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Factors affecting reproductive efficiency of cattle

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FACTORS AFFECTING REPRODUCTIVE EFFICIENCY OF CATTLE

Melanie J. Starbuck

**Dissertation submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Reproductive Physiology**

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ABSTRACT

Factors Affecting Reproductive Efficiency of Cattle

Melanie J. Starbuck

Four experiments were conducted to evaluate two measures of reproductive efficiency in cattle, conception rate and retention of pregnancy. In experiment I, the relationship between concentrations of progesterone and retention of pregnancy was examined. The corpus luteum (CL) was removed on day 29 of pregnancy in 40 suckled, beef cows, and pregnancy was supported with exogenous progesterone to day 53. Concentrations of progesterone (1.8 ± 0.1 ng/mL between days 30 and 38), were below those normally recorded for pregnant cows. Retention of pregnancy (77%) was monitored frequently using ultrasonography until day 53 and was negatively associated with age of cow, but not directly related to concentrations of progesterone or estradiol, or body condition score. On day 53, a new CL was induced using hCG and exogenous progesterone support was withdrawn (experiment II). Pregnancy was maintained to term in all 20 cows that formed a new CL, including four in which the new CL was contralateral to the uterine horn of pregnancy. Effects of a single treatment with rbST at artificial insemination on conception rates and retention of pregnancy in dairy cows and heifers and beef cows were examined in experiment III. Conception rates were increased only in dairy cows, specifically those > 100 days in milk. Retention of pregnancy was not affected by treatment with rbST. In experiment IV, the relationship between the number of antral follicles that developed within a follicular wave and fertility was examined. Development of follicles ≥ 4 mm in diameter was monitored by ultrasonography on alternate days from day 6 of the cycle until ovulation or diagnosis of pregnancy in beef cows and heifers before and after insemination during two (Trial 1) or three (Trail 2) estrous cycles. Cows were artificially inseminated 12 h after second estrus (Trial I) or third estrus (Trial 2). Numbers of follicles ≥ 4 mm in diameter differed with estrous cycle, follicular wave, and cow, but the average number of antral follicles per wave did not affect conception rates. Additional research is needed to determine the specific roles of age of cow and concentration of progesterone on reproductive efficiency.

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REVIEW OF LITERATURE

Introduction

Reproductive inefficiency due to infertility is not only a source of frustration to dairy and beef producers, but substantially and directly reduces farm profitability. In general, infertility results in decreased profits to producers and increased culling of otherwise productive females. In 1990, it was estimated that each pregnancy lost resulted in an average loss of \$640 on dairy farms (Thurmond et al., 1990). The loss of pregnancy in beef herds might be even more important economically, because most income is generated through a single source, the number of calves sold. “The fundamental causes of infertility may be advantageously considered as: fertilization failure, embryonic death, abortion, and failure to come in heat or some other abnormality preventing service” (Olds, 1969). Infertility can occur at any point in the reproductive process, even prior to insemination, and the birth of a live calf results only after successful completion of all developmental stages from fertilization to parturition. Two factors that contribute significantly to reproductive efficiency are conception rate and loss of pregnancy.

Lucy (2001) stated that reproductive efficiency has declined in the lactating dairy cow, as measured by conception rate, rate of estrous detection, and days open, while milk production per cow has increased steadily. Only in cows with the highest levels of milk production does there appear to be a direct negative effect of level of milk production on conception rate. Conception rate is defined as the percentage of animals pregnant of the animals inseminated. Average conception rates for artificially-inseminated beef cows are usually greater than those for dairy cows, averaging between 65 and 80% (Patterson, 2003). First service conception rates of dairy cows to artificial insemination decreased

from approximately 65% in 1951 to 40% in 1996 (Butler, 1998). Conception rates were approximately 55% in parous dairy cows in the 1950's and 1960's (Johnson et al., 1958; Mares et al., 1961), while current rates are approximately 35%, or lower for timed insemination (Drost et al., 1999; Cartmill et al., 2001; Pancarci et al., 2002). Many factors influence conception rate including time of insemination and quality of the oocyte and spermatozoa.

Calving rates to a single insemination were 52 to 57% in dairy cattle (Mawhinney and Roche, 1978) and approximately 55% in beef cattle (Diskin and Sreenan, 1980). Fertilization failure tends to be low, accounting for only a small portion of the loss of potential offspring and is usually reported as the percentage of recovered normal ova that were fertilized. In fact, to a single breeding, fertilization rates of 82 to 100% can be expected for beef heifers (Diskin and Sreenan, 1980; Maurer and Chenault, 1983). Fertilization rates of 100% have been reported for parous beef females following natural service (Maurer and Chenault, 1983), while those for parous dairy cattle inseminated artificially with fresh semen were 85 to 89% (Kidder et al., 1954; Boyd et al., 1969).

In cattle, more pregnancies are lost during the embryonic period (conception to the end of organogenesis) than during the fetal period (end of organogenesis to parturition). The fetal period was designated to begin on day 42 of gestation, the end of the differentiation stage, by the Committee on Bovine Reproductive Nomenclature (1972). For parous beef females slaughtered at intervals up to day 16, Maurer and Chenault (1983) reported that 67% of embryonic mortality to that time occurred by day 8. In beef heifers, the majority of embryonic loss occurred by day 16 (Diskin and Sreenan, 1980) or as early as day 14 (Dunne et al., 2000). While 40% of pregnancies are lost early

in the embryonic period between day 8 and 18, loss of pregnancy after day 15 of gestation, during or following maternal recognition of pregnancy, results in extended interestrus intervals (Northey and French, 1980), making it a more frustrating problem for cattle producers. The occurrence of late embryonic and early fetal loss in dairy cattle between days 24 and 75 of gestation was estimated at 7% or 12% based on concentrations of progesterone in the milk (Kummerfield et al., 1978; Bulman and Lamming, 1979). More recently, pregnancy has been evaluated using ultrasonography and after a review of recent literature, Santos et al. (2004) estimated that late embryonic and early fetal mortality occurs in 10.4% of lactating dairy cows, a loss rate of 0.85% per day between days 30 and 45, while rarely exceeding 4% in beef cattle without an incidence of infectious abortion (Dunne et al., 2000).

Many factors affect embryonic and fetal loss in the cow and the causes can be classified into two broad categories, environmental factors and genetic factors (Ayalon, 1978; King, 1991; Kastelic, 1994). Environmental factors can be further divided into internal and external factors. The internal environment is perhaps the most studied, including physiology of the uterine environment, the hormones secreted by the dam to maintain pregnancy or stimulate embryonic development, and the hormones secreted by the embryo to ensure its own survival. External factors such as nutrition, disease, temperature or other environmental stressors can affect embryonic survival. Genetic factors that originate from either the male or female gamete might result in altered or improper development of the embryo or placenta, which might lead to loss of pregnancy.

Estrous Cycle

Reproductive cyclicity allows the female repeated opportunities to conceive. Each estrous cycle begins with a defined period of sexual receptivity when the chance of conception is greatest, called estrus. In cattle, the average length of the estrous cycle is 21 days (Hansel and Convey, 1983), with a normal range of 17 to 24 days (Chapman and Casida, 1937) or 19 to 23 days (Hawk et al., 1980). The estrous cycle consists of two phases, the follicular phase and luteal phase (Hansel et al., 1973). Throughout the estrous cycle, most follicles that begin to grow during the luteal phase undergo atresia. However, some follicles grow, complete maturation during the follicular phase, and ovulate.

Follicular Growth and Development

Primordial Germ Cells. Primordial germ cells (PGC's) are larger than somatic cells and have large, round nuclei with prominent nucleoli. Primordial germ cells originate in the inner lining of the yolk sac during embryogenesis, and then migrate to and colonize the urogenital ridge, specifically the primitive gonad. In the mouse, they appear first in the embryo by day 8 and begin migration toward the primitive gonad by day 12 (Ginsburg et al., 1990). Migration of PGCs is an active process, and they possess pseudopodia that allow them to travel using ameboid-like movements. After reaching the undifferentiated gonad, PGC's proliferate quickly by mitotic division and are then transformed into oogonia, which are larger and have a different distribution of cytoplasmic organelles than PGC's. An egg is considered to be within a follicle as soon as a definite layer of cells becomes arranged around it as a uniform capsule of follicular epithelium (Mossman and Duke, 1973).

Folliculogenesis. Folliculogenesis is the process of growth and development of follicles until ovulation or atresia (reviewed by Ireland, 1987) that begins with development of primordial follicles. Prior to reproductive senescence, ovaries have a pool of non-growing primordial follicles in which the oocyte is generally considered to be arrested in prophase I of meiosis, specifically the late diplotene or dictyate stage of prophase I. Meiosis begins first at the inner border of the ovary, where interactions between germ cells and somatic cells first occur. Proliferation of oogonia usually is restricted to prenatal development (e.g. ruminants, primates) or occurs shortly after birth (e.g. rodents, rabbits; Greenwald and Roy, 1994). The pool of primordial follicles will be depleted progressively during the reproductive years. However, it was reported recently that germline stem cells maintain oogenesis in postnatal mouse ovaries (Johnson et al., 2004). Adult mouse ovaries rapidly generate hundreds of oocytes, despite a small premeiotic germ cell pool which appear to be originating in the bone marrow (Johnson et al., 2005). The fertility and developmental competency of these oocytes has not yet been determined.

Primordial follicles leave the non-growing pool continuously (Pederson, 1969), presumably by conversion into primary follicles. The primordial oocyte is characterized as being surrounded by a single layer of squamous pregranulosa cells that transform from flattened to cuboidal forms as the oocyte develops to the primary stage. Of the approximately 150,000 primordial follicles present at birth in a heifer (Erickson, 1966), fewer than 100 will mature and ovulate during the lifetime of an average cow. The majority of follicles present on the ovary will degenerate by a process known as atresia, literally meaning 'failure to open'.

Primary follicles increase in size 400 to 600 fold and, depending on species, hundreds may enter the growing pool in each reproductive cycle. The epithelium of a growing primary follicle is mitotically active and forms two to six layers of granulosa cells surrounding the oocyte; the follicle is now designated as pre-antral stage, secondary follicle. What causes primordial follicles to enter the pool of growing primary follicles and subsequently, what initiates development of primary follicles to the stage of secondary follicle is not known. However, development was independent of gonadotropin support, because hypophysectomy does not prevent follicles from moving to the next stage of development (reviewed by Richards, 1980).

At various points, secondary follicles become surrounded by theca, develop a vascular supply, acquire receptors for FSH and LH, and become dependent on gonadotropins for continued development (reviewed by Scaramuzzi et al., 1993). The zona pellucida, a glycoprotein layer surrounding the oocyte, also is acquired at this stage. Follicular fluid is secreted by the epithelial cells of a secondary follicle, forming a space called the antrum, within the follicle.

With the appearance of an antral cavity, the structure is called a tertiary follicle (Greenwald and Roy, 1994). Follicular cells of tertiary follicles are mitotically active and continue to produce follicular fluid that accumulates in the antrum. Maturation of a tertiary follicle results in preovulatory growth, leading to ovulation when exposed to an ovulatory surge of LH. Final maturation from early antral stage to preovulatory size takes about 22 days in the cow (Staigmiller and England, 1982).

Concepts in Folliculogenesis

Recruitment. Recruitment is the process whereby a cohort, a group of early antral follicles (Goodman and Hodgen, 1983), begins growth together within a short period of time (Schwartz, 1974). During recruitment, follicles acquire the ability to respond to gonadotropins and become dependent on them for continued development (Goodman and Hodgen, 1983). It begins with an increased frequency of pulsatile secretion of LH, following priming by FSH. Factors influencing the number of follicles recruited will be discussed later.

Selection. Selection reduces the number of follicles in a growing cohort and is complete when the number of healthy follicles in a growing cohort, or pool, equals the number of follicles that will ovulate (Goodman and Hodgen, 1983). It was originally defined by Hodgen (1982) to begin coincident with recruitment of a cohort of primordial follicles. Potentially, all follicles are capable of ovulation. Depending upon the species, only one or some of the recruited follicles will acquire dominance and escape atresia, ending the process of selection. Selection is associated with appearance of LH receptors on granulosa cells (Ireland and Roche, 1982, 1983) and increased steroidogenic activity within the follicle that together cause increased production of estradiol. Plasma concentrations of FSH decline, but the frequency of LH pulses increases (McNeilly et al., 1992) and production of inhibin increases.

Dominance. Each period of follicular growth is associated with the recruitment of a group of follicles, from which one is selected to become the dominant follicle; in the cow, the single largest follicle that suppresses the growth of other follicles (Ireland and Roche, 1987; Turzillo and Fortune, 1990). Deviation, the divergence of growth rates

between what will become the dominant follicle and the subordinate follicles, is associated closely with dominance. The end of the common growth phase and the beginning of deviation occurs when the future dominant follicle reaches a mean diameter of 8.5 mm in Holstein heifers and occurs in a period of less than 8 hours (Ginther et al., 2001a). Dominant follicles affect development of other follicles through production of hormones (e.g. estradiol, inhibin, activin and follistatin), either directly or indirectly.

Early studies by Dufour and co-workers (1972), followed development of follicles marked with India ink and showed no correlation between the size of the largest follicle and the stage of the estrous cycle after day 9 and that the largest follicle present five days before estrus was not the ovulatory follicle in heifers. In fact, selection of the ovulatory follicle occurs between days 16 and 17 of the estrous cycle, 3 to 4 days before ovulation, when growth patterns of the largest and second largest follicles begin to diverge (Pierson and Ginther, 1988). A cohort including the ovulatory follicle emerged on day 16 in cows with three follicular waves, but the ovulatory follicle emerged on day 10 or 11 in cows with two waves (Ahmad et al., 1997). Follicular waves will be discussed in the next section.

Ultrasonography has allowed rapid advancement in the understanding of follicular development. In the cow, there are both ovulatory and non-ovulatory dominant follicles, usually described as such because each is the largest follicle on the ovary at some point. The first dominant follicle can be identified between days 2 and 4 of the estrous cycle using ultrasonography and will reach a maximum diameter of 6 to 8 mm. It will remain this size for a short period, then undergo atresia after emergence of the second follicular

wave (Sirois and Fortune, 1988; Knopf et al., 1989; Ginther et al., 1989a; Driancourt et al., 1991).

Follicular waves. Growth and atresia of ovarian follicles occurs in a sequential or wave-like manner throughout the bovine estrous cycle (Rajakoski, 1960), including the period after breeding (Ginther et al., 1989b). During the estrous cycle, between one (Savio et al., 1988) and four (Sirois and Fortune, 1988) waves develop, but the occurrence of two or three follicular waves in a single estrous cycle is most common (Ginther et al., 1989c; Knopf et al., 1989; Fortune, 1993). Thus, usually one or two dominant non-ovulatory follicles develop prior to an ovulatory dominant follicle, which suppresses the growth of other follicles larger than 4 mm in size (Savio et al., 1993). Because duration of the luteal phase is one to two days longer in estrous cycles with three rather than two follicular waves, length of the luteal phase of the estrous cycle appears to, in part, determine the number of follicular waves (Ginther et al., 1989c; Fortune, 1993). In fact, experimentally prolonging the luteal phase with exogenous progesterone produced estrous cycles with four or five follicular waves in heifers (Sirois and Fortune, 1990).

Ireland et al. (2000) defined the beginning of a follicular wave as the first day of the estrous cycle that a growing follicle ≥ 5 mm in diameter in a new wave is detected. A wave ends in either ovulation or atresia of the dominant follicle. New waves typically begin on day 1 (day of ovulation) and day 10 or days 1, 9 and 16 in cows with two or three follicular waves, respectively (Ginther et al., 1989c; Ahmad et al., 1997). The day of detection of ovulatory follicles was earlier in the estrous cycle for animals with two waves rather than three waves (12.3 vs. 16.5; Ahmad et al., 1997).

