 1 %) via the comparative C_T method as previously described by Hettinger et al. (2001) with 18S RNA as the normalization control (18S control kit; 43108993E; Applied Biosystems, Foster City, CA), which contains VIC as the 5' reporter dye and TAMRA as the 3' quencher dye. Normalization control reactions for 18S RNA occurred in separate wells on the same 96 well plate with identical reaction concentrations and conditions. Data were analyzed by assigning an arbitrary threshold cycle (C_t) to amplification plots, and the C_t for a sample was the cycle that its amplification plot crossed the threshold. The C_t was assigned above the baseline or background levels that occur in early cycles and below the plateau of amplification; the C_t was always in the log-linear range of amplification. The aromatase C_t was normalized to the 18S C_t and this value was termed the ΔC_t. Fold differences in mRNA were determined by the formula: $2^{-\Delta\Delta Ct}$, with the $\Delta\Delta C_t$ of a treatment being its mean ΔC_t minus the mean ΔC_t of the treatment with the least mRNA (which had the largest ΔC_t).

Concentrations of estradiol (intra- and interassay CV were 6 and 12 %, respectively; Spicer and Enright, 1991), androstenedione (intraassay CV was 8 %; Stewart et al., 1996), progesterone (intraassay CV was 7 %; Coat-A-Count progesterone kit, Diagnostic Products Corp., Los Angeles, CA), and IGF-I (intraassay CV was 5 %; Echternkamp et al., 1990) in FF were quantified by RIA. The progesterone RIA was validated for bovine FF, and 108 and 112 % of 0.125 and 0.5 ng were recovered when added to 25 μL of bovine FF. The inhibition curve generated by adding 25, 50, 75, or 100 μL of bovine FF to assay tubes was parallel to the standard curve. Concentrations of IGF-I (intra- and interassay CV were 13 and 11 %, respectively; Echternkamp et al., 1990) and progesterone (intra- and interassay CV were 5 and 8 %, respectively; Coat-A-Count progesterone kit, Diagnostic Products Corp., Los Angeles, CA; Vizcarra et al., 1997) in plasma were quantified by RIA. The relative amounts of IGFBP were evaluated by one-dimensional SDS-PAGE in 4 μL of FF (3 gels) and plasma (6 gels; inter-gel CV was 25.0 %; Echternkamp et al., 1994). Cows were blocked by time of aspiration and BCS and randomly assigned to gels. Briefly, samples were heat denatured (3 min at 100°C) and electrophoresed on a 12% polyacrylamide gel. Proteins were then transferred to nitrocellulose paper (Midwest Scientific, St. Louis, MO) and ligand-blotted overnight with ^{125}I-IGF-I and ^{125}I-IGF-II. The nitrocellulose blots were exposed to X-ray film at -80°C for 2 d (IGFBP-3 and -2) or 7 d (IGFBP-4 and -5), and band intensity on autoradiographs was determined using scanning densitometry (Molecular Analyst, Bio-Rad).

Statistical Analysis

The effects of reproductive status on concentrations of progesterone, androstenedione, estradiol, IGFBP, and IGF-I in FF, IGF-I in plasma at aspiration, size of the DF, and aromatase real-time RT-PCR ΔC_t values were analyzed in a completely randomized block design using a mixed model procedure (Proc Mixed procedure; SAS Inst. Inc., Cary, NC). Reproductive status (anovulatory at 30 d postpartum, anovulatory at 47 d post partum, and ovulatory at proestrus) was included in the model as fixed effects. Assay or gel number was included in the model as a random effect for analysis of changes in concentrations of IGF-I in plasma and IGFBP in FF. Homogeneity of variance for traits between cows was determined with Levene's test (SAS Inst. Inc., Cary, NC). Log transformation was used for concentrations of estradiol, progesterone, androstenedione, and aromatase mRNA because variances were not homogenous. The actual least squares means and standard errors are presented when data were transformed for analyses. When DF were aspirated 30 and 47 d postpartum, cows had similar lengths of time from aspiration to estrus (34 ± 9 d). Therefore, time of follicular aspiration was classified as > 35 d or < 35 d before first postpartum estrus, and results were re-analyzed with days relative to first estrus and ovulation (> 35 d before the first estrus, < 35 d before the first estrus, and at proestrus) included in the model as fixed effects.

Changes in concentrations of IGF-I and IGFBP in plasma were analyzed in a completely randomized design with either six (IGF-I; one sample per wk for each cow during the 6 weeks before first ovulation) or three (IGFBP in plasma at 30 ± 2 d postpartum, 47 ± 2 d postpartum, and proestrus for each cow) repeated measures across time using a mixed model procedure (Proc Mixed procedure; SAS Inst. Inc., Cary, NC)

with assay or gel number included in the model as a random effect. Concentrations of IGFBP in plasma were also evaluated by days relative to first estrus and ovulation with three repeated measures across time (> 35 d before the first estrus, < 35 d before the first estrus, and at proestrus) and gel number was included in the model as a random effect. Degrees of freedom for the pooled error term were calculated by Kenward-Roger's approximation (SAS Inst. Inc., Cary, NC). The covariance structure for the repeated measure was modeled by a first order autoregressive function with lag equal to one. Time effects were tested using the pooled residual error term, and significant treatment effects were separated using a student's t test with the PDIFF procedure of SAS (SAS Inst. Inc., Cary, NC). Partial correlation coefficients for anovulatory cows, adjusted for DF that were collected 30 and 47 d postpartum, were calculated among concentrations of follicular fluid constituents and days from aspiration to the first postpartum estrus (GLM procedure and MANOVA/PRINTE statement; SAS Inst. Inc., Cary, NC).

Results

Sixty percent of cows had a short luteal phase (< 8 d), 13 % of cows had a normal luteal phase, and 27 % of cows had no luteal activity preceding the first postpartum estrus with 90 % of cows having a normal luteal phase after first estrus. Cows lost 61.7 ± 10.3 kg from precalving to the day of follicle aspiration but maintained BCS (BCS = 4.5 ± 0.1 at both calving and aspiration). Maximum size of DF did not differ for anovulatory and cyclic cows (Table 1) and averaged 12.8 ± 0.6 mm in diameter. Concentrations of IGF-I, progesterone, and IGFBPs in FF and aromatase mRNA in granulosa cells were not different for DF from anovulatory cows aspirated 30 and 47 d postpartum and from DF at proestrus after cows had initiated estrous cycles (P > 0.1; Table 1 and 2). Concentrations

of IGF-I and IGFBPs in plasma were not different at 30 and 47 d postpartum before ovulation and at proestrus ($P > 0.1$; Table 1 and 3). However, concentrations of estradiol were 4- to 6-fold greater ($P < 0.01$) in preovulatory DF from ovulatory cows than in DF from anovulatory cows aspirated 30 and 47 d postpartum (Table 1). When DF were aspirated 30 and 47 d postpartum, cows had similar days from aspiration to estrus (34 ± 9 d; Table 1).

The average interval from aspiration to first postpartum estrus was 36 d longer for cows aspirated > 35 d before first estrus (mean = 56 d; range = 36 to 120 d) than cows aspirated < 35 d before estrus (mean = 20 d; range = 6 to 34 d; Table 4; $P < 0.01$). All DF were estradiol active and had an estradiol:progesterone ratio > 1. Dominant follicles were not different ($P > 0.1$) in size when aspirated > 35 or < 35 d before first estrus, or at proestrus (Table 4), and follicles averaged 12.9 ± 0.6 mm in diameter at aspiration. Proestrous DF had 5- to 7-fold greater concentrations of estradiol in FF than follicles aspirated > 35 d or < 35 d before first estrus (Figure 1; $P < 0.01$), and concentrations of estradiol were similar in follicles aspirated at > 35 or < 35 d before first estrus. Dominant follicles aspirated < 35 d before first estrus had a 2.5-fold greater concentrations of progesterone in FF (Figure 2; $P < 0.01$) and tended to have a 3-fold greater concentrations of androstenedione in FF (Figure 3; $P = 0.07$) than follicles aspirated > 35 d before first estrus. Dominant follicles collected < 35 d before first estrus and at proestrus had similar concentrations of progesterone in FF (Figure 2). Concentrations of androstenedione in FF were greater in DF aspirated from proestrous cows than from anovulatory cows (Figure 3; $P < 0.01$). Concentrations of estradiol and androstenedione in follicular fluid of anovulatory cows, adjusted for period of aspiration, were positively correlated ($P <$

0.02; Table 5). Aromatase mRNA in granulosa cells was not significantly different ($P > 0.1$; Table 4 and Figure 4) for DF aspirated from proestrous cows and anovulatory cows > 35 and < 35 d before estrus.

Concentrations of IGF-I in FF and plasma and IGFBPs in plasma were not different (Table 4 and 6; $P > 0.1$) at < 35 and > 35 d before first estrus and ovulation and at proestrus. Concentrations of IGF-I in plasma did not change ($P > 0.1$) during the 6 wk prior to first postpartum estrus and ovulation (Figure 5). Amount of IGFBP 2 (34 kDa), 4a (22 kDa), and 5 (27 and 29 kDa) in FF were not influenced (Table 7) by days from aspiration to first postpartum estrus; however, the amounts of IGFBP 3 (42-44 kDa) and 4b (20 kDa) in FF were greater (Table 7; $P = 0.06$; $P < 0.05$, respectively) in proestrous follicles and DF aspirated < 35 d before first estrus than in DF aspirated > 35 d before estrus. Concentrations of IGF-I and IGFBP-3 in FF were positively correlated ($P < 0.02$; Table 5). Concentrations of hormones and IGFBP in follicular fluid and aromatase mRNA in granulosa cells and days from aspiration to estrus were not correlated ($P > 0.1$; Table 5).

Discussion

The postpartum anestrous interval was 74 ± 8 d, which is average for cows calving with a BCS of 4.5 (Richards et al., 1986; Lents et al., 2000; Looper et al., 2003). Postpartum anovulatory cows lost an average of 61.7 ± 10.3 kg between precalving and time of follicle aspiration, which included calf weights. Cows maintained BCS from calving to aspiration. Size of DF at aspiration was not different for ovulatory proestrous and postpartum anovulatory cows. Similarly, size of the largest follicle did not differ between 7 and 56 d postpartum in suckled beef cows (Spicer et al., 1986a). However,

others have observed an increase in maximum size of DF with successive follicular waves from calving until end of the postpartum interval (Murphy et al., 1990; Stagg et al., 1995). In other studies, follicles were measured in multiple follicular waves prior to the first estrus, but in the present study, DF size was determined only at 30 and 47 d after calving in anovulatory cows and at proestrus of ovulatory cows. Size of the dominant follicle could have been less during follicular waves prior to 30 d postpartum.

Proestrous cows, after the first postpartum ovulation, had greater concentrations of estradiol in FF of DF than anovulatory cows. Similarly, large follicles recovered from cows exsanguinated 28 d postpartum had a 4-fold increase in concentrations of estradiol compared with cows at 14 d postpartum (Spicer et al., 1986a). Estradiol causes the LH surge and induces ovulation of DF (Hobson and Hansel, 1972; Short et al., 1973; Kesner et al., 1981), so inadequate estradiol synthesis by DF, may not initiate an LH surge and ovulation. Progesterone and androstenedione are precursors for estradiol synthesis (Lacroix et al., 1974; Fortune, 1986), and limited concentrations of these steroids may reduce synthesis of estradiol in DF of anovulatory cows. Dominant follicles that were aspirated < 35 d prior to the first postpartum estrus had greater concentrations of progesterone and tended to have greater concentrations of androstenedione in FF than DF that were aspirated > 35 d before the first estrus. Dominant follicles collected < 35 d before first estrus and from ovulatory cows at proestrus had similar concentrations of progesterone in FF, but concentrations of androstenedione in FF were greater at proestrus. An increase in progesterone production may be necessary for maximum androstenedione production by the DF (Lacroix et al., 1974; Fortune, 1986). Spicer et al. (1986a) observed that concentrations of progesterone in FF increased 3-fold from 7 to 14

d after calving, but concentrations of androstenedione in FF did not change from 7 to 56 d postpartum. In our study, ovaries were evaluated daily to identify the DF and ensure that FF was collected when DF were at their maximum size for all treatments. Since the previous report was conducted before ultrasound technology was available, DF could have been collected at different stages of follicular growth. Anovulatory and ovulatory cows had DF with similar amounts of aromatase mRNA in granulosa cells in the current study. Greater estradiol secretion by DF from ovulatory compared to anovulatory cows is probably due to increased concentrations of androstenedione and progesterone in FF of DF of ovulatory cows. Concentrations of androstenedione and estradiol in FF of anovulatory cows were positively correlated in this study. Pulsatile infusion of GnRH to nutritionally induced anovulatory cows increased concentrations of androstenedione, estradiol (Prado et al., 2002), and IGF-I (Hamilton et al., 1999) in FF. Enzymes for converting progesterone to androstenedione may be lacking in DF of postpartum anovulatory cows. Increased estradiol secretion by bovine preovulatory DF is associated with greater concentrations of androstenedione in FF, P450 17α-hydroxylase mRNA in theca cells, P450 side-chain cleavage mRNA in theca and granulosa cells, and 3β-hydroxysteroid dehydrogenase mRNA in theca and granulosa cells (Tian et al., 1995). Aromatase mRNA in preovulatory DF did not increase with estradiol secretion (Tian et al., 1995). Increased synthesis of progesterone and androstenedione but not aromatase mRNA are precursors for maximum estradiol secretion by bovine preovulatory DF, and during postpartum anovulation, decreased concentrations of progesterone and androstenedione in FF of DF may prevent maximal estradiol secretion.

Insulin-like growth factor-I stimulates proliferation of bovine granulosa cells and progesterone and estradiol secretion in vitro (Spicer et al., 1993; Spicer et al., 2002a). Concentrations of IGF-I in plasma and FF of DF were similar for proestrous and anovulatory cows in the current study. Concentrations of IGF-I in plasma did not change during six weeks prior to first estrus, which is consistent with previous reports in primiparous beef cows (Ciccioli et al., 2003). In contrast, concentrations of IGF-I in plasma increased prior to first postpartum ovulation when cows were fed a nutrient restricted diet (Roberts et al., 1997; Stagg et al., 1998). Discrepancies between studies could be due to differences in nutritional status of cows, because nutrient intake influences concentrations of peripheral IGF-I in the bovine (Rutter et al., 1989; Bossis et al., 1999; Ciccioli et al., 2003). Changes in plasma IGF-I during the postpartum interval may also be influenced by genotype (Spicer et al., 2002b).

The physiological actions of IGF-I are controlled by a complex system of IGFBP, IGF-I receptors, and IGFBP proteases (reviewed by Spicer and Echternkamp, 1995; Fortune et. al., 2001; Mihm and Austin, 2002) allowing similar concentrations of total IGF-I in FF to have different effects on follicles. Four IGFBPs were described in the bovine ovary, using a ligand blot with immunoprecipitation and immunoblot to identify bands as IGFBP-2 (34-35 kDa), IGFBP-3 (40-49 kDa), IGFBP-4 (22-28 kDa), and IGFBP-5 (30 kDa; Echternkamp et al., 1994; de la Sota et al., 1996). The molecular weights of IGFBPs in this study are similar to those previously reported in bovine FF (Echternkamp et al., 1994; Stewart et al., 1996; Prado et al., 2002) and serum (Funston et al., 1996; Roberts et al., 1997). The relative concentrations of IGFBP in plasma did not differ for anovulatory and ovulatory cows. Postpartum beef cows with a postpartum

anovulatory interval greater than 20 wk had less peripheral IGFBP-3 at 2 wk postpartum than cows with a postpartum anovulatory interval less than 20 wk (Roberts et al., 1997). Consistent with this finding, ovulatory proestrous cows and cows < 35 d before the first estrus tended to have greater IGFBP-3 in FF of DF than cows > 35 d before estrus, in the present study. Follicular fluid from follicles aspirated from anovulatory cows that were less than 35 d before the first postpartum estrus, and fluid from follicles of ovulatory proestrous cows also had greater concentrations of the low molecular weight from of IGFBP-4 (20 kDa) than fluid aspirated from follicles of cows greater than 35 d before first estrus. The 20 kDa from of IGFBP-4 is probably the non-glycosylated form of the IGFBP, but the physiological consequences of glycosylation of IGFBP are not known (Jones and Clemmons, 1995). Postpartum anovulatory cows and ovulatory proestrous cows had similar concentrations of IGFBP-2, -5, and the 22 kDa form of IGFBP-4 in FF of DF. Concentrations of IGFBP-3 in FF were not influenced by atresia and were similar in dominant and subordinate follicles (de la Sota et al., 1996; Spicer et al., 2001). Insulin-like growth factor binding proteins transport IGFs throughout the body, increase the half-life of IGFs, sequester IGFs in certain tissue, alter the interaction of IGFs with their receptors, and have IGF independent actions (Jones and Clemmons, 1995). Increased IGFBP-3 in FF of DF may sequester IGF in the follicle and increase its biological half-life. Bovine DF have less IGFBP-2, -4, and -5 than subordinate follicles (de la Sota, 1996; Mihm et al., 1997; Mihm et al., 2000; Rhodes et al., 2001), and these binding proteins increase with atresia (de la Sota et al., 1996). The present study evaluated DF at different times before the first postpartum ovulation, and concentrations of IGFBP in subordinate and atretic follicles were not evaluated.

Bovine follicles that were > 8 mm and healthy had less IGFBP-4 in FF but greater IGFBP-4 mRNA and protein in granulosa cells when compared with smaller or atretic follicles (Roberts and Echternkamp, 2003). Luteinizing hormone increased IGFBP-4 mRNA in ovine granulosa cells and bovine theca cells in vitro (Armstrong et al., 1996; Armstrong et al., 1998), but LH decreased IGFBP-4 concentrations in cultured granulosa cells from large bovine follicles (Spicer and Chamberlain, 2002). Luteinizing hormone may increase IGFBP-4 mRNA but decrease IGFBP-4 protein and is likely due to LH stimulation of IGFBP proteases. In primates, IGFBP-4 mRNA was expressed only in granulosa cells of preovulatory DF that also had LH receptor mRNA (Zhou et al., 2003). Insulin-like growth factor binding protein-4 mRNA was expressed in theca cells of growing porcine follicles and in granulosa cells with LH receptor mRNA during later stages of follicular growth (Zhou et al., 1996). Luteinizing hormone increased IGFBP-4 mRNA in primate follicles in vivo (Zhou et al., 2003). These results support the hypothesis that IGFBP-4 mRNA is expressed in theca and granulosa cells of DF during late follicular growth in cells that also express LH receptor genes; so expression of IGFBP-4 mRNA may be induced by LH. Because concentrations of LH increase with time postpartum (Rawlings et al., 1980; Garcia-Winder et al., 1984; Stagg et al., 1998), increased low molecular weight IGFBP-4 in FF of DF collected from ovulatory proestrous and anovulatory cows < 35 d before the first estrus may be due to greater secretion of LH in these cows. However, treating nutritionally anovulatory beef cows with GnRH did not alter concentrations of IGFBP-5, 4, 3, or 2 in FF of large (> 5 mm) or small follicles (< 5 mm; Hamilton et al., 1999; Prado et al., 2002). Gonadotrophins may not alter IGFBP concentrations in nutritionally induced anovulatory cows because IGF-I

concentrations are reduced and IGF-I is needed to activate pregnancy-associated plasma protein-A (PAPP-A), a zinc dependent metalloproteinase that may be responsible for IGFBP proteolysis (Mazerbourg et al., 2001; Monget et al., 2003).

In conclusion, inadequate concentrations of androstenedione in DF of anovulatory cows may limit estradiol synthesis. Only DF from anovulatory cows < 35 d before first estrus had concentrations of progesterone in FF similar to proestrous follicles. Synthesis of progesterone and androstenedione in dominant follicles of anovulatory cows must increase before maximum estradiol synthesis occurs. Concentrations of IGF-I and IGFBPs in plasma and IGF-I in FF of DF were not influenced by reproductive status. Concentrations of low molecular weight IGFBP-4 were greater and concentrations of IGFBP-3 tended to be greater in FF of DF from cows that were ovulatory or < 35 d before their first postpartum ovulation than in DF of cows > 35 d before their first postpartum ovulation. Increased concentrations of IGFBP-3 and -4 in FF of DF during postpartum anovulation could influence functions of the DF. Future research is warranted to identify hormones and factors that regulate IGFBP during the postpartum interval.

Implications

A better understanding of the mechanisms causing postpartum anovulation may allow development of treatments and management decisions to decrease the length of the interval from calving to conception in beef cows. Minimal concentrations of androstenedione and progesterone in follicular fluid of dominant follicles during postpartum anovulation may limit estradiol synthesis, which could prevent final maturation and ovulation of dominant follicles. Cows that calve with good body

condition have decreased postpartum anovulatory intervals. Additional research is needed to determine if body condition score at calving alters postpartum reproduction by altering the IGF-I system to influence synthesis of androstenedione, progesterone, and estradiol in follicular fluid of dominant follicles.