Each follicular wave was preceded or accompanied by a rise in FSH that reached peak concentrations, defined as a 50 to 75% increase in serum concentrations, when follicles were 4 to 5 mm in diameter (Adams et al., 1992). Nadir concentrations occurred 10 to 24 hours after the end of the common growth phase, when one follicle of the growing pool became the dominant follicle (Ginther, 2000; Ginther et al., 2001a). The decline of circulating FSH is a result of negative feedback from estradiol and inhibin produced by the emerging follicles (Adams et al., 1992, 1993; Gibbons et al., 1997) and these products prevent emergence of a new wave (Bergfeldt et al., 1994). Inhibition or delay of the rise of FSH delayed the next follicular wave (reviewed by Fortune, 1994). Likewise, immunization of heifers against GnRH or the continuous infusion of a GnRH agonist suppressed the rise in FSH and blocked emergence of a new follicular wave (Gong et al., 1996; Prendiville et al., 1995, 1996).

Number of follicles per wave. In cattle, high numbers of antral follicles are associated positively with an increased responsiveness to gonadotropin treatment during superovulation (Kawamata et al., 1994; Cushman et al., 1999; Taneja et al., 2000; Singh et al., 2004). This response is associated with a greater recovery of oocytes, a greater percentage of normal embryos, more transferable embryos and higher pregnancy rates after *in vitro* fertilization (Cushman et al., 1999; Taneja et al., 2000). Burns and co-workers (2005) reported a high repeatability within cow (0.95) of numbers of antral follicles ≥ 3 mm in diameter during both ovulatory and non-ovulatory follicular waves, as speculated by others (Boni et al., 1997; Singh et al., 2004). Despite the high repeatability of numbers of antral follicles per follicular wave within cow, there was a high degree of variability among cows that was attributed primarily to the numbers of smaller follicles

(Burns et al., 2005). The number of subordinate follicles, antral follicles that did not become the dominant follicle, that developed within a wave was variable among cows and did not affect the interval to emergence of the subsequent follicular wave (Ginther et al., 1989b).

While the numbers of antral follicles in the ovary remained relatively constant until cattle were 8 to 10 years of age (Erickson, 1966; Katska and Smorag, 1984), the number of antral follicles decreased thereafter, as fewer primordial follicles remained in the ovary in older cows (Erickson, 1966). The numbers of follicles developed in each follicular wave did not vary with age of cow, despite the loss of ovarian reserve as cattle aged (Burns et al., 2005). Thus, there might be a compensatory mechanism that maintains high repeatability of numbers of follicles developed during follicular waves in cattle, like that for the number of ovulatory follicles that develop during each estrous or menstrual cycle of different species (Law of follicular constancy; Lipschutz, 1928).

Factors that regulate recruitment

Requirements for Continued Development. Hirshfield (1991) defined a generation as the time required to double the number of granulosa cells visible in the largest cross section through the follicle. Using this method with rat follicles, she reported that little atresia occurred during the first seven generations of granulosa cells and that most become atretic during the 8th or 9th generation. Thus, Hirshfield (1991) described rat follicles at the 8th or 9th generation of granulosa cells as “hitting a brick wall,” which cannot be overcome unless signals are received at precisely the time they reach this normal stopping point in development. The point during antral follicular

development at which most atresia occurs during the estrous or menstrual cycle is dependent upon species, with most follicular atresia occurring around the time of antrum formation in the rat, but not until well beyond formation of the antrum in cattle (Lussier et al., 1987) and human beings.

Because antral follicles develop in groups or cohorts, it appears that recruitment of follicles is accomplished through signals that allow them to continue to develop. More follicles developed beyond the normal stage of atresia when the patterns of plasma LH and FSH during the early follicular phase were mimicked in cynomolgus monkeys (Zelevnik and Kubik, 1986).

Follicle Stimulating Hormone. As stated earlier, recruitment of primordial follicles and some growth of a reduced number of pre-antral follicles occurs in hypophysectomized animals. While gonadotropins are not required for recruitment and early growth, when they were administered to hypophysectomized animals, both recruitment and growth rate of follicles were improved (Greenwald and Roy, 1994). Messenger RNA for FSH receptors (FSHR) is localized specifically to the mural and cumulus granulosa cells and was detected in follicles with only one or two layers of granulosa cells (Xu et al., 1995a). The receptors for FSH appeared to be active during pre-antral development, because culture of pre-antral bovine or hamster follicles in serum-free media supplemented with FSH led to an increased number of granulosa cells (Roy and Greenwald, 1989; Hulshof et al., 1995). Additionally, granulosa cells from large pre-antral follicles can synthesize cAMP or lactate in response to treatment with FSH *in vitro* (McNatty et al., 1992; Boland et al., 1993). Expression of mRNA for the LH receptor is localized to the thecal cells during pre-antral and early antral stages of

growth and is first detected when the theca interna forms around the granulosa cells (Xu et al., 1995a,b; Bao et al., 1997a). The incorporation of increasing concentrations of FSH into protocols used for superovulation also resulted in greater numbers of recruited follicles.

Growth Hormone and Insulin-Like Growth Factor -1 (IGF-1). Follicular development is associated with many other factors other than gonadotropins. Mice lacking the receptor for growth hormone (GH) are fertile, but have reduced numbers of pre-ovulatory follicles and a reduced rate of ovulation (Zaczek et al., 2002). Fewer follicles developed to > 7 mm in diameter in heifers (Cohick et al., 1996) or to > 200µm in diameter in mice (Bachelot et al., 2002) when concentrations of GH were reduced. Treating heifers with pharmacological doses of recombinant GH increased the number of antral follicles without affecting concentrations of gonadotropins, and direct administration of GH into the ovarian artery did not affect secretion of ovarian steroids in sheep with autotransplanted ovaries (Campbell et al., 1995). Messenger RNA for the GH receptor has been identified in ovaries of cattle, specifically within oocytes and granulosa cells of primordial and primary follicles (Kolle et al., 1998).

Growth hormone can act through both insulin-like growth factor-1 (IGF-1) and insulin. *In vitro*, IGF-1 is a potent stimulator of steroidogenesis and proliferation of granulosa and thecal cells (Campbell et al., 1996; 1998). Mice that are null for IGF-1 have follicles that arrest at the late preantral or early antral stages and do not respond to gonadotropins (Baker et al., 1996; Zhou et al., 1997). Thus, IGF-1 appears unnecessary for follicular development until the early antral stage in mice.

The availability of IGF-1 is regulated by its association with specific binding proteins (IGFBP's), which are affected by nutritional status (Webb and Armstrong, 1998). Expression of both IGFBP-2, -4 and -5 have been detected in bovine follicles (Armstrong et al., 1998; Webb and Armstrong, 1998). Decreased concentrations of the various IGFBP's around the time of follicular deviation have been reported in follicles that become dominant (Armstrong et al., 1998).

Additional Growth Factors. As reviewed by McNatty and co-workers (1999), one of the most important growth factors affecting the recruitment of follicles is the tyrosine kinase receptor c-kit and its ligand, stem cell factor (SCF). The transformation from primordial to primary follicle is prevented when the interaction between c-kit and SCF is inhibited in mice (Huang et al., 1993; Yoshida et al., 1997). Interestingly, proliferation of granulosa cells ceased when antibody to c-kit was administered to mice (Yoshida et al., 1997).

Some very early signals from the oocyte might be required for proliferation of the granulosa cells; two likely candidates are growth differentiation factor-9 (GDF-9) and epidermal growth factor (EGF). Numbers of primary and small pre-antral follicles increased and numbers of primordial follicles decreased during culture of slices of bovine ovarian cortex in media free of gonadotropins (Wandji et al., 1996; Braw-Tal and Yossefi, 1997). Therefore, factors involved in initiating and controlling follicular growth are most likely paracrine or autocrine rather than endocrine in nature. Recruitment of follicles may be limited by another growth factor, follistatin. Overexpression of follistatin in transgenic mice inhibited follicular growth beyond the primary stage of development (Guo et al., 1998).

Steroidogenesis. Expression of mRNA for the steroidogenic enzymes, cytochrome P450 side-chain cleavage (P450scc), cytochrome P450 17 α -hydroxylase (P450c17), and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) was detected soon after formation of the theca interna. The thecal cells also expressed mRNA for steroid acute regulatory protein (StAR; Bao et al., 1998). As early antral follicles developed, expression of P450scc, P450c17, 3 β -HSD and StAR increased (Xu et al., 1995a,b; Bao et al., 1997a,b). The simultaneous initiation of expression of mRNA for both P450scc and cytochrome P450 aromatase (P450arom) in granulosa cells of most follicles 4 to 6 mm in diameter is associated with follicular recruitment in cattle (Xu et al., 1995a,b; Bao et al., 1997a). When follicles reach 6 to 9 mm in diameter, all follicles in the cohort express those two enzymes, however, expression was not evident in some smaller follicles, 4 to 5 mm in diameter, that were apparently recruited within the same cohort. The number of follicles that express P450scc and P450arom during recruitment is similar to the number of follicles that continue growth and undergo further development. Thus, expression of P450scc and P450arom in granulosa cells is associated with growth of follicles beyond 4 to 6 mm in diameter (reviewed by Webb et al., 1999), when concentrations of estradiol secreted from follicles increases. Bovine follicles \leq 5 mm in diameter do not secrete estradiol (Skyer et al., 1987).

Establishment of Pregnancy

One or two follicles from each cycle will ovulate near the end of the follicular phase. High concentrations of estradiol are secreted from the ovulatory follicle coincident with the expression of estrus. With natural service, mating occurs during

estrus, and artificial insemination is usually done 12 hours after the onset of estrus. Ovulation of the dominant follicle takes place approximately 28 to 32 hours after the onset of estrus in the cow (Trimberger, 1948; Walker et al., 1996). Ovulation occurs in response to a surge of luteinizing hormone secreted from the anterior pituitary (reviewed by Hansel and Convey, 1983). The oocyte is released from the follicle and the remnants of the follicle reorganize and form a glandular structure called the corpus luteum (CL), which secretes progesterone that is required for the maintenance of pregnancy. Fertilization occurs in the ampulla of the oviduct. The zygote will journey through the isthmus of the oviduct and finally to the uterus, where it will develop and remain until parturition.

Early Pregnancy

Maternal Recognition of Pregnancy. During the estrous cycle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the luminal epithelium and superficial glandular epithelium of the uterine endometrium is secreted and causes regression of the CL. The secretion of $PGF_{2\alpha}$ is controlled by progesterone, estrogen and oxytocin (OT) and is most studied in the ewe. Oxytocin from the CL and the anterior pituitary binds its receptors to elicit episodic release of $PGF_{2\alpha}$ (Flint et al., 1986). Estrogen, acting through the estrogen receptor alpha ($ER\alpha$), increases gene expression for OT receptors (OTR; Hixon et al., 1987). Progesterone acts to suppress both $ER\alpha$ and OTR in the endometrium, but progesterone can down-regulate its own receptor, and thus, the block of $ER\alpha$ and OTR is temporary. Briefly, after the expression of PR is reduced, estradiol from developing follicles acts via

ER α to increase expression of OTR, which collectively causes release of PGF $_2\alpha$ in a pattern that induces luteolysis of the CL (McCracken, 1980; Beard and Lamming, 1994).

The rate limiting enzyme in the production of prostaglandins is the cyclooxygenase (COX) enzyme, which converts arachadonic acid into prostaglandin H $_2$, the common substrate for synthesis of other prostaglandins. Other prostaglandins are synthesized via specific prostaglandin synthases. COX-2 is an inducible enzyme that is associated with many reproductive pathways. Female mice lacking the COX-2 enzyme were infertile and had abnormalities in ovulation, fertilization, decidualization and implantation (Dinchuk et al., 1995; Langenbach et al., 1995; Lim et al., 1997).

Maternal recognition of pregnancy requires communication between the conceptus and the dam during early pregnancy in order to prevent luteal regression in response to episodic secretion of PGF $_2\alpha$ from the uterine endometrium, thus, extending the life of the CL beyond that of the estrous cycle (reviewed by Bazer et al., 1997). Beginning on day 12, bovine embryos secrete interferon tau (IFN- τ ; Farin et al., 1990). In the ewe, IFN- τ had no effect on the expression of the COX-2 enzyme in the endometrium. Kim et al. (2003) concluded that the antiluteolytic effects of IFN- τ were to inhibit transcription of the ER α and the OTR, which prevented production of luteolytic pulses of PGF $_2\alpha$ and maintained the CL. Besides alterations to the secretory pattern of PGF $_2\alpha$, there appear to be additional mechanisms inherent within the CL of pregnancy to prevent luteal regression. Enzymatic ability of the CL to metabolize PGF $_2\alpha$ to its inactive metabolite PGFM was greater in CL from pregnant than non-pregnant ewes (Silva et al., 2000). Messenger mRNA for prostaglandin synthase F, the synthase that produces PGF $_2\alpha$, was lower in CL from pregnant than non-pregnant ewes (Costine et al., 2004).

The local nature of the relationship between the conceptus and the CL during maternal recognition of pregnancy is well-documented (Thatcher et al., 1986; Bazer, 1992) and continues into the second month of gestation (Lulai et al., 1994; Bridges et al., 2000). In cows with replacement CL induced on the ovary contralateral to the gravid horn by treatment with either hCG or LH before day 53, pregnancy was maintained in one of five (Lulai et al., 1994) or none of five (Bridges et al., 2000) cows. Pregnancy rate was greater in recipient dairy heifers following transfer of day-6 embryos to the uterine horn ipsilateral to the ovary containing the CL than contralateral (75% vs. 0%; Kastelic et al., 1991b). While some embryos transferred into the uterine horn contralateral to the ovary containing the CL could prevent luteal regression during maternal recognition of pregnancy, 33% or less were maintained to days 42 to 50 (Christie et al., 1979; Del Campo et al., 1983).

Embryonic development – Days 20 to 42. Gestation can be divided into two periods, the embryonic period and the fetal period. In the cow, the embryonic period usually refers to the time period between conception (day 0) until day 42, when organogenesis is complete. While the conceptus (the embryo plus its extraembryonic membranes) was visible on day 13, the embryo proper was not detected with ultrasonography until day 19 (Curran et al., 1986a). Ultrasonographically, the embryo appears initially as a short, straight line approximately 3 to 4 mm in length and then takes on a C-shape around day 25, and an L-shape around day 33 (Curran et al., 1986b). The bovine embryo weighed approximately 0.15 g, 1.38 g and 14.43 g on days 25 to 29, 40 to 44, and 60 to 64, respectively (Eley et al., 1978).

Using ultrasonography, the embryonic heartbeat can be detected on day 19 or 20, and is more rapid than in the adult. The heartbeat decreases between days 20 and 26 of gestation and then remains relatively constant, near 150 beats per minute, until day 60 (Curran et al, 1986b). The amnion, the layer of the placenta directly surrounding the embryo, was first observed with ultrasonography on day 29, but the amnion of embryos from cows that were slaughtered completely surrounded the embryo by day 17 or 18 (Greenstein and Foley, 1958; Salisbury et al., 1978).