Table 1. Concentrations of hormones in follicular fluid (FF), aromatase mRNA in granulosa cells, and size of dominant follicles (DF) from anovulatory cows at 30 or 47 d postpartum (PP) and from ovulatory proestrous cows

	Time of aspiration		
	Anovulatory		Ovulatory
Criteria	30 d PP	47 d PP	proestrus
Cows, No.	7	10	4
Size of DF, mm	12.4 ± 0.6	13.1 ± 0.6	13.0 ± 0.7
IGF-I in FF, ng/mL	26 ± 4	22 ± 3	28 ± 4
IGF-I in plasma, ng/mL	22 ± 4	22 ± 5	31 ± 6
Estradiol in FF, ng/mL	107 ± 63[a]	68 ± 53[a]	435 ± 79[b]
Progesterone in FF, ng/mL	47 ± 12	62 ± 16	85 ± 11
Androstenedione in FF, ng/mL	9 ± 5[a]	5 ± 4[a]	35 ± 10[b]
Aromatase mRNA, C_t[c]	22.9 ± 1.5	23.4 ± 1.5	21.7 ± 1.8
18S rRNA, C_t	16.7 ± 0.6	16.7 ± 0.4	16.8 ± 0.6
Aromatase mRNA, ΔC_t	6.2 ± 1.3	6.7 ± 0.9	4.9 ± 1.5
Aspiration to estrus, d	34.0 ± 9.0	34.4 ± 8.0	-

[a,b] Means in a row without a common superscript differ ($P < 0.01$). Statistical analysis were conducted on log transformed data.

[c] the C_T for a sample was the cycle that its amplification plot crossed an arbitrary threshold assigned in the log-linear range of amplification. $\Delta C_T = C_T$ for aromatase - C_T for normalization control, 18S.

Table 2. Relative concentrations[a] of IGFBP in follicular fluid of dominant follicles from anovulatory cows aspirated at 30 d or 47 d postpartum and from ovulatory cows at proestrus

	Time of aspiration		
	Anovulatory		Ovulatory
Criteria	30 d postpartum	47 d postpartum	proestrus
IGFBP-3 (42-44 kDa)	47.8 ± 13.1	46.5 ± 12.8	58.4 ± 13.8
IGFBP-2 (34 kDa)	2.4 ± 0.9	3.4 ± 0.8	2.7 ± 1.1
IGFBP-5 (29 kDa)	2.3 ± 1.0	1.6 ± 0.9	1.5 ± 1.2
IGFBP-5 (27 kDa)	1.3 ± 0.6	0.6 ± 0.5	0.4 ± 0.8
IGFBP-4a (22 kDa)	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
IGFBP-4b (20kDa)	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.3

[a] arbitrary densitometric units/4 μL

Table 3. Relative concentrations[a] of IGFBP in plasma of cows at 30 d and 47 d postpartum before ovulation and at the first proestrus

	Anovulatory		Ovulatory
Criteria	30 d postpartum	47 d postpartum	proestrus
IGFBP-3 (42-44 kDa)	64.1 ± 8.2	58.9 ± 8.2	58.3 ± 8.2
IGFBP-2 (34 kDa)	9.0 ± 1.2	9.3 ± 1.2	9.0 ± 1.3
IGFBP-5 (29-31 kDa)	7.5 ± 1.6	7.8 ± 1.6	7.2 ± 1.6
IGFBP-4a (28 kDa)	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4
IGFBP-4b (24 kDa)	1.6 ± 0.4	1.6 ± 0.4	1.3 ± 0.4

[a] arbitrary densitometric units/4 μL

Table 4. Concentrations of IGF-I in follicular fluid and plasma at aspiration, aromatase mRNA in granulosa cells, and size of DF from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus

	Time of aspiration		
	Anovulatory		Ovulatory
Criteria	> 35 d before first estrus	< 35 d before first estrus	proestrus
Cows, no.	7	10	4
Days postpartum at aspiration	39 ± 4	41 ± 3	49 ± 5
Aspiration to estrus, d	56 ± 6[a]	20 ± 6[b]	-
Size of DF, mm	12.3 ± 0.6	13.2 ± 0.6	13.0 ± 0.7
IGF-I in plasma at aspiration, ng/mL	23 ± 5	23 ± 4	32 ± 6
IGF-I in FF, ng/mL	23 ± 4	24 ± 3	28 ± 4
Aromatase mRNA, C_t[c]	22.1 ± 1.3	24.1 ± 1.2	21.7 ± 1.8
18S rRNA, C_t	17.0 ± 0.5	16.4 ± 0.4	16.8 ± 0.6
Aromatase mRNA, ΔC_t	5.1 ± 0.9	7.7 ± 0.9	4.9 ± 1.5

[a,b] Means in a row without a common superscript differ ($P < 0.01$).

[c] the C_T for a sample was the cycle that its amplification plot crossed an arbitrary threshold assigned in the log-linear range of amplification. $\Delta C_T = C_T$ for aromatase - C_T for normalization control, 18S.

Table 5. Correlation coefficients among days from aspiration to first postpartum estrus and concentrations of estradiol, insulin-like growth factor-I (IGF-I), androstenedione (andro), progesterone (prog), IGFBP-3, and IGFBP-4b in follicular fluid and aromatase mRNA (Arom) in granulosa cells of postpartum anovulatory cows

Variable	Estradiol	IGF-I	Andro	Prog	IGFBP-3	IGFBP-4b (20 kDa)	Arom ΔC_T
Aspiration to estrus, d	-0.22	-0.22	-0.32	-0.46	0.01	-0.11	-0.11
Estradiol	-	-0.30	0.63[a]	0.24	-0.042	-0.14	-0.06
IGF-I	-	-	-0.42	0.07	0.81[a]	0.04	-0.37
Andro	-	-	-	0.27	-0.23	-0.18	-0.12
Prog	-	-	-	-	-0.08	0.36	-0.23
IGFBP-3	-	-	-	-	-	0.09	-0.29
IGFBP-4b (20 kDa)	-	-	-	-	-	-	0.11

[a] $P < 0.02$, $n = 17$

Table 6. Relative concentrations[a] of IGFBP in plasma of cows at > 35 d and < 35 d before first postpartum estrus and ovulation and at the first proestrus

Criteria	Anovulatory		Ovulatory
	> 35 d before the first estrus	< 35 d before the first estrus	proestrus
IGFBP-3 (42-44 kDa)	61.4 ± 8.4	59.6 ± 8.3	58.3 ± 8.2
IGFBP-2 (34 kDa)	8.9 ± 1.2	8.0 ± 1.2	9.0 ± 1.3
IGFBP-5 (29-31 kDa)	7.1 ± 1.7	8.1 ± 1.7	7.2 ± 1.6
IGFBP-4a (28 kDa)	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4
IGFBP-4b (24 kDa)	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4

[a] Arbitrary densitometer units/4 μL

Table 7. Relative concentrations[a] of IGFBP in follicular fluid of dominant follicles from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus

	Time of aspiration		
	Anovulatory		Ovulatory
Criteria	> 35 d before first estrus	< 35 d before first estrus	proestrus
IGFBP-3 (42-44 kDa)	40.6 ± 13.7 [b]	52.3 ± 13.8 [c]	59.6 ± 14.5[c]
IGFBP-2 (34 kDa)	3.1 ± 0.8	2.8 ± 0.9	2.6 ± 1.1
IGFBP-5 (29 kDa)	1.0 ± 1.1	2.6 ± 1.0	1.7 ± 1.3
IGFBP-5 (27 kDa)	0.5 ± 0.7	1.3 ± 0.6	0.6 ± 0.8
IGFBP-4a (22 kDa)	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
IGFBP-4b (20kDa)	0.2 ± 0.3[d]	0.5 ± 0.3[e]	0.5 ± 0.3[e]

[a] Arbitrary densitometer units/4 μL

[b,c] Means without a common letter tend to differ (P = 0.06).

[d,e] Means without a common letter differ (P < 0.05).

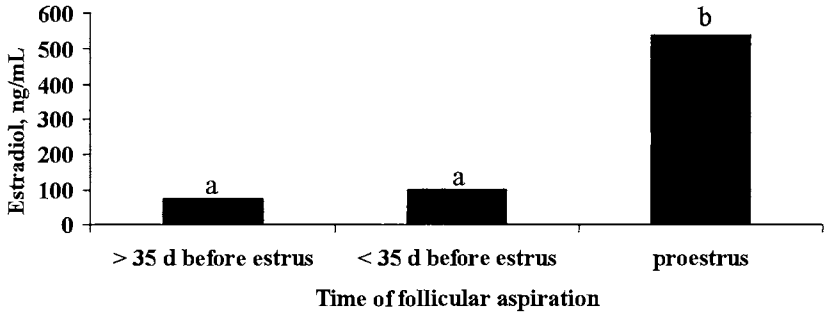

Figure 1. Concentrations of estradiol in follicular fluid of dominant follicles from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus. Pooled SE = 65. [a,b] Means with different superscripts differ ($P < 0.01$).

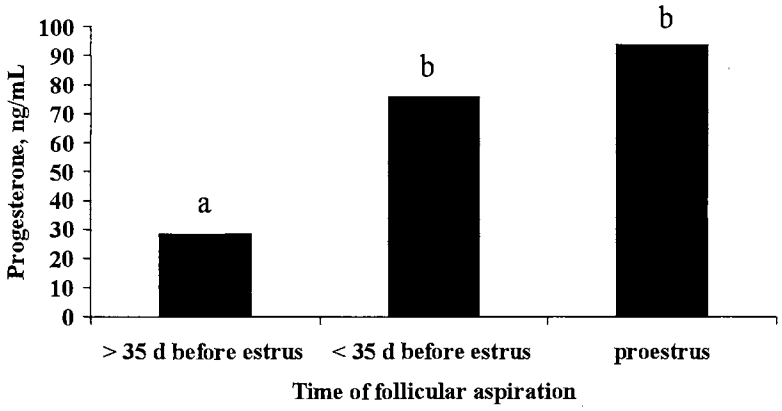

Figure 2. Concentrations of progesterone in follicular fluid of dominant follicles from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus. Pooled SE = 13. ^{a,b} Means without a common letter differ ($P < 0.01$).

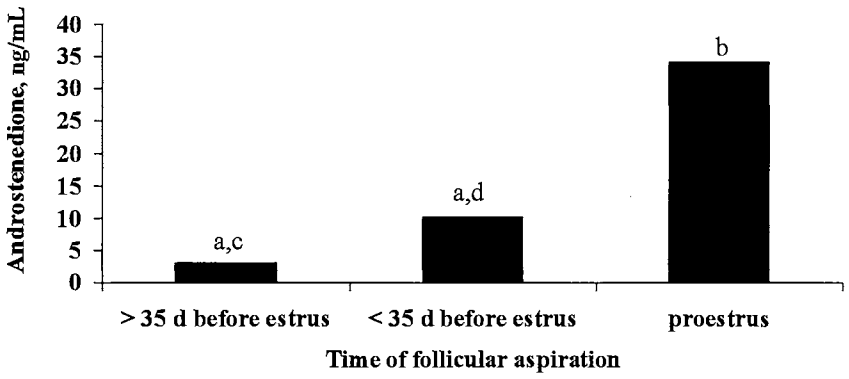

Figure 3. Concentrations of androstenedione in follicular fluid of dominant follicles from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus. Pooled SE = 6. ^{a,b} Means without a common letter differ ($P < 0.01$). ^{c,d} Means without a common letter tend to differ ($P = 0.07$).

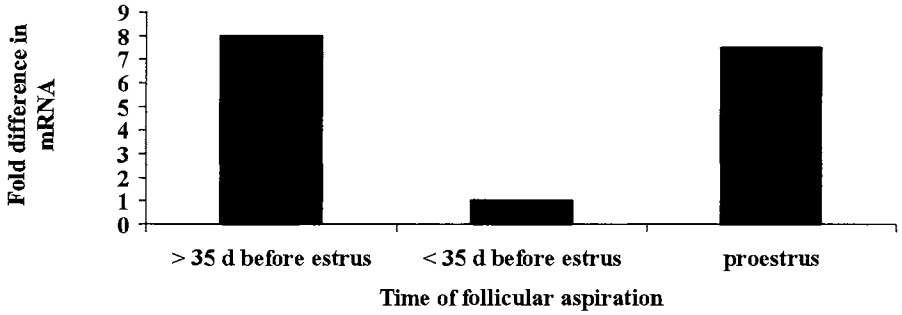

Figure 4. Aromatase mRNA in granulosa cells of dominant follicles from anovulatory cows aspirated > 35 d or < 35 d before first postpartum estrus and from ovulatory cows at proestrus.

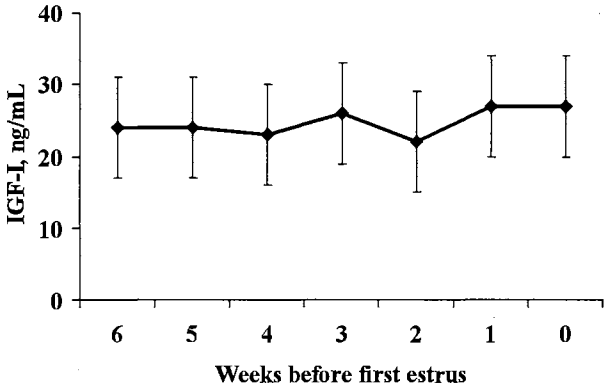

Figure 5. Concentrations of IGF-I in plasma of all postpartum cows during 6 wk before first estrus.

CHAPTER IV

Effects of body condition score at calving and length of the postpartum anovulatory interval on concentrations of steroids, insulin-like growth factor-I, insulin-like growth factor binding proteins, and mRNA for aromatase, leptin receptor, and pregnancy-associated plasma protein-A.

ABSTRACT: Effects of body condition score (BCS) at calving and length of the postpartum anovulatory interval (PPI) on IGF-I, IGFBP, and steroids in follicular fluid (FF), and aromatase, leptin receptor, and pregnancy-associated plasma protein-A (PAPP-A) mRNA in granulosa cells of dominant follicles (DF) were evaluated in Angus x Hereford cows. Growth of follicles was monitored by daily ultrasonography commencing 15 d postpartum until DF were 2 mm larger than subordinate follicles. The DF was collected by transvaginal ultrasound-guided follicular aspiration the following day (22.0 ± 0.7 d after calving). Follicular fluid and granulosa cells were obtained from cows (n = 14) with either a good (≥ 5.5) or thin (≤ 4.5) BCS at calving. In addition, FF was collected from DF of ovulatory cows (n = 6) after deviation during the first wave of an estrous cycle. Anovulatory cows were classified as having a PPI > 58 d or < 58 d. Cows with a PPI > 58 d had a PPI of 85 ± 7 d, and cows with a PPI < 58 d had a 50 ± 7 d PPI ($P < 0.01$). Cows calving with good and thin BCS had a PPI of 54 ± 11 d and 72 ± 9 d, respectively ($P > 0.1$). BCS at calving and length of the PPI did not influence

concentrations of estradiol, androstenedione, IGF-I, and IGFBP in FF or amounts of aromatase and PAPP-A mRNA in granulosa cells of DF. At 22 d postpartum, anovulatory cows with a PPI > 58 d tended ($P < 0.1$) to have lower concentrations of progesterone in FF than ovulatory cows and anovulatory cows with a PPI < 58 d. Cows calving with a thin BCS tended ($P < .07$) to have DF with 4 times more leptin receptor mRNA in granulosa cells than cows with a good BCS. Dominant follicles of cows during the first wave of an estrous cycle were 1.0 ± 0.3 mm larger ($P < 0.05$), had 3.4-fold more estradiol ($P < 0.001$), and 7.6-fold more androstenedione ($P < 0.05$) in FF than DF of anovulatory cows. Anovulatory and ovulatory cows had similar concentrations of IGF-I in FF and aromatase and PAPP-A mRNA in granulosa cells of DF. In conclusion, DF of postpartum anovulatory cows had a decreased ability to synthesize androstenedione and estradiol. Body condition score at calving does not influence the PPI by altering concentrations of steroids, IGF-I, or IGFBP in FF or PAPP-A mRNA in granulosa cells at 1 d after DF deviation.

Key Words: Beef Cows, Body Condition Score, Insulin-like Growth Factor-I, Insulin-like Growth Factor Binding Proteins, Estradiol, Leptin Receptor

Introduction

Nutritional intake regulates reproductive performance of cattle. Body condition score at calving (1=emaciated and 9=obese; Wagner et al., 1988) is the most important factor that determines duration of the postpartum anovulatory interval (Richards et al., 1986) and if cows will become pregnant during the breeding season (Selk et al., 1988). Cows calving with moderate BCS (≥ 5) resume estrous cycles sooner after calving than

cows calving with thin BCS (BCS < 5; Richards et al., 1986; Looper et al., 2003). Primiparous cows with a greater BCS at calving had increased pregnancy rates and a greater percentage in estrus by the end of the breeding season (Spitzer et al., 1995). The mechanisms by which body energy reserves of beef cows affect postpartum reproductive function have not been elucidated.

Nutrient intake of beef cattle influences follicular growth; restricting dietary energy intake decreased size of follicles in pre-pubertal (Bergfeld et al., 1994) and post-pubertal heifers (Murphy et al., 1991; Bossis et al., 1999). Concentrations of IGF-I in plasma are altered by nutrient intake of cows (Bossis et al., 1999; Ciccioli et al., 2003), and IGF-I modulates steroid synthesis and granulosa cell proliferation in follicles (Spicer et al., 1993; Spicer et al., 2002a). The biological activity of IGFI-I is regulated by IGF binding proteins (IGFBP) and IGFBP proteases (reviewed by Spicer and Echternkamp, 1995; Fortune et al., 2001; Mihm and Austin, 2002). Pregnancy-associated plasma protein-A (PAPP-A) is a zinc dependent metalloproteinase that may be responsible for IGFBP-2, -4, and -5 degradation in bovine follicles (Mazerbourg et al., 2001; Monget et al., 2003; Rivera and Fortune, 2003b). Pregnancy-associated plasma protein-A mRNA is expressed in bovine granulosa cells and was positively correlated with LH receptor and aromatase mRNA (Mazerbourg et al., 2001). Plasma concentrations of leptin are also influenced by nutrient intake and body energy reserves of ruminants (Delavaud et al., 2000; Ehrdardt et al., 2000; Wettemann et al., 2003). Leptin is secreted by adipose tissue (Zhang et al., 1994; Amstalden et al., 2000) and could alter reproduction via an ovarian mechanism. Bovine granulosa cells have receptors for the leptin (Spicer and Francisco,

1998), and leptin decreased in vitro steroid production by granulosa cells (Spicer and Francisco, 1997; Spicer et al., 2000b).

Concentrations of androstenedione, progesterone, and IGFBP in follicular fluid (FF) of dominant follicles (DF) change as postpartum cows approach their first ovulation (White et al., 2004), but concentrations of hormones were evaluated when the DF cease to grow. This sampling regime did not evaluate follicles before the maximum size of the follicle occurred. Concentrations of hormones in FF and gene expression during the growth phase of DF may provide insight into regulation of the DF of ovulatory and anovulatory cows. Objectives of this study were to evaluate effects of BCS at calving and length of the postpartum anovulatory interval on concentrations of estradiol, androstenedione, progesterone, IGF-I, and IGFBP in FF, and mRNA in granulosa cells for aromatase, PAPP-A, and leptin receptor in DF at 1 d after deviation in growth, and to compare concentrations of constituents in DF of postpartum anovulatory and ovulatory cows.

Materials and Methods

Animals and Experimental Protocol

Angus x Hereford cows (n = 14) were used to determine effects of body condition score (BCS) at calving and length of the postpartum anovulatory interval (PPI) on concentrations of IGF-I, IGFBP, estradiol, androstenedione, and progesterone in FF, and aromatase, leptin receptor, and PAPP-A mRNA in granulosa cells of DF. Cows (BW = 1162 ± 42; BCS = 5.0 ± 0.1) were blocked by BCS at 75 d of gestation and fed either a high or low diet so they would calve with good (≥ 5.5) or thin (≤ 4.5) BCS. The high diet consisted of free access to a ration (1.61 Mcal NE_m/kg DM, 0.90 Mcal NE_g/kg DM)

composed of rolled corn (39.7 % DM) ground alfalfa pellets (35.5 % DM) cottonseed hulls (22 % DM), cane molasses (2.5 % DM) and salt (0.3 % DM) that was fed for 96 d in a drylot, and then cows had access to dormant native grass pasture and were fed 2.3 kg/d of a 20 % crude protein supplement for 36 d followed by 1.4 kg/d of a 40 % CP supplement for 22 d. The low diet consisted of 0.4 kg of a 20 % crude protein supplement for 154 d and access to dormant native grass pasture. After 154 d on their respective diets, all cows were fed 1.4 kg/d of a 40 % crude protein supplement with access to the same dormant grass pasture and hay until calving. After calving, 1.4 kg/d of a 40% CP supplement was fed to maintain body condition.

Follicles were monitored daily by ultrasonography (7.5-MHz probe, Aloka, Corometrics Medical Systems, Wallingford, CT) from 15 d postpartum until recruitment of a new follicular wave was identified (a cohort of follicles larger than 5 mm in diameter that commence to grow simultaneously) and the DF became 2 mm larger than the subordinate follicles (defined as time of deviation). Ultrasound images of the ovary were recorded at each evaluation, and the diameter of the follicle was the average of the length and the width (Pierson and Ginther, 1988). The DF was aspirated one day after deviation (22.0 ± 0.7 d after calving). Local anesthesia was induced by caudal epidural treatment with 5 mL of 2% lidocaine. Ovaries were positioned against the vaginal wall, and a vaginal 5-MHz probe (Aloka, Corometrics Medical Systems, Wallingford, CT) was used to guide an 18 G, 55 cm needle (Cook Veterinary Products, Spencer, IN) to puncture the follicle and aspirate FF and granulosa cells to a 3-mL syringe. Anovulatory cows were classified as having a PPI > 58 d or < 58 d. Follicular fluid and cells were also collected from DF of ovulatory cows. Postpartum lactating ovulatory cows (n = 6; BCS of 5.0 ±

0.4) were fed 1.4 kg/d of a 40 % CP supplement after the first postpartum ovulation and had access to dormant grass pasture and hay. Ovulatory cows were treated with $PGF_{2\alpha}$ (i.m.; Lutalyse®, 25 mg; Pharmacia and Upjohn, Kalamazoo, MI) during the luteal phase of an estrous cycle (blood plasma progesterone > 1 ng/mL). Ovulation and follicular growth in ovaries of cows were evaluated daily by ultrasonography beginning two days after $PGF_{2\alpha}$. Then DF of the first wave of the new estrous cycle was aspirated, using the same criteria described for anovulatory cows.