Placental Attachment. A developing embryo is dependent on secretions from uterine glands that diffuse through unattached membranes until day 23 of gestation (Marion and Gier, 1958), when placental attachments become functional. Establishment of a functional placenta for transfer of nutrients and waste involves apposition and adhesion between the trophoblast and uterine surfaces in a gradual process. Non-echogenic vesicles, spherical or oblong in shape and 2 to 3 mm in size, appear in the horn of pregnancy, ipsilateral to the CL, as early as 10 days after ovulation (Curran et al., 1986a). Villous regions that develop on these vesicles, called cotyledons, will attach to caruncles in the uterine endometrium, creating placentomes and making possible direct transfer of nutrients to the developing extra-embryonic circulation. Caruncles, discrete, highly vascularized areas on the uterine epithelium, are distinct attachment sites. These sites can be identified on the uterine surface before attachment even commences and begin to elevate from the surface of the endometrium of pregnant cattle around day 20. The cotyledonary attachment is characteristic of ruminants, and the bovine placenta is described further as epitheliochorial. When viewed with light microscopy the chorionic surface appeared to be in direct contact with the connective tissue of uterine mucosa,

therefore, bovine and ovine placentas were classified previously as syndesmochorial (Chang, 1952).

The process of placental development and attachment is gradual, beginning with apposition of the endometrial and trophoblastic tissues on days 18 through 21 (Leiser, 1975; King et al., 1980), continuing with adhesion of the tissues in the intercaruncular regions near the embryo between days 22 to 27 (Leiser, 1975), and ending when actual contact is made between the surface of the caruncle and the chorion just before day 30 (King et al., 1979). A layer of epithelial cells covers caruncular tissue in the non-pregnant cow; this layer is lost as the first response of the caruncles to the embryonic membrane (Melton et al., 1951). Early on day 24 of gestation, the maternal cell types of the caruncular epithelium range from tall columnar cells to thin, elongated cells with some excessively large multinucleated cells, which become uniform cuboidal cells by day 29, as the epithelium changes from smooth to undulated (King et al., 1980).

Apposition first occurs in the area nearest the embryo around day 18, and by day 22, when adhesion has begun, has spread 10 to 15 cm (Leiser, 1975). Attachment also begins near the embryo with the proliferation of trophoblastic cells, forming cotyledons on the chorionic membrane over the caruncular areas. This process begins by day 30 and has spread to the body of the gravid uterine horn by day 35 and to the contralateral horn by day 38 (Melton et al., 1951). Where caruncular and cotyledonary tissue meet, initiating creation of a placentome, villi and crypts will begin to form. Inside each placentome, the epitheliochorial surface folds to create microvilli by day 24 and leads to intimate attachment of the two tissues by day 27, then to formation of villi and crypts after day 29 (King et al., 1980). At day 33 of gestation, when placentomes are first

visible by ultrasonography (Curran et al., 1986b) as smooth, flattened, semicircular elevations on the surface of the uterine lumen, the villi are not branched and range from 120 to 520 μm in length. Within three days they become branched, increasing contact, and by day 42 are approximately 1200 μm in length. Few structural changes take place in placentomes after day 42 (King et al., 1979). As in the ovine placentome, maternal arteries enter the maternal surface of the placentome and run toward the fetal surface, giving off branches to the main blood vessel. On the fetal side, each villus has one central arteriole with few branches, reaching the tip of the villus (Carter, 1975).

The bovine placenta consists of three layers: amnion, allantois and chorion. The outermost layer, chorion, fuses with the allantois to develop into a highly vascularized membrane that mediates exchange of heat and matter with the dam. The allantois was first visible on day 23 as a faint echogenic circle attached to the midventral portion of the embryo. One to two days later, the boundaries of the allantois became indistinct as it moved toward the chorion and was not visible as a separate structure after day 27. Expansion of the allantois occurs rapidly and Marion and Gier (1958) reported that on days 23, 25 and 27 of gestation the allantois expanded from 20 mm to 80 mm and 160 mm, respectively.

Hormones and Pregnancy

Estradiol. The ovary is the primary source of estrogens between days 20 and 60 of gestation, as the placenta contributed little until approximately day 50 (Robertson and King, 1979; Patel et al., 1999). In the ewe, approximately 80% of circulating estradiol was of uterine origin during the middle of gestation (Weems et al., 1994).

Concentrations of estradiol varied widely, from 4 to 10 pg/mL, in cattle during the second month of gestation (Eley et al., 1979). The dosage of melengestrol acetate, an orally-active progestin, which maintained pregnancy in beef heifers bilaterally ovariectomized at 42 days of gestation, was not adequate to maintain pregnancy in unilaterally-ovariectomized heifers (Zimbelman and Smith, 1966). In beef cows, Pritchard et al. (1994) found a negative linear association of pregnancy rate with concentrations of estradiol in peripheral blood on day 14 to 17 post estrus. In beef cows in which a new CL had been induced, more pregnancies were retained when concentrations of estradiol were lower at 31 to 35 days of gestation (Bridges et al., 2000). However, others have reported that concentrations of estradiol and size of the largest, or two largest follicles during the fifth week of gestation did not affect retention of pregnancy in dairy cows (Starbuck et al., 2004; Starbuck et al., 2005).

Progesterone. Pregnancy can be maintained in ovariectomized cattle and sheep treated with progesterone alone (Alexander and Williams, 1966; Zimbelman and Smith, 1966; Inskeep and Baker, 1985). In cattle, the CL is required for retention of pregnancy throughout most of gestation and either lutectomy or ovariectomy during late gestation hastened parturition (McDonald et al., 1953; Estergreen et al., 1967). Tanabe (1970) reported that the amount of exogenous progesterone required to maintain pregnancy in lutectomized cows decreased as pregnancy continued, most likely due to increased production of progesterone by the placenta. An ovarian source of progesterone is required for only the first 50 to 60 days of gestation in the ewe (Casida and Warwick, 1945).

As stated earlier, prevention of luteal regression is essential for maintenance of pregnancy beyond the duration of the estrous cycle, a process known as maternal recognition of pregnancy. Humblot (2001) suggested that late embryonic mortality occurred when the CL was maintained beyond maternal recognition of pregnancy and cows returned to estrus after day 24. Luteal regression typically preceded loss of the pregnancy when embryonic mortality occurred before day 25 of gestation, but followed loss of the pregnancy when embryonic mortality occurred between days 25 and 40 in dairy heifers (Kastelic, 1991b). In dairy cattle monitored frequently with ultrasonography, embryonic death preceded luteal regression, despite peripheral concentrations of progesterone that were adequate to maintain pregnancy in other animals (Starbuck et al., 2004). Experimentally-induced luteal regression on day 28 or 42 of gestation resulted in death of the embryo within 2.4 days and ovulation 2.6 days after embryonic mortality (Kastelic, 1989). After pregnancy was ended either abruptly, by rupture of the amniotic vesicle, or slowly, by colchicine injection, luteal regression did not occur for 35 or 22 days, respectively (Kastelic, 1989).

Synthesis of Progesterone. Progesterone is a steroid hormone composed of a ring complex containing three cyclohexane rings and one cyclopentane ring. Cholesterol, from either dietary intake or hepatic biosynthesis, is the starting point for all steroid hormones, including progesterone. The side chain of cholesterol is cleaved by cholesterol side chain cleavage enzyme (P450_{scc}) within the mitochondria of steroidogenic cells to yield pregnenolone, This reaction is the rate-limiting step in the production of steroid hormones and is controlled by gonadotropins. To form progesterone, pregnenolone is acted upon by the enzyme 3 β -hydroxysteroid

dehydrogenase (3 β -HSD). Synthesis and secretion of steroid hormones is controlled by a series of positive and negative feedback loops. Progesterone is synthesized primarily by the CL in the cow. In the pregnant animal, production of progesterone by the CL continues until a functional placenta is formed. Once formed, the placenta will assume the role of primary-producer of steroids in the ewe. A CL is necessary for production of progesterone and maintenance of pregnancy until approximately day 200 in the cow (reviewed by Niswender et al., 2000).

Catabolism of Progesterone. The concentration of a hormone in circulation is related to both production and catabolism. The liver is the primary site of metabolism for progesterone (Bedford et al., 1973; Parr et al., 1993) and over 90% of progesterone in hepatic portal blood is metabolized during the first pass through the liver (Parr et al., 1993). The rate of clearance of progesterone from the body influences the circulating concentrations, which might affect conception rates or retention of pregnancy. An inverse relationship exists between peripheral concentrations of progesterone and feed intake in sheep (Williams and Cumming, 1982; Parr et al., 1993) and swine (Prime and Symonds, 1993; Miller et al., 1999). High rates of dry matter intake, as in the high-producing, lactating dairy cow (Harrison et al., 1990), have been associated with reduced reproductive efficiency. Increased DMI during early gestation was associated with increased rates of embryonic loss in sheep (Parr et al., 1987) and in swine (den Hartog and van Kempen, 1980). Increased intake of feed before AI decreased embryonic survival in beef heifers (Dunne et al., 1999).

Metabolic clearance rate and dry matter intake are correlated directly (Parr et al., 1993; Miller et al., 1999). Metabolic clearance rate increased quickly and acutely after

consumption of feed, and the increase persisted longer in cows with greater DMI (Sangsritavong et al., 2002). As dry matter intake increased, blood flow to the liver increased (Sangsritavong et al., 2002) and in sheep, oxygen consumed by the liver also increased (Burrin et al., 1989). Greater blood flow to the hepatic portal vein was reported for sows with higher feed intake (Symonds and Prime, 1989). While acute feeding reduced circulating concentrations of progesterone in the pregnant, lactating cow, feeding smaller meals (25% of the daily amount of total mixed ration at six hour intervals) eliminated the acute effect of feeding on concentrations of progesterone (about a 25% reduction in circulating concentrations; Vasoncelos et al., 2003).

Liver blood flow and metabolic clearance rate of steroids are elevated acutely after consumption of feed and are two possible factors that affect concentrations of progesterone in the lactating dairy cow. Higher levels of feeding in ovariectomized dairy cows treated with controlled internal drug releasing devices (CIDR) inserts reduced plasma concentrations of progesterone and increased fecal excretion of 20α -pregnane (Rabiee et al., 2002a). While concentrations of progesterone were lower in lactating than non-lactating cows (2.43 vs. 3.53 ng/mL; Sangsritavong et al. 2002), level of milk production did not affect plasma concentrations of progesterone or the excretion rate of fecal metabolites of progesterone in lactating dairy cows (Rabiee et al., 2002b). Concentration of progesterone did not differ with levels of dry matter or metabolisable energy (Rabiee et al., 2002c); however, concentrations of progesterone were reduced in pasture-fed, non-lactating cows with ad libitum access to feed compared to those with restricted access (Rabiee et al., 2001).

Classic Progesterone Receptors. Steroid hormones cause biological effects by altering patterns of gene expression within hormone-responsive cells. Because steroids are lipophilic, they diffuse easily across the plasma membranes of cells and once inside, interact with their receptors. Like other steroid receptors, the genomic progesterone receptor (PR) is a member of the ligand-inducible DNA-binding superfamily of nuclear receptors. Binding of progesterone to its receptor induces a conformational change of the receptor proteins (Allan et al., 1992) which causes dimerization of two ligand receptor complexes (Tsai et al., 1988; Tsai and O'Malley, 1991), increases phosphorylation (Weigel et al., 1995), and binding of receptor dimer to specific hormone responsive DNA elements located in the promoter regions of target genes (Gronemeyer, 1991; Tsai and O'Malley, 1991). Genes that are responsive to progesterone contain response elements specific for progesterone (PREs) upstream of their promoters, which mediate binding of the receptor dimer to the DNA target. Once bound to the PRE, the PR interacts with additional cellular machinery to affect the rate of mRNA production and thus, protein production.

Two distinct isoforms of progesterone receptors exist, PR-A and PR-B, but they arise from a single gene. PR-B contains an additional NH₂-terminal stretch of 165 amino acids, which encodes a transactivation function in specific target genes that can be activated only by PR-B. Each isoform displays different transcriptional regulatory activities, and Mulac-Jericevic and coworkers (2000) used selective ablation of PR-A in mice to demonstrate that the PR-B isoform regulates a specific set of target genes.

PR-B strongly activates transcription in some cell types in which PR-A is inactive. The ratio of expression of PR-A to PR-B differs with stage of development and

hormonal status. The progesterone receptor knock-out (PRKO) mouse is infertile, exhibits uterine dysfunction and impaired sexual behavior, and is anovulatory (Lydon et al., 1995). The PRAKO and PRBKO mouse models allow researchers to assign specific functions to the independent types of PRs. The response of PRAKO mice to superovulatory stimuli is reduced compared to wild type mice, a trait that is unaffected in PRBKO mice.

The uterus is a complex organ consisting of three primary cellular components: a central luminal epithelium that branches into and forms the glandular epithelium, the underlying stroma, which is comprised of many cell types including fibroblasts and endothelial cells, and finally, the myometrium, a surrounding layer of both circular and longitudinal muscle. While receptors for both progesterone and estrogen can be found in all three layers, each layer has a distinct response to steroids. Progesterone receptors are induced by estrogen, but progesterone down regulates its own receptor during the luteal phase (Rexroad, 1981a,b).

Three distinct single nucleotide polymorphisms were detected in the PR gene and might be associated with recurrent abortion. The frequency of occurrence of an SNP-containing allele was greater in women that previously had suffered multiple miscarriages than in those with no history of miscarriage (Schweikert et al., 2004).

Membrane-bound Progesterone Receptors. While there is evidence that supports progesterone's autocrine action in the CL, classical PR's have not been detected. Recently, putative membrane-bound progesterone receptors (mPR) were identified in rats (Cai and Stocco, 2005) and ewes (Ashley et al., 2005). The cDNA for the putative mPR in the ewe contains 1052 base pairs that encode for 350 amino acids, which are distinct

from those of the intracellular PR. Computer structural analysis predicted that the receptor possesses a seven-transmembrane domain, which is characteristic of G-protein coupled receptors (Ashley et al., 2005). In the ewe, expression for mPR was detected in the hypothalamus, pituitary, uterus, ovary and CL (Ashley et al., 2005). In the rat, five potential isoforms of the mPR have been detected: mPR α , mPR β , MPR γ , PR membrane component 1 (PRMC1), and protein RDA288 (Cai and Stocco, 2005).

The molecular mechanism of how mPR's function is not fully determined. Forced expression of RDA288 in spontaneously immortalized rat granulosa cells that do not express classical PR's, increased binding of progesterone and reduced apoptosis (Peluso, 2005). RDA288 is localized to the extracellular surface of the plasma membrane of spontaneously immortalized rat granulosa cells, but lacks a transmembrane domain. In order to elicit a response, RDA288 interacts with the transmembrane protein PRMC1 to form a functional complex, which increases activity of protein kinase G (Peluso, 2005).

Progesterone and Uterine Receptivity. Receptivity of the uterus for arrival of the embryo(s) from the oviduct and establishment and maintenance of pregnancy in mammals is dependent on the actions of progesterone. The progesterone antagonist RU486 prevents implantation and can be used to terminate an established pregnancy. Within the uterus, PR are expressed in the endometrial epithelium and stroma. Therefore, direct regulation of many genes by progesterone is possible. However, continuous exposure of the endometrium to progesterone reduced expression of protein for PR. Expression of protein for PR was undetectable in the luminal and glandular epithelium of the pregnant ewe after days 11 and 13 of gestation (Spencer and Bazer, 1995) and

throughout most of gestation can be detected only in the stroma and myometrium (Spencer et al., 2004). Loss of PR in the uterus immediately prior to implantation is common among mammals (Carson et al., 2000; Spencer et al., 2004). The loss of PR by the endometrium is correlated directly with reduced expression of specific genes that are induced by progesterone.