Blood plasma was obtained every 3 or 4 d from 15 d postpartum until 23 d after the first postpartum estrus and ovulation. Progesterone in plasma was used to access luteal activity. The first postpartum ovulation and end of the PPI was defined as the first of three consecutive plasma samples with progesterone > 0.5 ng/mL (≥ 8 d).

Hormone and mRNA Analysis

Fluid aspirated from follicles was cooled on ice for 10 min after collection and then centrifuged at 2000 x g for 7 min to separate fluid and granulosa cells. Granulosa cells and fluid were stored in separate 5 mL cryogenic polypropylene conical vials. TRIzol (500μL; Invitrogen Corp., Carlsbad CA) was added to vials containing granulosa cells to extract RNA. Follicular fluid and granulosa cells with TRIzol were frozen within 20 min after aspiration in liquid nitrogen. Follicular fluid was stored at –20°C and granulosa cells were stored at –80°C until samples were analyzed. For extraction of RNA, vials containing granulosa cells and TRIzol were brought to 20°C, and 100 μL of chloroform (molecular biology grade) was added to each vial. Samples were incubated for 3 minutes and then were centrifuged at 3500 x g for 30 min at 4°C. The upper

aqueous phase was transferred to a fresh tube and 250 μL of isopropanol (molecular biology grade) was added followed by incubation at 20°C for 10 min. Samples were centrifuged for 10 min at 4°C to precipitate RNA, and the supernatant was removed. RNA was washed in 500 μL of 75 % ethanol and centrifuged at 3500 x g for 30 min at 4°C. The supernatant was decanted and the RNA was stored in RNAase free water (Qiagen, Valencia, CA) at –80°C until analyzed (< 4 months after extraction). The quantity of RNA was determined using a fluorescent nucleic acid stain (RiboGreen; Molecular probes Inc., Eugene, OR). Briefly, a standard curve from 0 to 1000 μg/μL was created by diluting RNA stock (2 μg/mL) in TE buffer (10 m*M* Tris-HCL, 1m*M* EDTA; pH = 7.5). Samples were diluted 1:50 in TE buffer, and 50 μL of sample and 50 μL of RiboGreen reagent (diluted stock solution 1:200 in TE buffer) were incubated for 3 min at 20°C. Fluorescence of samples and standards were measured on a microplate reader (Wallac 1420, Perkin Elmer, Boston, Ma; excitation at 480 nm and emission at 520 nm; intraassay CV was 8 %).

Relative concentrations of mRNA in granulosa cells were quantified by real-time RT-PCR, and conditions were optimized for each target gene (Table 1). Pimers and probes were created with Primer Express™ software (Foster City, CA) as described by Voge et al. (2004), and the GeneBank accession numbers that were used to create unique primers and probes for PCR analysis of aromatase, leptin receptor, and PAPP-A are presented in Table 1. A BLAST search (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) for each primer and probe was conducted to ensure that only target genes were evaluated. The aromatase primer and probe were previously validated (White et al., 2004). RT-PCR product for leptin receptor (reaction conditions described bellow) and PCR marker (50 to

1000 bp; Promega, Madison, WI) were electrophoresed on a 2 % agarose gel to further validate that size of product was consistent with the predicted size. This resulted in a single DNA product, which was cut out, extracted (Qiagen, Valencia, CA), and then sequenced at the biochemistry core facility to validate that the sequence of the product was homologous to bovine leptin receptor. To validate primers for PAPP-A, RT-PCR product (reaction conditions described bellow) and PCR marker were electrophoresed on a 3 % agarose gel, and 6 μL of the same product was treated with 0.5 μL Shrimp Alkaline phosphatase (E70092Y, Amersham Biosciences, Amersham, UK), 0.5 μL of exonuclease I (E700732, Amersham Biosciences, Amersham, UK), incubated at 37°C for 30 min then 85°C for 15 min, and sequenced.

A master mix was created for quantification of mRNA and the following reagents were added for each reaction: 12.5 μL of RT-PCR master mix (Qiagen, Valencia, CA), 0.25 μL of reverse transcriptase mix (Qiagen, Valencia, CA), 1 μL of probe (see Table 1 for final concentrations in reactions), 1 μL of both forward and reverse primers (see Table 1 for final concentrations in reactions) and 8.25 μL of RNAase-free water (Qiagen, Valencia, CA). Reactions also included 1 μL (25 ng) of total RNA, so reactions contained 25 μL. The probes for aromatase, leptin receptor and PAPP-A contained a 5'reporter dye (TET, 6-FAM, and 6-FAM, respectively) and 3' quencher dye (TAMRA) that allowed amplification of RNA to be evaluated on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction conditions consisted of 1 cycle to reverse transcribe RNA (50 °C for 30 min then 95 °C for 15 min) proceeded by 45 cycles to amplify cDNA (94°C for15 s then 60°C for 60 s). The relative concentrations of mRNA were determined in duplicates (intraassay CV was 1 %) via the

comparative C_t method as previously described by Hettinger et al., 2001, and 18S RNA was the normalization control (18S control kit; 43108993E; Applied Biosystems, Foster City, CA). The 18S probe contained a 5' reporter dye (VIC) and 3' quencher dye (TAMRA). Reactions for 18S occurred on the same multi-well plate as target genes but in separate wells, and final reaction concentrations for primer and probe were 400 and 200 n*M*, respectively. Data were analyzed by assigning an arbitrary threshold to amplification plots, and the cycle when a samples amplification plot crosses the threshold was defined as the threshold cycle (C_t). The C_t was assigned above the background levels that occur in early cycles and bellow the plateau of amplification; so, the Ct was always in the log-linear range of amplification. Samples with greater concentrations of a target gene had amplification plots that crossed the threshold cycle sooner and as a result had smaller C_t. The ΔC_t was calculated by subtracting a sample's target C_t from its 18S C_t, and ΔC_t of samples were used for statistical analysis.

Concentrations of estradiol (intraassay CV was 9%; Spicer and Enright, 1991), androstenedione (intraassay CV was 5%; Stewart et al., 1996), progesterone (intraassay CV was 8%; White et al., 2004), and total IGF-I (intraassay CV was 13 %; Echternkamp et al., 1990) in FF were quantified by RIA. Concentrations of progesterone in plasma were quantified by RIA (intra- and interassay CV were 4 and 5%, respectively; Vizcarra et al., 1997). The relative amounts of IGFBP in 4 μL of FF were evaluated via one-dimensional SDS-PAGE as previously described by Echternkamp et al. (1994), and cows were blocked by BCS at calving and randomly allotted to gels (2 gels, inter-gel CV was 19%). Briefly, samples were heat denatured (3 min at 100°C) and electrophoresed on a 12% polyacrylamide gel. Proteins were then transferred to nitrocellulose paper (Midwest

Scientific, St. Louis, MO) and ligand-blotted overnight with ^{125}I-IGF-I and ^{125}I-IGF-II. The nitrocellulose blots were exposed to X-ray film at -80°C for 2 d (IGFBP-3 and -2) or 7 d (IGFBP-4 and -5), and band intensity on autoradiographs was determined using scanning densitometry (Molecular Analyst, Bio-Rad). Effects of length of the PPI on concentrations of IGFBP in FF could not be evaluated because treatments were confounded within gel.

Statistical Analysis

The effects of body condition score at calving and length of the PPI on concentrations of progesterone, androstenedione, estradiol, and IGF-I in FF, size of the DF, and aromatase mRNA, PAPP-A mRNA, and leptin receptor mRNA in granulosa cells were analyzed in a completely randomized design using a mixed model procedure (Proc Mixed procedure; SAS Inst. Inc., Cary, NC). Postpartum ovulatory status/BCS at calving (ovulatory, anovulatory good BCS, and anovulatory thin BCS) were included in the model as fixed effects. Effects of postpartum ovulatory status/length of the PPI (ovulatory, anovulatory with a PPI > 58 d, anovulatory with a PPI < 58 d) were also analyzed in a separate model as fixed effects. A mixed model procedure was also used to evaluate the effects of ovulatory status and BCS at calving on concentrations of IGFBP in FF, and gel number was included in the model as a random effect. Homogeneity of variance for traits between cows was determined with Levene's test (SAS Inst. Inc., Cary, NC). Log transformation was used for concentrations of estradiol, androstenedione, progesterone, and IGFBP because variances were not homogenous. The actual least squares means and standard errors are presented when data were transformed for analyses. Changes in concentrations of mRNA of target genes were analyzed by

evaluating treatment affects on ΔC_t, and fold differences in mRNA are presented when treatment effects were significant. Fold differences in mRNA were determined by the formula: $2^{-\Delta\Delta Ct}$, with the $\Delta\Delta C_t$ of a treatment being its mean ΔC_t minus the mean ΔC_t of the treatment with the least mRNA (which had the largest ΔC_t). Significant treatment effects were separated using a student's t test with the PDIFF procedure of SAS (SAS Inst. Inc., Cary, NC). Correlation coefficients for anovulatory cows were calculated among concentrations of follicular fluid constituents, target gene mRNA, length of the PPI, and BCS (CORR procedure; SAS Inst. Inc., Cary, NC).

Results

Cows with good BCS at calving had a greater ($P < 0.05$) BCS and BW at calving (Table 2) than thin BCS cows. Good BCS cows lost one-half a BCS and 89 kg BW from calving to aspiration while thin BCS cows maintained BCS from calving to aspiration and lost 49 kg (BCS at aspiration = 4.3 ± 0.1; BW = 498 ± 24 kg). Although cows with a good BCS at calving resumed luteal activity an average of 18 d earlier than thin BCS cows, length of the PPI was not significantly different (Table 2). Cows were also classified by length of the PPI, and cows with a PPI > 58 d averaged 35 d longer to first luteal activity than cows with a PPI < 58 d (Table 3; $P < 0.01$).