Progesterone and Secretions from the Uterine Glands. During the peri-implantation period, conceptuses are bathed in secretions (histotroph) composed of proteins, carbohydrates, sugars, lipids and ions produced by the endometrial glands that are necessary to sustain the conceptus. The protein components of the histotroph are important for conceptus-maternal interaction, specifically the processes of elongation of the trophoblast, recognition of pregnancy, implantation, and placentation (reviewed by Spencer et al., 2004a,b). Expression of many components of the histotroph is under steroid control, particularly by progesterone and estrogen. The implantation cascade involves rearrangements of multiple cells, made possible through contributions from the extracellular matrix. One specific glycoprotein involved in this process is osteopontin (OPN).

Osteopontin is a highly phosphorylated, acidic 70-kDA glycoprotein that stimulates cell to cell adhesion, increases cell to extracellular matrix communication and promotes cell migration via changes in cytoskeletal structure (Denhardt and Guo, 1993; Butler et al., 1996; Sodek et al., 2000). OPN is a member of the small integrin-binding ligand, N-linked glycoproteins (SIBLING) family of related extracellular matrix proteins that was originally isolated from bone (Franzen and Heinegard, 1985). Integrin heterodimers $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$ bind to the Arg-Gly-Asp

(RGD) amino sequence found on OPN (Miyachi et al., 1991; Hu et al., 1995; Bayless et al., 1998).

To date, expression of OPN has been shown in the uterus, placenta and conceptus of five species: human beings (Young et al., 1990), baboons (Fazleabas et al., 1997), mice (Nomura et al., 1988), sheep (Johnson et al., 1999a), and pigs (Garlow et al., 2002). Expression of OPN in the uterus was induced by progesterone in the ewe (Johnson et al., 2000) and human being (Omigbodun et al., 1997). Expression of OPN in the ewe is associated with a loss of PR in the glandular epithelium (Spencer et al., 1999). Treatment of ovine endometrial explants maintained in culture for 24 hours with progesterone induced the expression of OPN mRNA in the glandular epithelium of the endometrium and increased secretion of the 45-kDa fragment of cleaved OPN protein; effects that were ablated by additional treatment with an antagonist of the progesterone receptor (Johnson et al., 2000). Likewise, in the pig, expression of OPN was induced in the glandular epithelium by progesterone, but was induced in the luminal epithelium by estrogen (White et al., 2005).

In the pregnant ewe, expression of OPN mRNA increased in the uterine glands beginning on day 13 and was present in uterine flushings on day 15 (Johnson et al., 1999a). Immunoreactive OPN was localized to the luminal and glandular epithelium and trophoderm of day 19-conceptuses (Johnson et al., 1999b). Osteopontin secreted from the uterine glands was then available as a ligand for its specific integrin receptors. Similar expression of OPN was reported in the pregnant pig during the period of peri-implantation, however only weak expression of OPN was shown by the trophoderm (Garlow et al., 2002).

The exact constituents of the bovine uterine histotroph have not been determined, however, many of the progesterone-induced proteins are similar to those found in sheep (Leslie et al., 1990). As stated earlier, secretions from uterine glands are essential for proper development of embryos. Embryos developed to day 9 of gestation in ewes without uterine glands (uterine gland knock out), but by day 14, they are either reabsorbed or severely growth-retarded (Gray et al., 2001, 2002). Aberrant production of progesterone-induced proteins in the histotroph, like OPN, due to low concentrations of progesterone, might cause late embryonic mortality in the high-producing dairy cow, but this possibility has not been evaluated.

Prostaglandin F₂ alpha (PGF₂α). The effects of prostaglandins on embryo quality and viability have been evaluated most in the early postpartum cow. Premature increases in secretion of PGF₂α are associated with first postpartum ovulation and lead to a shortened estrous cycle (Ramirez-Godinez et al., 1981; Cooper et al., 1991). Even when exogenous progesterone was provided, cows bred at the first ovulation postpartum failed to maintain pregnancy (Breuel et al., 1993a). However, approximately half as many pregnancies were maintained when day-6 embryos from cows with short estrous cycles were transferred to cows with cycles of normal length as when embryos from normal cows were transferred to cows with normal cycles (Schrick et al., 1993). Additionally, when the reciprocal transfer was completed and cows with short cycles received supplemental progesterone, pregnancy rates following transfer of good embryos were half those in cows with normal cycles. Because pregnancy could be maintained in some beef cows during short cycles when good quality embryos from normal cows and exogenous progesterone were provided, Butcher et al. (1992) concluded that a factor in

addition to the short-lived CL, later shown to be PGF₂α, contributed to reduced pregnancy rates in cows with short cycles.

Schrick et al. (1993) reported greater concentrations of PGF₂α in the uterine lumen of cows with short cycles than in cows with normal cycles. Cows with greater concentrations of PGF₂α had lower quality embryos (Schrick et al., 1993; Hockett et al., 1998). Pregnancy rate also was decreased when PGF₂α was administered between days 4 to 7 or 5 to 8 after mating despite supplemental progesterone in cows (Buford et al., 1996; Seals et al., 1998) and ewes (Costine et al., 2001). However, embryonic survival was not compromised when PGF₂α was administered later, during maternal recognition of pregnancy, to progesterone-supplemented cows (Seals et al., 1998). Increased concentrations of uterine PGF₂α have been reported in cows with negative energy balances (Butler et al., 1998), experimentally-induced mastitis (Hockett et al., 2000) or heat stress (Malayer et al., 1990), which in each case might lead to increased embryonic death.

In contrast, Bridges et al. (2000) reported that embryonic survival increased with higher concentrations of PGF₂α in the inferior vena cava in cows dependent upon a replacement CL induced by hCG between days 28 and 36 of pregnancy. They proposed that PGF₂α might play a role in attachment of the embryo. Rodents require high concentrations of PGF₂α for implantation and treatment with indomethacin on day 5 of pregnancy decreased weights of implantation sites and extended the length of pregnancy (Kennedy et al., 1977). Similarly, in the hamster, indomethacin decreased the weight of implantation sites and the associated increase in PGE (Evans and Kennedy, 1978) and in the mouse blocked the decidual reaction and corresponding increases in PGF₂α (Rankin

et al., 1979). Thus, high concentrations of $\text{PGF}_2\alpha$ might be detrimental during early embryonic development, but beneficial during attachment in the cow. Stewart et al. (2003) observed increases in body temperature and in $\text{PGF}_2\alpha$ in ewes that were treated with peptidoglycan on day 5, which decreased pregnancy rate.

Induced Corpora Lutea

Corpora lutea have been induced in both cows (Pratt et al., 1982) and ewes (Inskeep et al., 1964) using treatment with either human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH), or luteinizing hormone (LH). Induced CL were intended to increase conception rates by increasing concentrations of progesterone when hCG was given before (Breuel et al., 1990), at the time of, or five to seven days after insemination (Rajamahendran and Sianangama, 1992); although, the results have been inconsistent. Corpora lutea have been induced in postpartum (Pratt et al., 1982), cycling (Howard and Britt, 1990), lactating (Rajamahendran and Sianangama, 1992), and pregnant cows (Wright et al., 1994). Treatments with LH (Lulai et al., 1994) or hCG (Wright et al., 1994; Bridges et al., 2000) induced ovulation and thus, formation of a new CL, in pregnant cows in which the CL of pregnancy had been removed and pregnancy maintained with exogenous progestogens.

Human chorionic gonadotropin, derived from the chorion, is LH-like and preferentially binds to the α subunit of the LH receptor (Hong et al., 1999). When the receptor complex, located on the small luteal cells (Mamluk et al, 1998), is bound by either hCG or LH, it acts through the adenylate cyclase pathway, catalyzing the conversion of ATP to cAMP (Saxena et al., 1986). Some actions of hCG include a

luteotropic effect similar to LH (Donaldson and Hansel, 1965) and stimulation of synthesis of progesterone by the CL *in vitro* (Veenhuizen et al., 1972) and *in vivo* (Breuel et al., 1989). Merz et al. (1981) reported that ^{125}I hCG binds to the theca of nearly all follicles, regardless of health of the follicle, and to the granulosa cells in a variable manner.

Diagnosis of Pregnancy using Ultrasonography

Repeated, non-invasive evaluations of pregnancy can be made on the same animal using ultrasonography. An experienced ultrasound technician can detect the bovine embryo as early as d 19 of gestation (Curran et al, 1986a,b), 15 to 20 days earlier than pregnancy can be diagnosed easily and accurately with the traditional method of rectal palpation. While detection of the embryo proper is the only guaranteed indication of pregnancy, increases in fluid within the uterus also can be used to indicate early pregnancy. However, the accuracy of pregnancy diagnosis by this method in dairy heifers was less than 50% before day 18 with a 5.0 MHz probe, or day 16 with a 7.5 MHz probe (Kastelic et al., 1991a).

Viability of the embryo, as determined by presence of a heartbeat, can be determined in most cows starting on day 20 of gestation (Curran et al, 1986). In addition to earlier detection of pregnancy, ultrasonography has many advantages. Because it is less invasive than palpation, it might prevent any palpation-induced abortions, particularly those caused by using the membrane slip technique, when detecting early pregnancy (Vaillancourt et al., 1979). Additionally, retention of pregnancy was not

associated with day of pregnancy diagnosis (Baxter and Ward, 1997) or frequency of examinations using ultrasonography (Starbuck et al., 2004).

Ultrasonography provides an opportunity for visual appraisal of the entire conceptus throughout early fetal development. For example, heart rate can be measured and a rapid decline in heart rate over a short period may indicate impending death of the embryo (Kastelic et al., 1991b). During this time, the embryo and fetus will progress through many critical stages of development, for example: expansion of the allantois, placentation, and organogenesis, which must be completed successfully for pregnancy to continue. While pregnancy can be diagnosed earlier using ultrasonography compared to palpation, 9 to 10% of early pregnancies diagnosed by ultrasonography are lost before day 35 (Mee et al., 1994; Nation et al., 2003), making it necessary to recheck pregnant cows at approximately 60 days of gestation. Thus, ultrasonography makes it possible to study late embryonic mortality soon after maternal recognition of pregnancy. In the lactating dairy cow, such losses may be substantial, on average 12% (Santos et al., 2004).

Factors that influence loss of pregnancy

Number of Follicular Waves. The number of follicular waves affects fertility of the oocyte from the ovulatory follicle. Fewer cows with two waves became pregnant to a single breeding than those with three waves (70 vs. 96% in beef cows, Ahmad et al., 1997; 63 vs. 81% in dairy cows, Townson et al., 2002). As the number of follicular waves increased from two to three, the interval from emergence of the ovulatory follicle to ovulation was reduced from 10 or 11 days to 6 or 7 days (Sirois and Fortune, 1988; Ginther et al., 1989c). Retrospectively, the mean interval from emergence of the

ovulatory follicle to estrus was one day shorter in dairy cattle that became pregnant following insemination, than in those that did not conceive (Bleach et al., 2004). As the interval between emergence of the ovulatory follicle and estrus increased, pregnancy rates declined (Stock and Fortune, 1993; Mihm et al., 1994; Bleach et al., 2004). Likewise, extended duration of dominance of a persistent, ovulatory follicle reduced fertility, as reported for cows synchronized with low concentrations of progesterone (Mihm et al., 1994; Cooperative Regional Research Project NE-161, 1996).

The successive development of dominant follicles during the estrous cycle is related to luteal phase concentrations of progesterone (Bergfelt et al., 1991) and following endogenous luteolysis, can be maintained with treatments that achieve luteal phase concentrations of progesterone (Sirois and Fortune, 1990; Adams et al., 1992). However, treatment with sub-luteal concentrations of progesterone (1 to 2 ng/mL) extends the period of dominance of a follicle (Sirois and Fortune, 1990; Adams et al., 1992; Savio et al., 1992), and for several days it can still ovulate if progesterone is removed (Mihm et al., 1994).

Synchronization of Estrus. Lucy (2001), suggested that retention of pregnancy in lactating dairy cattle had declined as the use of fixed-time artificial insemination protocols increased. Pregnancy rates at 30 and 60 days post-insemination did not differ between cows that were inseminated following detection of estrous and cows that were inseminated on a timed basis when all cows were pre-synchronized previously (Rutigliano and Santos, 2005). Additionally, retention of pregnancy did not differ in lactating dairy cows inseminated following detection of standing estrus (86.8%) or after

being synchronized with OvSynch[®], a breeding protocol utilizing timed artificial insemination (89.6%; Chebel et al., 2004).

Not all cattle in a population are at the same stage of the estrous cycle prior to synchronization of estrus, which results in variability in the response and in fertility. In cows induced to ovulate by treatment with GnRH, smaller ovulatory follicle size at the time of timed artificial insemination reduced pregnancy rates, but follicle size had no effect on pregnancy rates when cows ovulated spontaneously and were bred by the AM:PM rule (Perry et al., 2005). As discussed later, this effect might be attributed to reduced production of progesterone between days 2 to 12 post ovulation by CL from smaller follicles that were induced to ovulate compared to that produced by larger ovulatory follicles (Busch et al., 2005). Macmillan et al. (2003) indicated that conception rates might be reduced in dairy cows following timed artificial insemination when standard doses of GnRH (100 µg) were used, because small follicles induced to ovulate formed CL that were less competent.

Cerri et al. (2005) compared embryo quality on day 6 among four synchronization protocols: detected estrus (GnRH on d 6 of the estrous cycle followed by prostaglandin 7 days later and insemination upon estrus), or OvSynch beginning at three different stages of the estrous cycle, day 3, day 6, or day of estrus (OV3, OV6 and OVE, respectively). Because the dominant follicles were smaller, fewer cows ovulated after administration of GnRH on day 3 (OV3) than in response to the other treatments. While overall synchronization was lower in the three 'OvSynch' groups than in the 'detected estrus' group, rates of fertilization were equal across method of synchronization (86.3%). Synchronization with OV3 resulted in fewer embryos of excellent and good quality as a

proportion of those collected, and embryos had fewer blastomeres than those from either OV6, OVE, or detected estrus. While insemination at detected estrus did not improve fertilization rates or embryo quality, treatment in preparation for timed AI beginning on day 3 of the estrous cycle compromised oocyte quality due to prolonged dominance of the ovulatory follicle at insemination.

Synchronization of estrous using long-term treatments with progestins caused prolonged persistence of the ovulatory follicle that reduced conception rate and increased pregnancy loss (Ahmad et al., 1995; Inskeep, 2002). Even when short-term treatments with progestins (≤ 8 days) were used, conception rates were greater in cows inseminated following spontaneous estrus (Xu et al., 1996; Chenault et al., 2003). Persistent dominant follicles formed when subluteal concentrations of progesterone induced increased frequency of pulses of LH during the estrous cycle before insemination (Inskeep, 2002; Bridges and Fortune, 2003). Conception rates were greater for smaller preovulatory follicles than for larger follicles with dominance maintained for an extended period prior to the surge of LH (91% vs. 36%; Breuel et al., 1993b). The resumption of meiosis occurred prematurely in oocytes exposed to high peak frequency of LH (Revah and Butler, 1996; Mihm et al., 1999) and led to embryo mortality before the 16-cell stage (Ahmad et al., 1995). After the occurrence and regression of a persistent follicle, fertility of the next follicle was unaffected (Smith and Stevenson, 1995). Low dietary intake of feed reduced the diameter and persistence of dominant follicles during the estrous cycle of beef heifers and tended to increase the proportion of estrous cycles with three dominant follicles (Murphy et al., 1991). Overall, while some synchronization protocols might affect conception rate, neither timed insemination nor insemination following

detected estrus appeared to influence late embryonic or early fetal mortality (Santos et al., 2004).

Placental Development. Fewer than one live animal is born per 100 reconstructed nuclear transfer (NT) embryos (Schnieke et al., 1997). First trimester losses of greater than 50% are common for pregnancies using NT or *in vitro* produced embryos. Placental abnormalities occur at a high incidence during both early and late gestation. While pregnancy rates at day 30 were similar for recipient cows carrying NT and control embryos, by day 90 of gestation few pregnancies with NT embryos remained. Abnormalities of the placenta, which included reduced vascularity, reduced number of cotyledons formed, and abnormal epithelium on the cellular level, appeared to be associated directly with the high rates of pregnancy loss for recipients carrying NT embryos (Hill et al., 2000).