All DF that were aspirated were estradiol active and had an estradiol:progesterone ratio > 1. Anovulatory and ovulatory cows had DF that grew at a similar rate from recruitment to deviation and were similar in size at deviation, but anovulatory cows had DF that were 1 mm smaller at follicular aspiration (1 d after deviation; $P < 0.01$; Tables 2 and 3) than DF collected from the first follicular wave of an estrous cycle. Dominant

follicles from the first follicular wave of an estrous cycle had 3.4-fold more estradiol ($P < 0.01$) and 7.6-fold greater androstenedione ($P < 0.01$) in FF than DF of anovulatory cows (Table 4 and 5). Concentrations of estradiol and androstenedione in follicular fluid of anovulatory cows were positively correlated ($P = 0.02$; Table 6). Concentrations of IGF-I (total; Table 4, and 5) and IGFBP (Table 7) in FF of DF were not significantly different for anovulatory and ovulatory cows, and concentrations of IGF-I, progesterone, and estradiol in FF of anovulatory cows were not correlated ($P > 0.1$; Table 6). Body condition score at calving (Table 4) and length of the PPI (Table 5) did not influence concentrations of estradiol, androstenedione, and IGF-I in FF of DF collected at 22 d postpartum; similarly, relative concentrations of IGFBP in FF were not affected by BCS at calving (Table 7). Concentrations of progesterone in FF were not different for anovulatory cows calving with good or thin body condition score (Table 4). Anovulatory cows with a PPI > 58 d tended ($P < 0.1$; Table 5) to have lower concentrations of progesterone in FF at 22 d postpartum than ovulatory cows and anovulatory cows with a PPI < 58 d.

Concentrations of mRNA for aromatase and PAPP-A in granulosa cells (Table 8 and 9) were not different for anovulatory and ovulatory cows. The amount of aromatase and PAPP-A mRNA in granulosa cells of DF from anovulatory cows at 22 d postpartum was not affected by BCS at calving or length of the PPI (Table 8 and 9). The relative concentrations of PAPP-A and aromatase mRNA in granulosa cells and estradiol in FF of anovulatory cows were correlated ($P < 0.05$; Table 6), and concentrations of estradiol increased with greater PAPP-A and aromatase mRNA (decreased ΔC_t). Concentrations of aromatase and PAPP-A mRNA in granulosa cells of anovulatory cows were positively

correlated ($P < 0.05$; Table 6). Length of the PPI did not influence leptin receptor mRNA in anovulatory cows (Table 9), but anovulatory cows with a thin BCS at calving and ovulatory cows tended (Table 8; $P < 0.07$) to have 4-fold more leptin receptor mRNA in granulosa cells than cows with a good BCS at calving.

Discussion

Body condition score is the most important factor determining length of the PPI (Richards et al., 1986) and if a cow becomes pregnant during the breeding season (Selk et al., 1988). Cows calving in good BCS (≥ 5) resume normal estrous cycles 20 to 35 d sooner after calving than cows calving with thin BCS (< 5; Richards et al., 1986; Looper et al., 2003). In the current study, cows calving with good BCS had PPI that were 18 d shorter, but not significantly different from thin BCS cows. The absence of a significant effect of BCS on the PPI could be due to a smaller number of cows per treatment.

Dominant follicles from anovulatory cows collected at 22 d postpartum were smaller in diameter 1 d after follicular deviation than DF from ovulatory cows collected 1 d after deviation during the first follicular wave of an estrous cycle. Maximum size of DF increased with successive follicular waves during the postpartum period (Murphy et al., 1990; Stagg et al., 1995). Body condition score at calving did not influence size of DF from anovulatory cows at 22 d postpartum. Maximum size of DF was larger for cows calving with good BCS than cows calving in thin BCS (Lents et al., 2000). In the current study, follicle size was determined at 22 d postpartum and 1d after deviation, and maximal size of the DF was not determined. A low energy diet decreased growth rate (Lucy et al., 1992; Armstrong et al., 2001) and maximum size of bovine preovulatory DF (Armstrong et al., 2001; Ciccioli et al., 2003), and ovulatory Hereford x Friesian heifers

fed to gain weight for 10 wk had larger preovulatory DF compared with heifers that maintained or lost BW (Spicer et al., 1991). In contrast, energy or fat intake did not alter size of bovine DF during postpartum anovulation or at the first postpartum ovulation (Stagg et al., 1995; Beam and Butler, 1998). The discrepancies between studies demonstrate the complexity of the effect of nutrition on reproduction, and could be due to differences in breed of cows, types and lengths of nutritional treatments, and ovulatory status of cows.

Insulin-like growth factor-I stimulates bovine granulosa cell proliferation and steroidogenesis in vitro (Spicer et al., 1993; Spicer et al., 2002a). The biological actions of IGF-I are controlled by IGFBP and the IGFBP protease, PAPP-A. Concentrations of IGF-I and PAPP-A may influence selection of bovine dominant follicles (reviewed by Spicer and Echternkamp, 1995; Fortune et al., 2001; Mihm and Austin, 2002). Insulin-like growth factor binding proteins sequester IGF-I and alter the biological activity of IGF, and IGF-I that is not bound to IGFBP (free IGF-I) is biologically active and can bind to its receptor. Proteolysis of IGFBP-4 and -5 was greatest in dominant estradiol active follicles (Rivera et al., 2001; Spicer et al., 2001) and was controlled by a zinc dependent metalloproteinase (Mazerbourg et al., 2001; Rivera et al., 2001; Monget et al., 2003; Rivera and Fortune, 2003b). Pregnancy-associated plasma protein-A is a zinc dependent metalloproteinase, and antibodies to PAPP-A inhibited IGFBP-2, -4, and -5 degradation in bovine preovulatory follicular fluid (Mazerbourg et al., 2001; Rivera and Fortune, 2003b; Monget et al., 2003). Concentrations of IGF-I (total), IGFBP, and PAPP-A mRNA in DF were similar at 22 d postpartum for anovulatory cows with a good or thin BCS at calving as well as for anovulatory and ovulatory cows. Concentrations of

FF IGF-I, FF IGFBP, and PAPP-A mRNA in DF were also similar for ovulatory cows and anovulatory cows with a PPI > 58 d or < 58 d, and thus do not appear to limit growth of DF in this study. Body condition score of cows and peripheral IGF-I were correlated (r = 0.4 to 0.5; Bishop et al., 1994; Lents et al., 2002; Spicer et al., 2002b), and concentrations of IGF-I in FF at 17 d postpartum were greater when beef cows had a greater BCS at calving (Ryan et al., 1994). Increasing energy intake of cows augmented peripheral IGF-I and decreased IGFBP-2 and –4 mRNA in healthy follicles (Armstrong et al., 2001). Primiparous beef cows fed to gain 0.9 kg/d for 11 wk after calving had greater plasma concentrations of IGF-I compared with cows fed to gain 0.4 kg/d (Ciccioli et al., 2003). However, ovulatory Hereford x Friesian heifers fed to gain, maintain, or loose weight had similar IGF-I in plasma and in FF of DF (Spicer et al., 1991). Beef cows fed 50 % of the diet allotted to control cows had similar IGF-I in FF of DF at 20 and 35 d postpartum (Rutter and Manns, 1991). Treating nutritionally induced anovulatory cows with GnRH did not alter concentrations of IGFBP in FF (Prado et al., 2002). Nutrient intake and body condition score may influence the IGF system, but concentrations of FF IGF-I, FF IGFBP, and PAPP-A mRNA in DF were not indicative of postpartum reproductive function in our study. Body condition score did not alter the IGF-I system in DF, 1 d after deviation, and final growth and ovulation of DF may be inhibited, or not stimulated, by other mechanisms.

Body condition score at calving and length of the PPI did not influence concentrations of estradiol or androstenedione in FF of DF at 22 d postpartum. Ovulatory cows had dominant follicles with 3-fold more estradiol and 8-fold more androstenedione in FF at 1d after deviation than anovulatory cows. Increased estradiol

synthesis by DF of ovulatory cows was not due to altered aromatase mRNA in granulosa cells but may be due to availability of androgens. Concentrations of estradiol and androstenedione in FF of anovulatory cows were positively correlated. Pulsatile infusion of GnRH to nutritionally induced anovulatory cows increased concentrations of androstenedione and estradiol in FF of follicles (Prado et al., 2002). When FF was aspirated from DF at their maximum size, concentrations of androstenedione in FF were greater in proestrous DF than in DF of anovulatory cows aspirated at > 35 d and < 35 d before the first postpartum estrus and tended to be greater in DF < 35 than in DF > 35 d before first estrus (White et al., 2004).

At 22 d postpartum, anovulatory cows with a PPI > 58 d tended to have lower concentrations of progesterone in FF than ovulatory cows and anovulatory cows with a PPI < 58 d. Concentrations of progesterone in FF were greater in DF collected at proestrus and in anovulatory cows < 35 d until first postpartum estrus than in DF > 35 d before first postpartum estrus (White et al., 2004). Body condition score at calving of anovulatory cows did not influence concentrations of progesterone in FF of DF. In agreement with our results, Ryan et al. (1994) found that BCS at calving did not influence concentrations of progesterone and estradiol in FF at 17 d postpartum. Spicer and coworkers (1991) concluded that concentrations of progesterone in FF of small and medium follicles, and estradiol in estrogen active follicles, were greater in Hereford x Friesian heifers fed to gain weight than in heifers fed to loose or maintain weight. Feeding heifers a high lipid supplement increased in vitro synthesis of progesterone of granulosa cells collected from preovulatory follicles (collected 19- 21 d postpartum; Wehrman et al., 1991). Nutritional intake may influence steroid synthesis of follicles in

heifers, but the effects of nutrient intake on steroid synthesis of follicles from postpartum mature cows has not been established. Ovulatory cows had greater concentrations of androstenedione in FF of DF. Luteinizing hormone or other treatments that increase androstenedione synthesis may allow DF of anovulatory cows to produce more estradiol resulting in ovulation.