Using RT-PCR and cDNA microanalysis, heat shock proteins have been identified as being potentially important for proper placental development (Niemann and Wrenzycki, 2000; Ushizawa et al., 2004). Heat shock proteins may disturb the coordination between the conceptus and endometrium in cattle and have been associated with early embryonic loss in cattle exposed to heat stress during maternal recognition of pregnancy (Putney et al., 1988). Mutant mice lacking HSP90 β had normal embryonic development until day 9 of gestation, including normal fusion of the allantois and chorion and invasion of the allantoic blood vessels into the chorion; however, the blood vessels in the allantois failed to expand and the trophoblast cells did not differentiate to form the placental labyrinth required for the pregnancy to continue (Voss et al., 2000).

Embryo Transfer. The transfer of embryos of known quantity and quality into recipient cows allows for the study of factors affecting retention of early pregnancy. While less than 50% of dairy cows were pregnant 27 to 30 days after embryo transfer (Drost et al., 1999; Sartori et al., 2003), 69 and 83% of beef cows were pregnant on day 37 after transfer of frozen or fresh embryos, respectively (Spell et al., 2001). Even embryos produced *in vitro* can be utilized for embryo transfer, however as reported by numerous researchers, zygotes developed *in vitro* have delayed development. Morulas produced *in vitro* show less compaction, exhibit variable amounts of coalescence of individual blastomeres, are grainy in appearance and have reduced perivitelline space when compared to those produced *in vivo*. Additionally, blastocysts appeared darker, had more granules and lipid droplets, and often had incomplete junctional complexes between the inner cell mass and trophoblast.

While the incidence of abortion beyond the second month of gestation is low (1 to 2 %), when *in vivo*-produced embryos are used, the incidence of spontaneous abortion between the second and seventh month of gestation was approximately 5% for *in vitro*-produced embryos (King et al., 1985; Hasler et al, 1987). Loss of pregnancy following transfer of *in vivo*-produced embryos often occurred before day 21 of gestation (Markette et al., 1985; Farin and Farin, 1999). Markette and coworkers reported that 54.7% of embryo transfer recipients (n = 910) were pregnant on day 60 of gestation. In that study, loss of pregnancy, as determined by observed returns to estrus, was reported to be 1.5% for days 2 to 16.5, 24.4% for days 17 to 24, 2.6% for days 24.5 to 34, 9.2% for days 34.5 to 46.5 and 7.5% after day 47. Thus, most loss of pregnancy occurs prior to maternal

recognition of pregnancy, but the period following attachment of the placenta is an additional time of substantial loss.

Body Condition Score. Body condition score (BCS) is a management tool that is useful for evaluating the nutritional status of cattle (Hady et al., 1994). Most frequently, reduced fertility is associated with low body condition score, but loss of pregnancy between weeks five and nine also has been associated with greater than ideal BCS (≥ 3.5) (Starbuck et al., 2004). Fertility is associated negatively with loss of BCS during the postpartum period in a linear manner (Gearhart et al., 1990; Domecq et al., 1997; Suriyasathaporn et al., 1998). Loss of pregnancy increased following a drop in BCS from parturition to 30 days postpartum; a one-unit decrease in BCS increased the risk of loss of pregnancy between days 38 and 90 by a factor of 2.4 (Lopez-Gatius et al., 2002) and increased days open between parturition and conception by more than 10 days, but did not affect conception rates (Lopez-Gatius, 2003). Similarly, Silke et al. (2002) observed that a one-unit decrease in BCS from days 28 to 56 had a 3.2-fold increase in the odds ratio for loss of pregnancy during that time. Poor fertility is associated with an abrupt decline in nutritional status during the postpartum period, which was related to the occurrence of metabolic and infectious diseases (Ruegg and Milton, 1995; Heuer et al., 1999), which might impair involution of the uterus (Loeffler et al., 1999).

The heritability of loss of body condition score between calving and early postpartum, prior to first service, ranged from 0.01 to 0.07. The genetic correlations between loss of body condition and days to first service ranged from 0.29 to 0.68 (Dechow et al., 2002). In addition to genetics, the loss of body condition score during early postpartum was related quadratically to the level of milk production (Waltner et al.,

1993). The relationship between loss of body condition and milk production might explain why the amount of condition lost was greater in cows with ≥ 4 lactations than in primiparous cows (0.9 vs. 0.3 BCS units change; Waltner et al., 1993).

Because cows in better condition would be expected to eat more feed to maintain condition and intake of feed is related to rate of metabolism, body condition score might affect retention of pregnancy through altered concentrations of hormones, as described in greater detail in the section on metabolism of progesterone.

Age of Cow. Increasing age in cattle and other mammalian species is associated with decreased reproductive efficiency (Erickson et al., 1976) and with a lower production and quality of transferable embryos following superovulation (Lerner et al., 1986). Generally, younger females have better conception rates than older females. Santos and co-workers (2004) compiled from literature that the overall rate of pregnancy loss for lactating dairy cows was 10.7%, compared to only 4.2% for dairy heifers. Other researchers have reported that lactating cows retained fewer pregnancies than heifers and that as age of the lactating cow increased, rate of pregnancy retention was either not affected (Jousan et al., 2005) or decreased (Ball, 1978; Starbuck et al., 2004; Starbuck et al., 2005).

Primiparous dairy cows had higher conception rates than multiparous cows (27.4 vs. 24.1%; Chebel et al., 2004). Infertility due to increasing age is not a common problem in the modern dairy industry, because most dairy cattle are culled from herds by the age of six. Canadian dairy producers listed old age as the primary reason for culling cows only 3.1% of the time (Visscher, 2001). Chebel and coworkers (2004) reported no differences in retention of pregnancy between primiparous and multiparous cows;

however, lower rates of pregnancy retention were reported for dairy cows 5 to 8 years old than for younger cows (Starbuck et al., 2004). Conception rates were greater in repeat-breeder dairy cows in second to fourth lactation, especially when treated with rbST at insemination and 10 days later (Morales-Roura et al., 2001). Thus, it appears that reproductive efficiency is peaking around the fourth lactation and decreasing in subsequent years.

In the beef industry, cows are commonly kept in production for 10 to 12 years. Bryner and co-workers (1990) studied the estrous cycle of young (5 to 7 years) and old (\geq 12 years) beef cows. Older cows had an earlier increase of FSH during midcycle, which was associated with an earlier increase and overall greater concentration of estradiol. Older cows also had reduced concentrations of progesterone on days 14 and 15 of the estrous cycle. Together, these alterations to the hormonal milieu during the estrous cycle lead to ovulation of a more advanced follicle with reduced fertility, which also has been reported in aged rats (Page and Butcher, 1982).

In general, there is a significant decrease in the number of follicles in older cows (Erickson et al., 1966; Katska and Smorag, 1984). Changes to the reproductive tract also occur, with older animals having increased collagen (Craig and Jollie, 1985) and decreased stromal cells (Wilcox, 1982) and estrogen receptors (Han et al., 1989). As mice age, the ability of endometrial cells to exhibit a decidual reaction is reduced (Otha, 1987). Microarray technology identified, in the mouse uterus, that the expression of 14 of the 297 estrogen-target genes studied was altered with aging (Khalyfa et al., 2003). Any of these genes might be associated with the age-related changes to reproductive tissues described above.

In human beings, as in other animals, fertility decreases with age. Fewer than 55% of women over the age of 40 achieve pregnancy within one year compared to 80% of women under 40 (reviewed by Fitzgerald, et al., 1998). It was estimated that in women 24 to 32 years of age, the total number of oocytes retrieved through assisted reproductive technologies declined by about 1.25 oocytes per year (Stolwijk et al., 1997). In addition to the age-related reduction in number of oocytes retrieved, quality of the oocytes declines. Thus, some patients opt for oocyte donation, which allows for the unique opportunity to evaluate age-related changes to both oocyte quality and uterine receptivity. If oocytes were donated by young women to older women, implantation rates for the most part were restored to normal, although some authors reported that pregnancy and implantation rates remain lower in older women than in younger women receiving young donor eggs (reviewed by Sauer, 1998). Thus, in the human being, the majority of the age-related decline in fertility appears to be due to reductions in oocyte quality.

Concentrations of progesterone. The relationship between concentrations of progesterone and establishment of pregnancy is well studied. Circulating concentrations of progesterone before (Folman et al., 1973; Fonseca et al., 1983; Folman et al., 1990) or after artificial insemination (Fonseca et al., 1983; Robinson et al., 1989) have been associated with differences in conception rates in cattle. Concentrations of progesterone in pregnant animals have been reported to increase over those in open animals beginning as early as 3 to 6 days after insemination (Maurer and Echterkamp, 1982; Albihn, 1991; Larson et al., 1997). Progesterone increased more rapidly in pregnant cows with embryos that produced measurable concentrations of IFN- τ on day 16, than in pregnant cows with

embryos that produced undetectable concentrations of IFN- τ (Mann and Lamming, 2001). In subfertile dairy cows, progesterone increased more slowly after estrus than in heifers (Shelton et al., 1990). However, Pritchard et al. (1994) reported no relationship among patterns of progesterone or estradiol on days 4 to 7 after insemination and conception rates in beef cows.

Increased concentrations of progesterone during the estrous cycle following insemination might (Fonseca et al., 1983; Meisterling and Dailey, 1987; Britt et al., 1989) or might not (Wiltbank et al., 1956; Hawk et al., 1963) predict improved fertility. However, in ovariectomized, recipient ewes, Parr et al. (1982) reported a positive, dose-dependent effect of progesterone treatment on the rate of embryo survival. Embryonic survival to day 30 of gestation was decreased when ewes were treated with epostane, an inhibitor of the steroid pathway enzyme 3 β -HSD, on days 9, 10 and 11 of gestation, an effect that was reduced when ewes were treated simultaneously with progesterone implants (Ashworth et al., 1987).

High producing dairy cows generally have lower circulating concentrations of progesterone than lower producing cows (Bilby et al., 1998; Lucy et al., 1998; Vasconcelos et al., 1999). In addition to affecting survival of the early embryo, concentrations of progesterone affected later retention of pregnancy through the early fetal stage. Retention of pregnancy to week 7 or 9 of gestation was related to concentrations of progesterone at week 5 of gestation in dairy cows. Cows with concentrations of progesterone in the low quartile were more likely to lose pregnancy than those with greater concentrations (Starbuck et al., 2004). In that study, the concentration of progesterone during week 5 of gestation at or below which only 50% of

pregnancies were retained was 2.8 ng/mL. Likewise, the value at or above which 95% of pregnancies were maintained to week 9 was approximately 6 ng/mL.

Supplementation of cows with exogenous progesterone after insemination increased pregnancy rates in some studies (Roberson et al., 1989; Folman et al., 1990; Lynch et al., 1999) and retention of pregnancy to day 90 of gestation (Lopez-Gatius et al., 2004). Exposure of embryos to higher than normal concentrations of progesterone during the early cleavage stage of development resulted in longer conceptuses (Garrett et al., 1988; Nephew et al., 1991) and sometimes heavier fetuses (Wilmot and Sales, 1981; Kleeman et al., 1994). This effect is probably mediated through altered uterine secretions, possibly because prolonged exposure to progesterone can increase IGFBP-specific proteases (Peterson et al., 1998). Exposure to high concentrations of progesterone during preimplantation development may influence patterns of de novo methylation of the embryonic genome (Razin and Shemer, 1995).

Treatments with GnRH, LH and hCG are used to induce ovulation and subsequent formation of a CL. Gonadotropin supplementation at breeding or within five to seven days thereafter has been recommended as a method to improve fertility in cattle by increasing concentrations of progesterone. Accessory CL were formed after treatment of cattle with hCG (Walton et al., 1990) or norgestomet, a synthetic progestogen, followed by hCG at norgestomet withdrawal (Coy and Garcia-Winder, 1991).

Treatment of heifers with hCG five days after estrus, increased concentrations of progesterone more than treatment with Buserelin, a GnRH agonist, while both were equally effective at inducing formation of a new CL (93%; Schmitt et al., 1996). Treatment with hCG, to induce formation of accessory CL during the diestrous period

after breeding, improved pregnancy rates (McDermott et al., 1986; Breuel et al., 1989). Similarly, pregnancy rates were improved in repeat-breeders after treatment with GnRH at breeding (Lee et al., 1983; Stevenson et al., 1984).

Interestingly, concentrations of progesterone were greater in cows with induced accessory corpora lutea, but not in those with two corpora lutea resulting from spontaneous ovulation. In cows with one or two CL, Pope et al. (1969) reported no difference in peak peripheral concentrations of progesterone during the estrous cycle. The decrease in weight per CL in cows treated with FSH to induce multiple ovulations (Echternkamp, 1992) might imply decreased output of progesterone per CL in cows with two CL. In ewes that had been superovulated, both weight per CL and progesterone per CL were correlated negatively with the number of CL, without a significant increase in rate of blood flow from the ovarian veins (Stormshak et al., 1963).

Twinning. In cattle, twinning increases the rate of late embryonic and(or) fetal mortality. Beef cows pregnant with twins had higher rates of pregnancy loss between pregnancy diagnosis by ultrasonography and parturition than cows pregnant with singles (12.4% vs. 3.5%; Echternkamp and Gregory, 1999). Pregnant dairy cows with two CL lost pregnancy more frequently between weeks 5 and 9 of gestation than those with one CL (27% vs. 9%; Starbuck et al., 2004). An estimated 38% of twin pregnancies terminated in either observable abortion (21%) or stillbirths (17%; Erb and Morrison, 1959). Mortality of twin embryos usually occurs prior to day 35 (Anderson, et al., 1982). However, in earlier work, Anderson et al. (1978) reported fetal loss of twin pregnancies between days 45 and 60 of gestation at a rate of 27.8%.

Unlike the sheep, a litter bearing species, and the horse, partial losses of pregnancies rarely happen in the cow, as death of one fetus in a twin pregnancy usually results in death of the other due to anastomosis of the placentas (Eckternkamp, 1992), which occurred in about 90% of twin pregnancies (Eckternkamp, 1987). In mares, twinning accounts for 10 to 30% of abortions and it is rare when live twin foals are born and thrive. Embryonic fixation, when the embryo contacts directly with the endometrium, occurs on days 16 and 17 of gestation in the mare. Seventy percent of twins' vesicles fix unilaterally, in the same horn. Interestingly, 85% of unilaterally-fixed twin pregnancies undergo natural reduction by day 40, while bilaterally-fixed twin pregnancies do not. Embryos are implanted prior to natural reduction, which often happens between days 17 and 38 (Ginther and Pierson, 1984). How and why one embryo is selected for natural reduction is not understood, although the close proximity of multiple embryos favors reduction from a twin pregnancy to a single pregnancy. In unilateral twins, there is often a loss of contact between the endometrial and trophoblastic surfaces from each embryo and in some cases the larger embryonic vesicle has been shown to completely surround the smaller one. Unilaterally-fixed twins also undergo natural reduction earlier than bilateral twins, when the yolk sac, a less efficient distributor of nutrients, is still the predominant placental membrane (Ginther, 1984). As stated above, partial loss of one of two embryos occurs rarely in cattle.

High producing dairy cows had significantly more ovulations than those of average and low milk production (Kinsel et al., 1998; Fricke and Wiltbank, 1999). Lactating cows exposed to heat stress for an entire estrous cycle had greater numbers of large (>10 mm) follicles during the first follicular wave (Wolfenson et al., 1995; Roth,

1998), and reduced dominance of the largest follicle, which allowed growth of a second large follicle, thus increasing the likelihood of multiple ovulations. Synchronization of estrus in cows resulted in the development of more co-dominant follicles when initiated during the first follicular wave of the cycle than in the second wave (Ginther et al., 2001b). Cattle selected for twinning had more secondary follicles, but equal numbers of primary and primordial follicles than those not selected for twinning (Cushman, et al., 2000). Six to 10% of twins are monozygotic twins, which result from the division of one fertilized oocyte (Johansson, 1932; Joubert, 1952).

STATEMENT OF THE PROBLEM

Production of beef and dairy cattle contributes significantly to the agricultural economy. Failure of a cow to become pregnant or maintain pregnancy is an important variable that accounts for a large percentage of reduced reproductive efficiency. While many authors have reported several general factors with which the occurrence of late embryonic and early fetal mortality is associated, most do not examine specific factors. Retention of pregnancy to weeks 7 and 9 of gestation was reduced in dairy cows with concentrations of progesterone in the lowest quartile (Starbuck et al., 2004). While loss of pregnancy during the period of placental attachment is less in beef cows than in dairy cows, a similar relationship between concentration of progesterone and maintenance of pregnancy has not been established. The direct effect of altered concentrations of progesterone during the period of attachment has not been evaluated in beef cows. In addition, beef cattle remain in production at greater ages than dairy cattle, which allowed for the effect of age of cow on retention of pregnancy to be evaluated.

Dairy producers often treat cows every 10 to 14 days throughout most of lactation with rbST to increase milk production. When initiation of treatment with recombinant bovine somatotropin (rbST) occurred coincident with first breeding to timed artificial insemination, conception rates were increased. While some producers might not be willing to initiate long-term treatment with rbST due to costs or perceived negative effects of extended use, those individuals might be willing to use a single treatment to increase conception rates in problem cows. However, the effect of a single treatment with rbST at insemination on retention of pregnancy has not been evaluated in either dairy or beef cattle.

Fertility has been associated positively with the number of ovarian follicular waves during the estrous cycle before breeding in both beef and dairy cows (Ahmad et al., 1997; Townson et al., 2002). Burns and co-workers (2005) reported a high repeatability within cow (0.95) of numbers of antral follicles ≥ 3 mm in diameter during both ovulatory and non-ovulatory follicular waves. It has not been determined if fertility of beef cattle is associated with the number of follicles that develop during the wave immediately prior to ovulation and subsequent insemination.

Four experiments were conducted to evaluate factors associated with conception rate and loss of pregnancy. The objective of experiment I was to determine whether the occurrence of late embryonic or early fetal mortality was associated directly with altered concentrations of progesterone during placental attachment. Pregnancy was maintained in lutectomized cows by exogenous progesterone. Additionally the association of late embryonic or early fetal mortality with concentrations of estradiol, age of cow, or body condition score were examined. Experiment II was a continuation of experiment I, and evaluated the ability of CL that were induced after day 53 to maintain pregnancy to term. The primary objective of experiment III was to determine if a single treatment with rbST, at insemination 12 h after observation of standing estrus, affected conception and (or) pregnancy retention rates in dairy cows, dairy heifers or beef cows. Finally, the main objective of experiment IV was to determine if fertility is associated with the number of antral follicles ≥ 4 mm in diameter that developed within the wave prior to ovulation and breeding. A second question was whether the numbers of follicles ≥ 4 mm in diameter that developed within waves in a cycle and within waves between cycles were repeatable within or among beef cows.

EXPERIMENT I:

**EFFECTS OF DECREASED PROGESTERONE DURING PLACENTATION ON
RETENTION OF PREGNANCY IN THE COW**

Abstract

Retention of pregnancy in dairy cows was associated positively with increased concentrations of progesterone at week 5 of gestation. The use of supplemental progesterone in early gestation to increase retention of pregnancy has had variable success. The objective of this experiment was to determine if induced variation in concentrations of progesterone beginning on day 28 affected late embryonic or early fetal loss in suckled beef cows. Cows were assigned randomly within age and uterine horn of pregnancy to receive progesterone from either two new ($n = 19$) or two used ($n = 21$) controlled internal drug releasing inserts (CIDR; replaced every 5 d) beginning on day 28 of gestation to produce varying concentrations of progesterone. Corpora lutea were enucleated from the ovary on day 29 by transrectal digital pressure. Pregnancy status was evaluated by ultrasonography daily between days 28 and 38 and generally every 5 d thereafter. Peripheral progesterone averaged 1.8 ± 0.1 ng/mL during days 30 to 38 and did not differ between progesterone treatments. The association of concentrations of progesterone or estradiol on days 30 to 33 or days 30 to 38, age or body condition on pregnancy retention to either day 38 or 53 was evaluated using logistic regression. Retention of pregnancy was not associated with concentrations of hormones or body condition score but was associated negatively with age of cow ($P < 0.05$). However, reduction of progesterone to a mean of 1.8 ng/mL increased loss of pregnancy from the normal of 2% for this herd, to 23%. In summary, retention of pregnancy was associated with age of cow, but not with concentrations of progesterone or estradiol during placentation.

Introduction

Loss of pregnancy during the late embryonic and early fetal periods of development is frequent in dairy cattle. In recent studies, 7 to 33% of pregnancies in lactating dairy cows were lost between days 28 and 98 of gestation (Inskeep, 2002; Santos et al., 2004). Dailey and coworkers (2002) postulated that most late embryonic and early fetal losses occur prior to day 45 of gestation. Progesterone is essential for maintenance of pregnancy and pregnancy can be maintained using only exogenous progesterone in cows that have no corpus luteum (CL; Zimbelman and Smith, 1966; Tanabe, 1970; Inskeep and Baker, 1985). Pregnancy retention to week 7 of gestation was associated positively with concentrations of progesterone during week 5 in dairy cows (Starbuck et al., 2004). Specifically, only half of the pregnancies were maintained when concentrations of progesterone were below 2.8 ng/mL during week 5 of gestation.

The objective of the present study was to evaluate directly the effect of altered concentrations of progesterone during late embryonic and early fetal development, specifically during placentation and the transition from embryo to fetus, on retention of pregnancy in suckled beef cows. To achieve this objective, cows underwent ovariectomy and replacement therapy with progesterone.

Materials and Methods

Preliminary Trial. In order to determine the treatments needed for the remaining experiment, suckled, Angus beef cows (N = 10) maintained on pasture at the West Virginia University Farm, Morgantown, WV, were assigned randomly to receive for six days one of four treatments: one previously-used (used for seven days and washed in

warm, soapy water; n = 3) controlled internal drug releasing device (CIDR; Pfizer Animal Health, New York, NY), one new CIDR (1.38 g progesterone; n = 2), one used and one new CIDR (n = 3), or two new CIDRs (n = 2). Cows were either 30 or 37 days pregnant on the day of CIDR insertion, designated day 0. Blood samples were collected daily until CIDR removal and assayed for concentrations of progesterone as described below.

Primary Experiment. This study used suckled beef cows (N = 40) of mixed breeds and ages (2 to 11 yrs; mean 4.3 ± 2.7) at the West Virginia Agricultural and Forestry Experiment Station, Aarthurdale, WV. Throughout the study, cows grazed rotationally on one of three pastures that contained primarily a mixture of orchard grass, tall fescue and red clover. Because one pasture had been grazed previously, cows were supplemented with hay during the first nine days of the experiment. At the start of the experiment, cows were 28 days pregnant to first-service artificial insemination (day 0) with a single embryo. Pregnancy was diagnosed via transrectal ultrasonography (Aloka 500 or 900 [Corometrics Medical Systems, Wallingford, CT] fitted with a 7.5 MHz rectal probe). Body condition scores were assigned on day 28 or 29 of pregnancy according to Whitman (1975) and averaged 5.9 ± 1.0 . The scale ranges from one to nine, with one being emaciated, six being ideal and nine being obese. Cows were assigned randomly within age class (2 to 3 yrs [2.1 ± 0.4 yrs; n = 22] and ≥ 4 yrs [7.2 ± 1.0 yrs; n = 18]) and uterine horn of pregnancy (right or left) to receive one of two treatments. Variability in concentrations of progesterone was achieved by implanting either two new (1.38 g progesterone per insert; n = 19) or two used (used previously for 7 d, then cleaned with warm, soapy water; n = 21) CIDRs, beginning on day 28 of gestation. The original CL of

pregnancy was removed by transrectal enucleation on day 29, thus maintenance of pregnancy was then dependent upon progesterone provided by the CIDRs. Two animals in which lutectomy was incomplete (detected by ultrasonography) received 25 mg PGF₂ α on day 30 (i.m.; Lutalyse, Pfizer Animal Health, New York, NY). Retention of pregnancy, based on visualization of an embryonic or fetal heartbeat, was evaluated daily by transrectal ultrasonography between days 28 and 38 and then on days 40, 43, 45, 48 and 53 or until loss of pregnancy. At each ultrasonographic examination, crown-rump length of the embryo or fetus, and diameters and locations of the two largest follicles \geq 5 mm and any corpora lutea on each ovary were recorded. The CIDRs were replaced every 5 d from day 33 until day 53 or loss of pregnancy.

Blood collection and radioimmunoassays. A 5-mL sample of jugular blood was collected at each ultrasonographic examination and placed immediately on ice for transportation to the laboratory. Samples were collected by an individual who did not handle CIDRs to reduce risk of possible contamination with progesterone. Samples were refrigerated (4°C) for 12 to 24 h before centrifugation. Serum was harvested and stored at -20°C until radioimmunoassayed for concentrations of progesterone and estradiol-17 β as described previously in our laboratory (Sheffel et al., 1982; Rozell and Keisler, 1990; Tortonese et al., 1990).

Statistical Analyses. The association of subsequent pregnancy status on either day 38 or 53 with mean concentrations of progesterone and estradiol from days 30 to 33 (the period after lutectomy, but before the first exchange of CIDRs) or days 30 to 38 (through the day of the second CIDR exchange), age of cow and body condition score were determined using the LOGISTIC procedure of SAS (1997). Day 38 and 53 were

chosen because they are near the conclusion of different developmental processes (placentation and the transition from embryo to fetus) that might affect retention of pregnancy. One cow lost both CIDRs after day 45 and lost pregnancy; those data were excluded from analyses after day 38.

Results

Preliminary Trial. Mean overall concentrations of progesterone for the CIDR treatments are shown in Figure 1. On days 1, 2 and 3 post CIDR insertion, there were high amounts of variability of concentrations of progesterone within cows receiving the same treatments. Two new CIDRs produced the greatest peak concentration of progesterone and was therefore selected as one treatment. One new CIDR produced the largest increase between initial concentration of progesterone on day 0 and the mean of days 2 through 5 post CIDR insertion (3.03 ± 0.01 ng/mL). Concentrations of progesterone between days 2 through 4 post insertion decreased in one of the cows with one used CIDR and increased only slightly (0.85 ± 0.04 ng/mL) in the remaining two animals, and therefore was expected to result in concentrations of progesterone too low to maintain pregnancy in any cows with no CL. Thus, two used CIDRs were selected as the second treatment.

Primary Experiment. Mean concentrations of progesterone did not differ between cows with two used or two new CIDRs when averaged over days 30 through 33 (1.8 ± 0.1 ng/mL for each) or days 30 through 38, the day of the second CIDR exchange (1.9 ± 0.1 and 1.8 ± 0.1 ng/mL, respectively). Thus, mean concentration of progesterone was included in the statistical model and not treatment. On day 28, mean concentration

of progesterone was 3.4 ± 0.3 ng/mL. Concentrations of progesterone increased 1.0 ± 0.2 ng/mL from day 28 to 29 following insertion of CIDRs, and decreased 2.3 ± 0.3 ng/mL from day 29 to 30, after lutectomy. Mean concentrations of progesterone for days 30 through 33 and 30 through 38 were 1.8 ± 0.1 (Figure 2). Mean concentration of estradiol between day 30 and 38 was 2.3 ± 0.1 pg/mL (Figure 2).

Pregnancy was lost from nine cows during progesterone replacement with CIDRs. Retention of pregnancy was 85% to day 38, and 77% to day 53. Thus, 66% of the total pregnancy loss occurred between days 28 and 38. Retention of pregnancy to day 38 or 53 was not associated with concentrations of progesterone (range 0.7 to 4.3 ng/mL) or estradiol (range 1.2 to 4.2 pg/mL) on days 30 through 33 or body condition score, but in each case was associated with age of the cow ($P < 0.05$; Figure 3). The prediction equation was $P38(\text{pregnant}) = 1 - (1/1 + e^{-(3.8301 + 0.3847 \times [\text{age}]})})$ or $P53(\text{pregnant}) = 1 - (1/1 + e^{-(3.2402 + 0.3926 \times [\text{age}]})})$, respectively.

After complete lutectomy and before day 38, a new CL was identified by ultrasonography in five cows, in each case on the ovary ipsilateral to the uterine horn of pregnancy.

Discussion

High rates of pregnancy loss do not usually occur in the suckled beef cow. However, manipulation of concentrations of progesterone in beef cows might be used to provide a model in which to study effects of sub-normal concentrations of progesterone in dairy cows. Overall loss of pregnancy between days 28 and 53 of gestation was 23%; the distribution of times of pregnancy loss was 66% and 33% between days 28 to 38, and

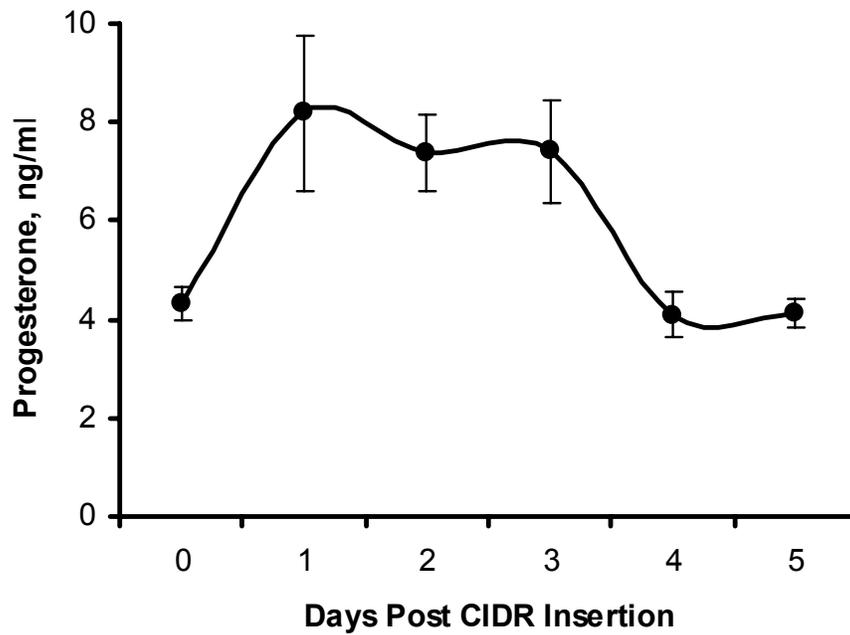


Figure 1. Mean effect of four progesterone treatments on jugular concentrations of progesterone in pregnant, suckled beef cows early in the second month of gestation (mean \pm SEM). Cows received either one used CIDR (n = 3), one new CIDR (n = 2), one used and one new CIDR (n = 3) or two new CIDRs (n = 2).