Granulosa cells have receptors for the leptin (Spicer and Francisco, 1997), and leptin had a negative effect on in vitro steroid production of granulosa cells (Spicer and Francisco, 1997; Spicer et al., 2000b). Gonadotropins regulate ovarian leptin and leptin receptor expression during the rat estrous cycle (Ryan et al., 2003). Cows calving with good BCS tended to have less leptin receptor mRNA in granulosa cells than cows calving with thin or moderate BCS. Leptin receptor mRNA is expressed in the hypothalamus of ewes (Dyer et al., 1997; Williams et al., 1999; Adam et al., 2002), and feed restriction (Dyer et al., 1997) and fasting (Adam et al., 2002) increased leptin receptor mRNA in the hypothalamus. Therefore, concentrations of leptin receptor mRNA in the hypothalamus and ovary may be sensitive to nutrient intake and/or body energy reserves of ruminants. In nutrient restricted ewes, cerebroventricular infusion of insulin and glucose decreased hypothalamic leptin receptor mRNA (Daniel et al., 2000), and exogenous insulin decreased leptin receptor message in a chicken hepatic cell line (Cassy et al., 2003). Steroids may also inhibit leptin receptor expression in the rat brain (Bennett et al., 1998) and human uterus (Koshiba et al., 2001). Body condition score and concentrations of leptin in plasma were correlated in gestating cows when cows were fed diets that resulted in a range of BCS of 3.5 to 7, but the relationship between BCS and concentrations of leptin in plasma were minimal when cows were fed the same diet (Wettemann et al.,

2003). Primiparous beef heifers calving with a BCS of 4 or 5 had similar concentrations leptin in plasma, but postpartum primiparous heifers fed to gain 0.9 kg/d had greater plasma leptin than those fed to gain 0.45 kg/d (Ciccioli et al., 2003). Exogenous leptin increased the size of LH pulses in ovariectomized estradiol treated cows that were fasted but not cows that were fed (Amstalden et al., 2002). Leptin augmented basal LH in vitro from perifused adenohypophysis from fasted but not normal fed cows (Amstalden et al., 2003). Acute nutrient restriction of heifers tended to decrease concentrations of leptin (14 % less) in plasma (White et al., 2001), and heifers fasted for 48 h had decreased leptin mRNA in fat, as well as, peripheral leptin concentrations (Amstalden et al., 2000). Increased concentrations of leptin may decrease leptin receptor expression and protein and provide a mechanism where the nutrient status of an animal controls the amount of leptin receptor in a tissue. Exogenous leptin decreased hypothalamic leptin receptor protein and mRNA in rats (Martin et al., 2000), and in vitro leptin decreased leptin receptor mRNA in chicken liver (Cassy et al., 2003), rat adrenal glands (Tena-Sempere et al., 2000), and neuroblastoma cells (Hikita et al., 2000). The importance of changes in plasma concentrations of leptin and its receptor in granulosa cells on follicular growth and maturation is not known, and further research is needed to evaluate if changes in plasma concentrations of leptin or its receptor in follicles alter postpartum reproductive performance.

In conclusion, DF from anovulatory cows had decreased ability to synthesize estradiol at 1 d after deviation compared with DF from the first follicular wave of an estrous cycle, which is probably due to inadequate concentrations of androstenedione. Concentrations of aromatase mRNA in granulosa cells of postpartum anovulatory cows

was similar to concentrations in granulosa cells of DF from ovulatory cows. The IGF-I system of DF at 1 d after deviation is not altered during postpartum anovulation and is probably not limiting follicular development. Ovulatory cows and anovulatory cows with a BCS $\leq$ 5 tended to have greater leptin receptor mRNA in granulosa cells than cows calving with a BCS > 5. Body condition score at calving did not affect the DF of anovulatory cows at 22 d postpartum and probably influences the PPI via other physiological mechanisms.

Implications

Body condition score is the most important factor determining if a cow will become pregnant during the breeding season, but the mechanisms linking a cow's energy stores to reproduction are not known. Body condition score at calving tended to influence leptin receptor mRNA in granulosa cells, but not concentrations of IGF-I, IGFBP, or mRNA of the IGFBP protease PAPP-A. Therefore, body condition score at calving may not have a direct effect on the postpartum ovary and must alter postpartum reproduction through other mechanism. Anovulatory cows had smaller dominant follicles that contained less androstenedione and estradiol than ovulatory cows, which is probably due to inadequate gonadotropin support. Dominant follicles of postpartum anovulatory cows may need to increase androstenedione synthesis to increase estradiol secretion.

Table 1. Primers and probes sequences and optimal reaction condition for target genes			
Gene	Sequence	Concentration in reactions	GeneBank Accession No.
Aromatase	FWD Primer 5'- CCTGGCCTGGTGCGC -3' (bp 645 to 659) REV Primer 5'- TCCAGCCTGTCCAGATGCTT -3' (bp 690 to 709) Probe 5'- TGGTGACCATCTGTGCTGATTCCATCA -3' (bp 661 to 687)	400 n*M* 400 n*M* 200 n*M*	Z32741
Leptin receptor	FWD Primer 5'- CAATGCAGCAGTGCTCAATTC-3' (bp 73 to 93) REV Primer 5'- GGGCTGTCTCCTGCTCTCAT -3' (bp 128 to 147) Probe 5'- CTCACAGGTTATGTCTGTGCTCTCAGCCTCA -3' (bp 96 to 126)	200 n*M* 200 n*M* 200 n*M*	U62385
Pregnancy-associated plasma protein-A	FWD Primer 5'- CAGATGTTGAGCAGCCCTGTAA-3' (bp 557 to 578) REV Primer 5'- GGGTTGACGGCTGAATTGG -3' (bp 602 to 620) Probe 5'- CCAGCCCGCACCTGGAGC -3' (bp 581 to 600)	200 n*M* 200 n*M* 200 n*M*	AF421141

Table 2. Size and growth rate of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows or from anovulatory cows with a thin or good BCS at calving

	Treatments		
	Anovulatory		Ovulatory
Hormone	Thin BCS	Good BCS	1st Wave
Cows, No.	9	5	6
BCS at calving	4.3 ± 0.1 [b]	6.7 ± 0.1 [c]	-
BW at calving, kg	547 ± 24 [b]	709 ± 29 [c]	-
BCS at aspiration	4.1 ± 0.1 [b]	6.2 ± 0.1 [c]	5.0 ± 0.4 [d]
BW at aspiration, kg	498 ± 24 [b]	620 ± 37 [c]	572 ± 29 [b,c]
Postpartum anovulatory interval, d	72 ± 9	54 ± 11	-
Size of DF at deviation, mm	9.1 ± 0.3	9.2 ± 0.3	9.3 ± 0.4
Size of DF at aspiration, mm	10.2 ± 0.2 [b]	10.4 ± 0.3 [b]	11.3 ± 0.3 [c]
Growth of DF, mm/d [a]	1.5 ± 0.1	1.6 ± 0.2	1.8 ± 0.2

[a] Growth from recruitment to deviation.

[b,c,d] Means in a row without a common superscript differ ($P < 0.05$).

Table 3. Size and growth rate of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows or from anovulatory cows with postpartum anovulatory intervals > 58 d or < 58 d

	Treatments		
	Anovulatory		Ovulatory
Criteria	PPI >58 d	PPI <58 d	1st Wave
Cows, No.	7	7	6
BCS at aspiration	4.8 ± 0.5	5.1 ± 0.5	5.0 ± 0.4
BW at aspiration, kg	483 ± 44	556 ± 29	572 ± 29
Postpartum anovulatory interval, d	85 ± 7[b]	50 ± 7[c]	-
Size of DF at deviation, mm	9.6 ± 0.3	8.7 ± 0.3	9.3 ± 0.4
Size of DF at aspiration, mm	10.3 ± 0.2[b]	10.2 ± 0.3[b]	11.3 ± 0.3[c]
Growth of DF, mm/d[a]	1.5 ± 0.1	1.6 ± 0.1	1.8 ± 0.1

[a] Growth from recruitment to deviation.

[b,c] Means in a row without a common superscript differ ($P < 0.01$).

Table 4. Concentrations of estradiol, androstenedione, progesterone, and IGF-I (ng/mL) in follicular fluid of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows or from anovulatory cows with a thin or good BCS at calving

	Treatments		
	Anovulatory		Ovulatory
Hormone	Thin BCS	Good BCS	1st Wave
Estradiol[a]	223 ± 40 [b]	266 ± 40 [b]	817 ± 139 [c]
Androstenedione[a]	3.8 ± 1.8 [b]	6.3 ± 2.9 [b]	37.0 ± 14.0 [c]
Progesterone[a]	53.0 ± 7.7	39.3 ± 2.0	63.1 ± 7.9
IGF-I	11.0 ± 2.1	13.1 ± 2.7	12.4 ± 2.4

[a] Data were log transformed for statistical analyses.

[b,c] Means in a row without a common superscript differ ($P < 0.01$).

Table 5. Concentrations of estradiol, androstenedione, progesterone, and IGF-I (ng/mL) in follicular fluid of DF aspirated 1 d after follicular deviation during the first wave of anestrous cycle of ovulatory cows or from anovulatory cows with postpartum anovulatory intervals > 58 d or < 58 d

	Treatments		
	Anovulatory		Ovulatory
Hormone	PPI >58 d	PPI <58 d	1st Wave
Estradiol[a]	240 ± 75[b]	239 ± 70[b]	817 ± 83[c]
Androstenedione[a]	3.7 ± 8.1[b]	5.6 ± 7.5[b]	37.0 ± 8.0[c]
Progesterone	40.2 ± 7.3[d]	54.2 ± 6.8[e]	63.1 ± 7.3[e]
IGF-I	13.8 ± 2.1	9.2 ± 2.7	12.4 ± 2.7

[a] Data were log transformed for statistical analyses.

[b,c] Means in a row without a common superscript differ ($P < 0.01$).

[d,e] Means in a row without a common superscript tended to differ ($P < 0.1$).

Table 6. Correlation coefficients among body condition score (BCS), length of the postpartum anovulatory interval (PPI), follicular fluid concentrations of estradiol, insulin-like growth factor-I, androstenedione (Andro), and progesterone (Prog), and mRNA for aromatase (Arom), leptin receptor (LR), and pregnancy-associated plasma protein-A (PAPP-A) in postpartum anovulatory cows

Variable	PPI	Estradiol	IGF-I	Andro	Prog	Arom, ΔC_t	LR, ΔC_t	PAPP-A, ΔC_t
BCS	-0.20	0.09	-0.09	0.01	-0.33	0.41	0.54	0.33
PPI	-	-0.29	0.44	-0.36	-0.39	0.52	-0.06	0.39
Estradiol	-	-	0.06	0.62[a]	-0.17	-0.61[a]	0.31	-0.84[a]
IGF-I	-	-	-	-0.36	0.37	0.06	0.16	-0.03
Andro	-	-	-	-	-0.18	-0.35	-0.07	-0.48
Prog	-	-	-	-	-	-0.25	-0.42	-0.09
Arom, ΔC_t	-	-	-	-	-	-	-0.02	0.84[a]
LR, ΔC_t	-	-	-	-	-	-	-	-0.27

[a] $P < 0.05$, $n = 14$

Table 7. Relative concentrations[a] of IGFBPs in follicular fluid of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows or from anovulatory cows with a thin or good BCS at calving

	Treatments		
	Anovulatory		Ovulatory
Criteria	Thin BCS	Good BCS	1st Wave
IGFBP-3 (42-44 kDa)	17.7 ± 2.2	19.4 ± 2.7	24.1 ± 2.5
IGFBP-2 (34 kDa)	1.8 ± 0.6	1.3 ± 0.7	0.8 ± 0.6
IGFBP-5 (29 kDa)	2.1 ± 0.7	1.7 ± 0.7	1.7 ± 0.7
IGFBP-5 (27 kDa)	1.1 ± 0.8	1.2 ± 0.8	1.1 ± 0.8
IGFBP-4 (22 kDa)	0.9 ± 0.7	1.1 ± 0.8	0.7 ± 0.8
IGFBP-4 (20 kDa)	1.3 ± 0.9	1.4 ± 0.9	1.3 ± 0.9

[a] arbitrary densitometric units/4 μL

Table 8. Aromatase, PAPP-A, and leptin receptor mRNA in granulosa cells of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows or from anovulatory cows with a thin or good BCS at calving

		Treatments		
		Anovulatory		Ovulatory
Target gene		Thin BCS	Good BCS	1st Wave
18S rRNA	C_t[a]	19.1 ± 0.4	16.6 ± 0.6	18.4 ± 0.6
Aromatase	C_t[a]	25.1 ± 0.9	22.0 ± 1.3	23.4 ± 1.1
	ΔC_t	6.0 ± 0.2	5.4 ± 0.2	5.0 ± 0.2
PAPP-A	C_t[a]	23.2 ± 0.5	21.0 ± 0.8	21.5 ± 0.8
	ΔC_t	4.1 ± 0.5	4.4 ± 0.5	3.1 ± 0.5
Leptin receptor	C_t[a]	30.6 ± 0.5	30.2 ± 0.6	29.6 ± 0.5
	ΔC_t	11.5 ± 0.5[b]	13.6 ± 0.6[c]	11.2 ± 0.6[b]

[a] The C_t for a sample was the cycle that its amplification plot crossed an arbitrary threshold assigned in the log-linear range of amplification. ΔC_t = C_t for target gene - C_t for normalization control, 18S.

[b,c] Means without a common letter tended to differ ($P < 0.07$).

Table 9. Aromatase, PAPP-A, and leptin receptor mRNA in granulosa cells of DF aspirated 1 d after follicular deviation during the first wave of an estrous cycle of ovulatory cows and from anovulatory cows with postpartum anovulatory intervals > 58 d and < 58 d

		Treatments		
		Anovulatory		Ovulatory
Target gene		PPI >58 d	PPI <58 d	1st Wave
18S rRNA	C_t[a]	18.5 ± 0.6	18.1 ± 0.6	18.4 ± 0.6
Aromatase	C_t[a]	23.9 ± 1.1	23.4 ± 1.1	23.4 ± 1.1
	ΔC_t	5.4 ± 0.2	5.3 ± 0.2	5.0 ± 0.2
PAPP-A	C_t[a]	22.9 ± 0.8	22.6 ± 0.8	21.5 ± 0.8
	ΔC_t	4.4 ± 0.5	4.5 ± 0.5	3.1 ± 0.5
Leptin receptor	C_t[a]	31.5 ± 0.5	30.8 ± 0.5	29.6 ± 0.5
	ΔC_t	13.0 ± 0.6	12.7 ± 0.7	11.2 ± 0.6

[a] the C_t for a sample was the cycle that its amplification plot crossed an arbitrary threshold assigned in the log-linear range of amplification. ΔC_t = C_t for target gene - C_t for normalization control, 18S.

CHAPTER V

SUMMARY AND CONCLUSIONS

Beef cows must produce a calf per year in order to decrease cost and increase efficiency of production. Beef cows have a period of time after calving without normal estrous cycles and ovulation, and postpartum anovulation is a major source of economic loss for beef producers. The mechanisms regulating length of the postpartum anovulatory interval are not completely known but may involve the hypothalamic-pituitary-ovarian axis. Hypothalamic and pituitary function during postpartum anovulation has been extensively studied leading to the hypothesis that decreased LH secretion after calving suppresses follicular growth and ovulation (Wettemann et al., 1980; Yavas et al., 2000; Wiltbank et al., 2002; Wettemann et al., 2003). Changes in ovarian function during postpartum anovulation are not well documented in the beef cow, and ovarian factors may act in synergy with gonadotropins to alter postpartum reproduction.

Body energy reserves and level of nutrient intake control length of the postpartum anovulatory interval. The nutrient status of animals influences the hypothalamic-pituitary-ovarian axis, and metabolic hormones are possible signals influencing reproduction (Wettemann and Bossis, 2000; Wettemann et al., 2003). Concentrations of peripheral IGF-I change with nutrient intake of cows and may influence reproductive processes. Insulin-like growth factor-I is an important modulator of follicular growth.

Understanding the mechanisms causing postpartum anovulation may lead to treatments and management decisions to decrease length of the postpartum anovulatory interval, and as a result, increase profitability of producers. Two experiments were conducted to evaluate ovarian function of postpartum anovulatory cows. In experiment 1, changes in follicular fluid hormones and granulosa cell aromatase mRNA of dominant follicles were evaluated at two times postpartum for anovulatory cows and during proestrus of a normal estrous cycle of ovulatory cows. The effects of body energy reserves at calving and length of the postpartum anovulatory interval on follicular fluid hormones and granulosa cell aromatase mRNA of dominant follicles were assessed in the second experiment.

In experiment 1, growth of dominant follicles was monitored daily in beef cows, and when dominant follicles reached their maximum size, follicular fluid and granulosa cells were collected in vivo from anovulatory cows at either 30 or 47 d postpartum. Follicular fluid and granulosa cells were also collected from ovulatory proestrous cows 48 h after cows were treated with $PGF_{2\alpha}$. Time of follicle aspiration of anovulatory cows was classified as > 35 d or < 35 d until the subsequent first postpartum estrus and ovulation, and changes in follicular fluid hormones and granulosa cell aromatase mRNA were evaluated.

Because concentrations of IGF-I (total) in plasma and follicular fluid and IGFBP in plasma were similar for anovulatory and ovulatory cows, their concentrations may not influence dominant follicle function of postpartum anovulatory cows. However, amounts of follicular fluid IGFBP-3, and the low molecular weight form of IGFBP-4 increased as cows approached the first postpartum estrus and ovulation. Anovulatory cows had dominant follicles with less estradiol in follicular fluid than ovulatory cows, and

differences may be due to reduced synthesis of androstenedione and progesterone, which are precursors for estradiol synthesis. Concentrations of progesterone in follicular fluid were greater, and concentrations of androstenedione in follicular fluid tended to be greater in anovulatory cows that were < 35 d from first postpartum estrus and ovulation than in cows that were > 35 d from first estrus. Concentrations of androstenedione in follicular fluid were greater in dominant follicles from ovulatory proestrous cows compared with dominant follicles from postpartum anovulatory cows.

Body energy reserves of beef cows are a major regulator of postpartum reproductive function, and body condition score (BCS) at calving may influence dominant follicle steroidogenesis and the IGF system. In experiment 2, effects of BCS at calving and length of the postpartum anovulatory interval on dominant follicle function were determined. Follicular growth was monitored daily by ultrasonography beginning 15 days postpartum for anovulatory cows, or during the first wave of an estrous cycle of ovulatory cows. Follicular fluid and granulosa cells were collected in vivo one day after the dominant follicle was at least 2 mm larger than the next largest subordinate follicle. Concentrations of estradiol, androstenedione, progesterone, IGF-I, and IGFBP in follicular fluid as well as aromatase, leptin receptor, and pregnancy-associated plasma protein-A mRNA in granulosa cells were determined. Anovulatory cows were classified as calving with a good (≥ 5.5) or thin (≤ 4.5) BCS. Body condition score at calving did not influence concentrations of estradiol, androstenedione, progesterone, IGF-I, or IGFBP in follicular fluid of dominant follicles from anovulatory cows at 22 d postpartum. The relative concentrations of aromatase and pregnancy-associated plasma protein-A mRNA were also similar for cows calving with good or thin BCS. Cows calving with

thin BCS tended to have more leptin receptor mRNA in granulosa cells than good BCS cows.

Cows were also classified as having a postpartum anovulatory interval that was > 58 d or < 58 d. Cows classified as having a postpartum anovulatory interval > 58 d averaged 35 d longer to first luteal activity. Concentrations of estradiol, androstenedione, IGF-I, and IGFBP were not different at 22 d postpartum for cows that resumed luteal activity > 58 d or < 58 d after calving. In experiment 1, concentrations of IGFBP-3 and -4 increased as cows approached their first postpartum estrus and ovulation, and differences between experiments could be due to time of follicular aspiration. Dominant follicles were collected when they reached their maximum size in the first experiment compared to 1 d after deviation in growth of dominant and subordinate follicles in the second experiment. Anovulatory cows that resumed luteal activity > 58 d after calving tended to have less progesterone in FF at 22 d postpartum, than ovulatory cows and anovulatory cows with a postpartum anovulatory interval < 58 d. Ovulatory cows had greater estradiol and androstenedione in follicular fluid of dominant follicles than anovulatory cows.

In conclusion, dominant follicles aspirated during the first wave of an estrous cycle of ovulatory cows and preovulatory dominant follicles collected during proestrus had greater concentrations of estradiol in follicular fluid than postpartum anovulatory cows. Estradiol synthesis by dominant follicles of postpartum anovulatory cows is repressed compared with dominant follicles of ovulatory cows both 1 day after deviation of dominant and subordinate follicles as well as when dominant follicles reach their maximum size. Reduced synthesis of estradiol by dominant follicles of postpartum

anovulatory cows may be due to reduced synthesis of progesterone and androstenedione. Increased progesterone synthesis as cows approach the first postpartum estrus and ovulation, may increase synthesis of androstenedione and estradiol allowing dominant follicles to produce sufficient estradiol for an LH surge and ovulation.

Body condition score at calving did not alter concentrations of follicular fluid steroids and the ovarian IGF-I system at 22 d postpartum, and thus BCS may influence postpartum reproduction through other mechanisms. Anovulatory cows with thin BCS at calving tended to have greater leptin receptor mRNA in granulosa cells, which is consistent with results in other species in which nutrient restriction enhanced leptin receptor mRNA in the brain. However, the biological significance of this observation is not known, and dominant follicles from ovulatory cows and anovulatory cows calving in thin BCS had similar leptin receptor mRNA in granulosa cells. Future research is needed to evaluate if changes in ovarian leptin receptor influence postpartum reproduction.

These results indicate that steroidogenesis of dominant follicles is dissimilar for ovulatory and anovulatory cows and changes as anovulatory cows approach the first postpartum ovulation. Changes in steroidogenesis of dominant follicles at 22 d postpartum could not be attributed to changes in body energy reserves or to changes in the IGF-I system. While body energy reserve at calving is the most important factor determining the length of the postpartum anovulatory interval, BCS at calving does not have a direct effect on the postpartum ovary at 22 d postpartum and must alter postpartum reproduction through other mechanisms. Cows should calve with a BCS ≥ 5 to maximize postpartum reproductive performance. A better understanding of the mechanisms controlling postpartum anovulation is essential to maximize potential

reproductive performance of beef cows. Research is needed to identify the mechanisms in which the body energy reserves of cows control postpartum anovulation in beef cows.

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VITA 2

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