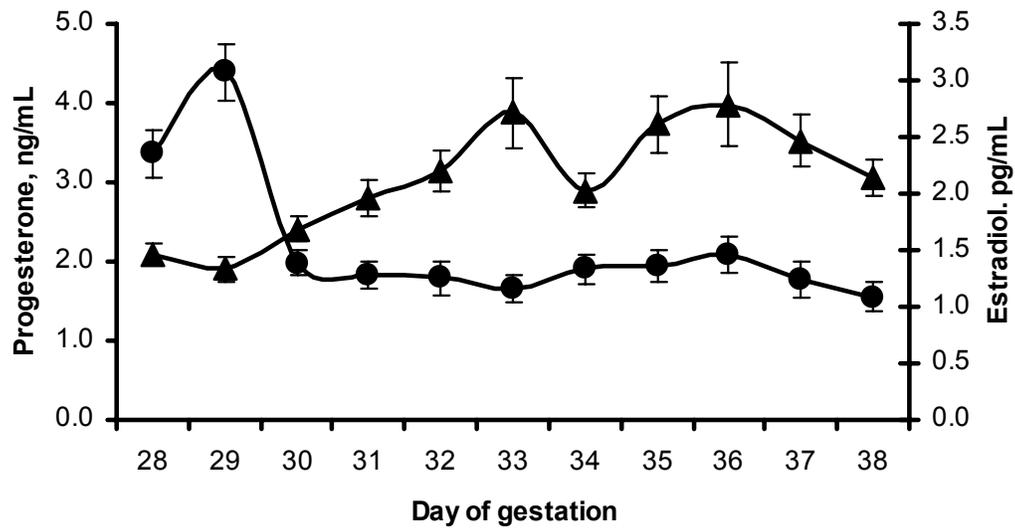


Figure 2. Concentrations of progesterone (●) or estradiol (▲) on days 28 through 38 in pregnant cows in which the CL of pregnancy had been removed on day 29 and pregnancy was maintained with progesterone from CIDR inserts (n = 40; mean ± SEM).

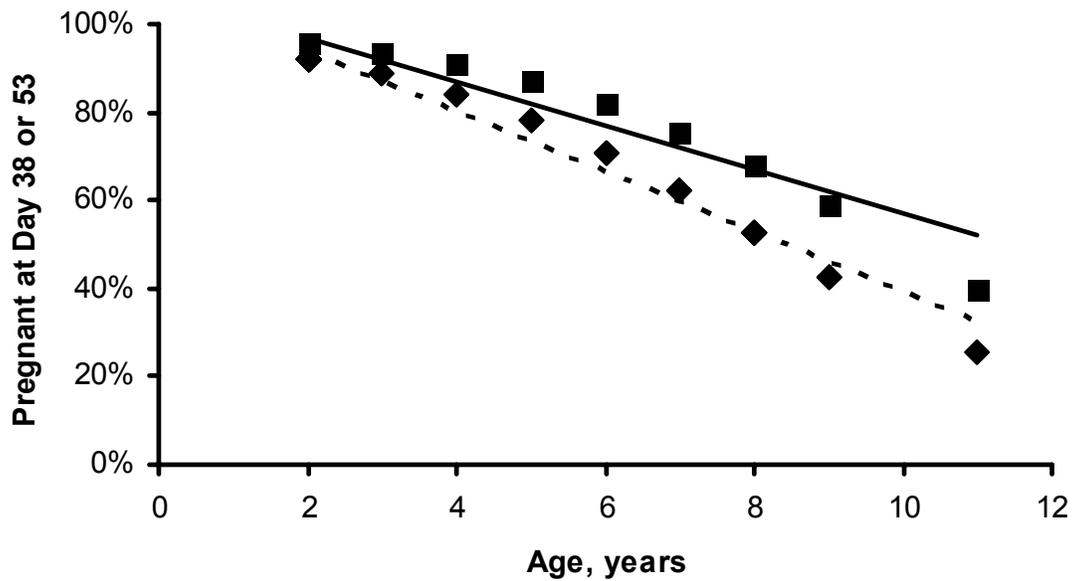


Figure 3. Predicted pattern of decrease in pregnancy retention to d 38 (■) or 53 (◆) with increasing age of cow (n = 40 and 39, respectively). The prediction equation was $P_{38}(\text{pregnant}) = 1 - (1/1 + e^{-(-3.8301 + 0.3847 \times [\text{age}]})})$ or $P_{53}(\text{pregnant}) = 1 - (1/1 + e^{-(-3.2402 + 0.3926 \times [\text{age}]})})$, respectively.

40 to 53, respectively. Thus, timing and distribution of loss of pregnancy appeared to be similar in suckled beef cows in which pregnancy was maintained with low concentrations of progesterone to patterns seen in lactating dairy cows (reviewed by Inskip, 2002 and Dailey et al., 2002). While retention of pregnancy was not associated with variation in concentrations of progesterone, the embryo and early fetus appeared to be affected by changes in maternal concentrations of progesterone before day 53, and specifically before day 38. Cows received equivalent concentrations of exogenous progesterone and the variation in peripheral concentrations of progesterone on days 30 to 33 were due to endogenous production of progesterone by CL that formed spontaneously after lutectomy in five cows. When those five cows were excluded, mean concentrations of progesterone on days 30 to 33 ranged from 0.7 to 2.5 ng/mL. If concentrations of progesterone had been more variable, an effect on retention of pregnancy might have been detected.

Under normal circumstances the incidence of late embryonic losses in beef cows ranged from 2 to 6% (reviewed by Inskip, 2004). In this study, mean concentration of progesterone was reduced by lutectomy followed by treatment with CIDRs to approximately half that reported for normal pregnant cows in the same stage of gestation (Erb et al., 1968; Donaldson et al., 1970) and loss of pregnancy was 23% compared to the approximately 2% loss in this herd from day 30 to term in the two years previous to this study. Nine of the 40 cows pregnant on day 28 lost pregnancy while on progesterone replacement. This loss might be comparable to the loss of pregnancy observed before detection of a decrease in luteal function (Kastelic et al., 1991b; Wolff, 1992).

Progesterone is essential for maintenance of pregnancy and retention of pregnancy between weeks 5 and 9 of gestation was related positively to concentrations of

progesterone during week 5 in the dairy cow (Starbuck et al., 2004). However, concentrations of progesterone during the fifth week of gestation did not affect retention of pregnancy in these suckled, beef cows, even though values were below the mean for dairy animals that experienced high rates of pregnancy loss between weeks 5 and 9 of gestation. Although concentrations of progesterone were well below the 2.8 ng/mL at which 50% of dairy cows lost pregnancy (Starbuck et al., 2004), pregnancy was maintained to day 53 in 77% of the animals. Of nine cows with mean concentrations of progesterone for days 30 to 33 below 1.0 ng/mL, eight maintained pregnancy to day 38 and seven to day 53. Thus, beef cows appear to require less progesterone for maintenance of pregnancy during placentation than dairy cows. Whether providing supplemental progesterone equivalent to normal concentrations found in pregnant cows would have restored maintenance of pregnancy to normal is not known.

Although variation in mean concentrations of progesterone during placental attachment might not be a primary cause of loss of pregnancy, supplemental progesterone provided to lactating, dairy cows for 28 days beginning on days 36 to 42 of gestation, improved retention of pregnancy to day 90 by 6.7 percentage points over control cows (Lopez-Gatius et al., 2004). Conversely, in the present study, reduction of progesterone to a mean of 1.8 ng/mL increased loss of pregnancy from the normal of 2% for this herd, to 23%.

As age of cow increased, retention of pregnancy decreased. Generally, younger females have better conception rates than older females. Santos and co-workers (2004) compiled from literature that the overall rate of pregnancy loss for lactating dairy cows averaged 10.7%, compared to only 4.2% for dairy heifers. Other researchers have

reported that lactating cows retained fewer pregnancies than heifers and that as age of the lactating cow increased, rate of pregnancy retention was either not affected (Jousan et al., 2005) or decreased (Ball, 1978; Starbuck et al., 2004; Starbuck et al., 2005). Age is confounded with many factors (mature body size, repeated pregnancies, changes to tissue of the reproductive tract with age, changes in metabolism, level of milk production, altered endocrine profiles, and other age-related changes) that make it difficult to pinpoint precise relationships that might lead to a decrease in retention of pregnancy for older females.

After lutectomy, a new CL was identified by ultrasonography on the ovary ipsilateral to the uterine horn of pregnancy in five cows. Concentrations of progesterone in these cows began to increase as early as day 33, above those recorded for the remainder of the cows, to concentrations found normally in pregnant cows. The new CL and pregnancy were maintained to day 53 in three of these cows. The lifespan of the CL from spontaneous luteinization was transient in the remaining two cows and was no longer visible by ultrasonography on day 40; one lost pregnancy and the other maintained pregnancy to day 53. The occurrence of spontaneous luteinization was unexpected and had not been seen in earlier studies in which dosages of progestogen were greater than those in our study (Bridges et al., 2000), but was reported recently to occur in cows with no CL treated with one new or used CIDR, within 48 h of CIDR insertion (Cerri et al., 2005b). Concentrations of progesterone might have been insufficient to inhibit an LH surge due either to 1) the rapid decline in concentration of progesterone following lutectomy or 2) an inadequate supply of progesterone from the CIDRs. When anestrous ewes were treated with $\text{PGF}_{2\alpha}$ on the day of insertion of medroxyprogesterone acetate

(MAP) sponges, four of six ovulated while the MAP sponge was in place (Davies et al., 2004). However, there were no apparent differences in overall changes of concentrations of progesterone following lutectomy or in the number of days with concentrations of progesterone below 1.0 ng/mL in cows that did or did not have spontaneous luteinization in the present study. Most likely, CIDRs provided inadequate concentrations of progesterone for cows with high rates of metabolic clearance of progesterone due to high rates of feed intake. Concentrations of progesterone provided by a single CIDR were adequate to maintain a first-wave dominant follicle in non-lactating dairy cows of low body condition, but not in cows of greater body condition, which ovulated the first-wave dominant follicle (Burke et al., 1998).

Concentrations of estradiol were not highly variable during days 30 to 33 or days 30 to 38 and did not affect retention of pregnancy. However, Bridges and co-workers (2000) reported that retention of pregnancy was improved in beef cows with no CL and pregnancy maintained with fluorogestone acetate (FGA), a synthetic progestin, following induction of a new CL and subsequent withdrawal of FGA support when estradiol was low and $\text{PGF}_{2\alpha}$ was high between days 31 and 35 of gestation. Working with beef cattle, Pritchard and co-workers (1994), found a negative linear association of pregnancy rate with concentrations of estradiol in peripheral blood on days 14 to 17 post-estrus. In contrast, retention of pregnancy between weeks 5 and 9 was increased in dairy cows with greater concentrations of estradiol at week 5 of gestation (Starbuck et al., 2004).

In this study, body condition score did not affect retention of pregnancy; however, few cows were in poor or excessive condition at pregnancy diagnosis. Dairy cows with greater body condition during the fifth week of gestation maintained fewer pregnancies

than cows with less condition (Starbuck et al., 2004). More often, low rates of reproductive efficiency have been attributed to low body condition, especially relative to the duration of postpartum anestrus (reviewed by Montiel and Ahuja, 2005). Cows with a one unit reduction in body condition score between breeding and pregnancy determination were 2.4 times more likely to lose pregnancy between days 38 and 90 (Lopez-Gatius et al., 2002).

In summary, lutectomized, suckled beef cows with pregnancy maintained on lower than normal concentrations of progesterone appear to be a useful model for the lactating dairy cow. Retention of pregnancy was associated with age of the cow. Concentrations of progesterone during the second and third months of gestation might not be the most important variable in loss of pregnancy, but reduced concentrations of progesterone appeared to reduce retention of pregnancy below the normal for beef cows (Zavy, 1994; Inskeep, 2004). Both age of cow and concentrations of progesterone remain good candidates to evaluate in order to further understand the problem of late embryonic and early fetal loss in dairy cattle. Additional studies are needed to determine the specific mechanisms by which age of cow and reduced concentrations of progesterone affect reproductive tissues or the conceptus that affect the likelihood of the pregnancy being maintained to term.

EXPERIMENT II:

**ABILITY OF INDUCED CORPORA LUTEA TO MAINTAIN PREGNANCY
FROM THE THIRD MONTH OF GESTATION TO TERM IN CATTLE**

Abstract

The local relationship between the pregnant uterine horn and the CL during maternal recognition of pregnancy is well-documented. It continues beyond that time; pregnancies were maintained in lutectomized cows when CL were induced on the ovary ipsilateral, but not contralateral, to the uterine horn of pregnancy during Days 28 to 53. This study evaluated factors affecting maintenance of pregnancy by CL induced after Day 53, in lutectomized cows that had received exogenous progesterone from Day 29 to 15 d after induction of a CL. Twenty-four suckled beef cows were lutectomized on Day 29 of gestation, and pregnancy was maintained with progesterone from two controlled internal drug releasing (CIDR) inserts, exchanged every 5 d. Beginning on Day 53, ovaries and viability of pregnancy were evaluated by ultrasonography every 5 d. When a follicle ≥ 10 mm in diameter was present ipsilateral to the fetus, each cow received 1,000 IU of hCG. Following induction of a CL (20 of 24), progesterone was reduced to a single CIDR for 5 d, then removed. Retention of pregnancy was confirmed by rectal palpation and calving. Cows with induced CL maintained pregnancy to term, including four with the CL contralateral to the fetus. Three cows failed to form normal CL by Day 98 and lost pregnancy after removal of exogenous progesterone. One cow that did not respond to hCG lost pregnancy during exogenous progesterone. In conclusion, CL induced after Day 53 maintained pregnancy to term, even when induced contralateral to the pregnant uterine horn.

Key words: progesterone, induced CL, cattle, pregnancy

Introduction

In cows that had no CL, pregnancy was maintained with exogenous progestogen (Tanabe, 1970; Inskip and Baker, 1985, Zimbelman and Smith, 1996). The local nature of the unilateral relationship between the conceptus and the CL during maternal recognition of pregnancy is well-documented (Thatcher et al., 1986; Bazer, 1992). Pregnancy was maintained in lutectomized cows when functional CL were induced early in the second month of gestation on the ovary ipsilateral to the horn of pregnancy (Lulai et al., 1994; Bridges et al., 2000). Retention of pregnancy was only 50% when the new CL was induced between Days 28 and 35 of gestation, but 100% when the new CL was induced after Day 35 (Bridges et al., 2000). The objective of this study was to evaluate factors affecting maintenance of pregnancy by CL induced after Day 53, in lutectomized cows that had received exogenous progesterone from Day 29 to 15 d after induction of a CL.

Materials and Methods

Twenty-four suckled, pregnant beef cows of mixed breeds and ages (3.4 ± 0.5 yr, range 2 to 9 yrs) and maintained on pasture at the West Virginia University Experiment Station, Arthur Dale, WV, were lutectomized by transrectal enucleation on Day 29 of gestation. Cows were inseminated artificially and were at least 45 d post partum on the day of artificial insemination (designated as Day 0 of gestation). Pregnancy was maintained through Day 53 with progesterone from two controlled internal drug releasing devices (CIDRs, Pfizer Animal Health, New York, NY, USA), which were inserted and exchanged every 5 d beginning on the day prior to lutectomy. That treatment maintained

progesterone at a mean concentration of 1.6 ± 0.1 ng/mL. Throughout the study, transrectal ultrasonography (Aloka 500 or 900, fitted with a 7.5 MHz rectal probe; Corometrics Medical Systems, Wallingford, CT, USA) was used to evaluate viability of the pregnancy (as determined by the appearance of a fetal heartbeat). Diameter and location of the two largest follicles ≥ 5 mm and any corpora lutea on each ovary were observed by ultrasonography at 5 d intervals after Day 53. Prior to the start of this experiment, cows had been evaluated daily by transrectal ultrasonography between Days 28 and 38 and then on Days 40, 43, 45 and 48 for retention of pregnancy.

On or after Day 53, cows were treated with human chorionic gonadotropin (hCG; 1,000 IU; i.m.; Sigma Chemical Co, St. Louis, MO, USA) to induce formation of a new CL when a follicle ≥ 10 mm was detected ipsilateral to the uterine horn of pregnancy. This amount of hCG was reported to readily induce formation of new CL in non-pregnant postpartum beef cows (Sheffel et al., 1982). The procedure was repeated until a new CL formed or until Day 98. If formation of a new CL was detected 10 d after treatment with hCG, exogenous progesterone was reduced to a single CIDR for 5 d and then reduced to none for 5 d, before a final ultrasonographic examination. On Day 98, two CIDRs were removed from each cow in which a functional new CL had not formed by that time. Retention of pregnancy was confirmed by rectal palpation approximately one month after conclusion of the study, and calving dates were recorded.

A 5-mL sample of jugular blood was collected at each ultrasonographic examination and placed immediately on ice for transportation to the laboratory. Samples were collected by an individual who did not handle CIDRs to reduce risk of contamination with progesterone. Samples were refrigerated (4°C) for 12 to 24 h before

centrifugation. Serum was harvested and stored at -20°C until radioimmunoassayed for concentrations of progesterone and estradiol-17 β as described previously in our laboratory (Sheffel et al., 1982; Rozell and Keisler, 1990; Tortonese et al., 1990). The intra- and inter-assay coefficients of variation for assays of progesterone were 10 and 8%, and for estradiol-17 β were 10 and 5%, respectively.

The effect of concentration of estradiol or progesterone on the day of first treatment with hCG on the formation of luteal tissue in response to the first treatment with hCG was examined using the LOGISTIC procedure of SAS (1997). Because numbers of animals were limited (n = 11), the effects of concentration of hormones on the day of second treatment with hCG on the formation of luteal tissue were not examined. The effects of age of cow, body condition score on Day 28 or 29 of gestation, and size of the largest follicle on the formation of luteal tissue in response to either the first or the first or second treatment(s) with hCG were examined using the LOGISTIC procedure of SAS (1997).

Results

Twelve of the 24 cows formed CL to a single treatment with hCG; the response tended to be affected positively by concentration of estradiol (P = 0.08; mean 2.0 \pm 0.2 pg/mL) but was not associated with concentration of progesterone (mean 1.3 \pm 0.2 ng/mL) at treatment. Eight of the remaining 12 cows formed luteal tissue after being treated a second time with hCG, 10 to 20 d later. Body condition score, diameter of the largest follicle at the time of treatment with hCG, and age of cow were not associated with the formation of luteal tissue. The CL formed after either the first or second

treatment with hCG were functional as shown by the increases in progesterone during withdrawal of exogenous progesterone at 5, 10, 15 and 20 d after treatment with hCG (Fig. 1). Luteal tissue formed on the ovary that was ipsilateral (n = 15) or contralateral (n = 4) to the fetus, or on both ovaries (n = 1). In these 20 cows, all CL were normal in appearance and echogenicity. Pregnancy was maintained to term in all 20 cows and length of gestation averaged 284 ± 7 d.

One cow lost pregnancy after failing to respond to one treatment with hCG and before a second treatment was given. After three (n = 2) or five (n = 1) treatments with hCG, three cows formed CL that, when viewed by ultrasonography, were less echogenic than CL that formed in the other cows. At the time of withdrawal of exogenous progesterone on Day 98, the CL were small and regressing in size in two of those three cows and the final cow had luteal tissue enclosing a fluid-filled cavity that had not reduced in size 15 d after treatment with hCG. Pregnancy was lost after withdrawal of progesterone in all three cows with abnormal CL.

Discussion

All cows that formed new functional CL during the second half of the first trimester retained pregnancy to term, as reported for cows with CL induced ipsilateral to the embryo after Day 35 and before Day 53 of gestation (Bridges et al., 2000). Additionally, concentrations of exogenous progesterone used to maintain pregnancy in ovariectomized cows from Day 29 to 53 were lower (mean of 1.56 ± 0.05 ng/mL between Days 30 and 38) than those normally found in pregnant cows (Erb et al., 1968;

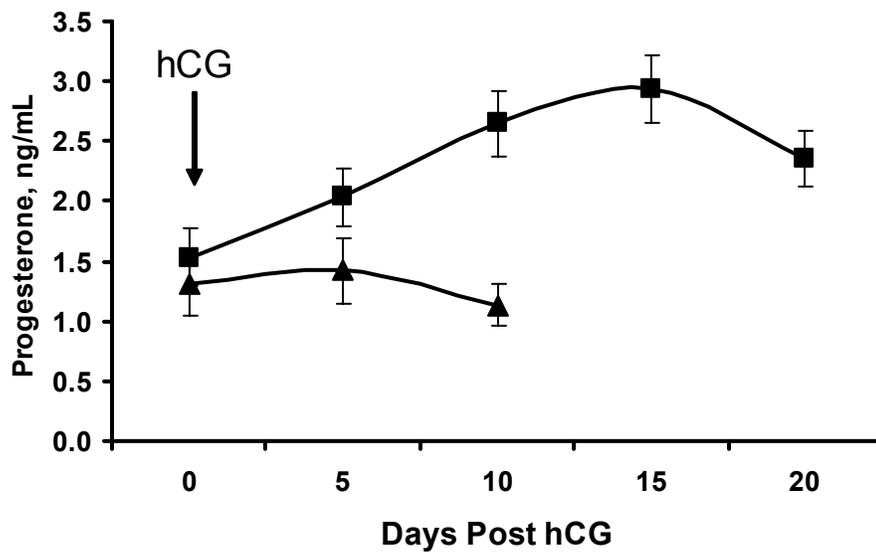


Figure 4. Concentrations of progesterone (mean \pm SEM) in lutectomized cows with pregnancy maintained by exogenous progesterone that formed a CL after either one or two treatments with hCG (\blacksquare ; n = 20), or did not form a CL in response to the first treatment (\blacktriangle ; n = 12). Cows with induced CL were treated with two CIDRs until day +10. Then supplementation was reduced to one CIDR, which was removed on day +15. The 20 cows with induced CL maintained pregnancy to term.

Donaldson et al., 1970). The local relationship between the conceptus and the CL during maternal recognition of pregnancy (Thatcher et al., 1986; Bazer, 1992) and early in the second month of gestation (Lulai et al., 1994; Bridges et al., 2000), appeared to dissipate by early in the third month. Retention of pregnancy was 0 of 5 (Bridges et al., 2000) or 1 of 5 (Lulai et al., 1994) for cows with replacement CL induced on the ovary contralateral to the gravid uterine horn before Day 53, but was 4 of 4 in cows with CL induced on Days 53 through 73. The final CIDR was removed from these four cows on either Day 68, 83, 88 or 88. When all three studies to date are considered, the ability of an induced CL to maintain pregnancy to term appears to be associated with stage of pregnancy during their formation and with location relative to the gravid uterine horn before Day 53. It apparently is not related to circulating concentrations of progesterone provided from exogenous sources before or during induction of the new CL.

While diameter of the largest follicle (mean diameter 12.8 ± 0.5 mm) on the ovary ipsilateral to the gravid horn was at least 10 mm (the minimum diameter expected to ovulate in anestrous beef cows; Smith et al., 1983) before the first treatment with hCG, only half of the cows formed a new CL in response to a single treatment with hCG. Ability to form luteal tissue tended to be associated positively with concentration of estradiol, but was not associated with other variables. In previous work, the incidence of formation of a replacement CL in response to treatment with hCG was not associated with concentrations of estradiol between Days 28 and 31 of gestation, but more cows with lower concentrations of estradiol between Days 31 and 35 formed CL in response to hCG (Bridges et al., 2000). The ovary is the primary source of estradiol during this period. Persistent follicles developed in beef heifers when concentrations of progesterone

were maintained at sub-luteal concentrations (1 to 2 ng/mL; Sirois and Fortune, 1988). Secretion of estradiol by persistent follicles is not constant and was elevated for only half the time that the persistent follicle was detectable by ultrasonography (Noble et al., 2000). Therefore, response to hCG might be associated with concentrations of estradiol only at certain stages in the lifespan of the follicle. Cows received equivalent concentrations of exogenous progesterone. Therefore, it is logical that the ability to form luteal tissue in response to hCG was not associated with concentrations of progesterone.

In summary, CL induced after Day 53 of gestation by either a first or a second treatment with hCG maintained pregnancy to term in lutectomized cows in which pregnancy was previously maintained by exogenous progesterone. Further, because pregnancy was maintained to term in cows with the new CL induced on either the ovary that was ipsilateral or contralateral to the uterine horn of pregnancy, it appears that the unilateral relationship between the CL and the conceptus may have dissipated by the third month of gestation.

EXPERIMENT III:

EFFECT OF A SINGLE GROWTH HORMONE (rbST) TREATMENT AT BREEDING ON CONCEPTION RATES AND PREGNANCY RETENTION IN DAIRY AND BEEF CATTLE¹

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Abstract

Initiation of long-term treatment with rbST (Posilac[®], Monsanto, St. Louis, MO) coincident with first insemination increased pregnancy rates in dairy cattle, but neither the efficacy of using only the initial injection, nor its effects on retention of pregnancy are known. Lactating dairy cows, dairy heifers, and lactating beef cows were assigned at random to treatment (rbST) or control. Dairy cows, dairy heifers, and beef cows received 500 mg rbST (n = 48, 35, 137 inseminations, respectively) at artificial insemination or were left untreated (n = 62, 33, 130 inseminations, respectively). Pregnancy was diagnosed by ultrasonography at 28 to 36 days. Treatment with rbST at insemination improved conception rates in dairy cows (60.4% vs. 40.3%; $P < 0.05$), but not in dairy heifers or beef cows. Conception rates did not differ in dairy cows at ≤ 100 days in milk (DIM), but were improved in cows treated with rbST after 100 DIM (64.3% vs. 25.8%; $P < 0.05$). Retention of pregnancy to ~ 60 days and sizes of CL, diameter of follicles ≥ 5 mm, and crown-rump lengths of embryos were not affected by treatment. The second objective was to examine the effects of rbST at insemination on birth weight and postnatal calf growth in beef cows. Birth and weaning weights of beef calves were not affected by treatment. In conclusion, a single treatment with rbST at insemination increased conception rates in dairy cows, specifically in those > 100 DIM.

Key words: rbST, conception rates, dairy cows, retention of pregnancy, beef cows, dairy heifers

Introduction

Reproductive efficiency in dairy cattle has declined over the last five decades. First service conception rates to artificial insemination decreased from approximately 65% in 1951 to 40% in 1996 in New York (Butler, 1998). Conception rates were approximately 55% in parous dairy cows in the 1950s (Johnson et al., 1958; Mares et al., 1961), while current rates are approximately 35%, or lower for timed insemination (Drost et al., 1999; Cartmill et al., 2001; Pancarci et al., 2002). In lactating dairy cows in which ovulation was synchronized with the Ovsynch[®] protocol (Pursley et al., 1997), initiation of long-term treatment with 500 mg recombinant bovine somatotropin (rbST) at insemination improved first service conception rates (Moriera et al., 2000a). The effect of a single treatment with rbST at insemination on conception rates has not been evaluated.

Seven to 33% of pregnancies in lactating dairy cows were lost between 28 and 98 days of gestation (reviewed by Inskoop, 2002). In contrast, only 4 to 6% of beef cows or heifers lost pregnancy during this same time period, unless there was an outbreak of disease (Dunne et al., 2000). Although the effects of rbST on the establishment of early pregnancy have been studied, its effects on retention of pregnancy during early attachment and fetal development have not been evaluated.

An improvement in conception and (or) pregnancy retention rates might result from effects of rbST on the embryo or on the reproductive tract. Luteolysis must be prevented to maintain early pregnancy. Only embryos ≥ 15 mm in length on days 15 to 17 of gestation produced measurable amounts of IFN- τ , a protein important for maternal recognition of pregnancy (Geisert et al., 1988). Because embryonic development in vitro

was stimulated when rbST was added to culture medium (Izadyar et al., 1997), treatment with rbST at insemination might increase the proportion of embryos able to signal for maternal recognition of pregnancy. Additionally, Badinga et al. (2002) reported that rbST decreased production of $\text{PGF}_{2\alpha}$ by cultured bovine endometrial cells, an effect which, if it occurred in vivo, might improve maintenance of the corpus luteum (CL) during maternal recognition of pregnancy.

Treatment with rbST increased concentrations of progesterone in cows during the estrous cycle (Schemm et al., 1990; Lucy et al., 1994; Morales-Roura et al., 2001), as well as increased day-17 luteal weights in lactating dairy cows (Lucy et al., 1995). Treatment with rbST increased the circulating concentration of IGF-I (Gallo and Block, 1990; Bilby et al., 1999), which has been shown to stimulate secretion of progesterone by the CL in vitro (Sauerwein et al., 1992). Dairy cattle with concentrations of progesterone in the lower quartile for a population of animals at week 5 of gestation had lower rates of pregnancy retention than cows with greater concentrations of progesterone (Starbuck et al., 2004). Thus, treatment with rbST might affect retention of pregnancy by increasing concentrations of progesterone during the period of attachment.

Lambs from rbST-treated ewes were heavier at birth and at days 30, 60 and 75 of age when the lambs were reared as singles, but not multiples (Costine et al., 2005). While birth weight of offspring was not affected when gilts were treated with pST early in gestation, the market-weight offspring had longer carcass sides (Kelley et al, 1995) and increased concentrations of muscle protein (Rehfeldt et al., 2001). Whether treating cows with rbST at insemination will improve birth or weaning weights of the resulting progeny has not been determined.

The primary objective of this study was to determine if a single treatment with rbST, at insemination 12 h after observation of standing estrus, affected conception and (or) pregnancy retention rates in dairy cows, dairy heifers or beef cows. The second objective was to determine whether birth weight or post-natal calf growth was affected by treatment with rbST in beef cattle.

Materials and Methods

Dairy Cows. Sixty-six lactating dairy cows (Holstein; n = 51 or Ayrshire; n = 15) milked twice daily were used. Cows were maintained in a free-stall barn with access to dry lots as weather permitted at the West Virginia University Farm in Morgantown, WV. No animals had received rbST before the study. Bi-monthly herd checks were performed to determine reproductive status of the herd. Animals with a palpable CL that were eligible for breeding or that were determined to be open from a previous insemination, and had not displayed estrus during the previous two-week period were synchronized for estrus using 25 mg PGF_{2α} (i.m.; Lutalyse, Pfizer Animal Health, New York, NY) followed 48 h later by 400 μg estradiol benzoate (i.m.; Sigma, Milwaukee, WI). A total of 110 artificial inseminations were recorded for the 66 lactating dairy cows used for this study. At each insemination, 12 h after spontaneous (n = 53 inseminations; 31 control and 22 rbST) or synchronized estrus (n = 57 inseminations; 31 control and 26 rbST), cows were assigned randomly within breed and service number, to receive either 500 mg rbST (s.c.; Posilac[®], Monsanto, St. Louis, MO; n = 48) or no treatment (n = 62). Cows averaged 75 ± 19 lbs of milk per day at insemination. A body condition score (scale 1 to

5; Wildman et al., 1982) was assigned to each animal at insemination. Characteristics for rbST-treated and untreated, control cows are shown in Table 1.

Cows were treated between March 2001, and November 2002, a time span which included the breeding periods of two lactations for some cows (n = 16). Twenty-two cows were reassigned after failing to conceive to the previous insemination or following loss of pregnancy, to receive either rbST or control during the same lactation. Regardless of first assignment, a mean of 61 ± 40 days passed between the first assignment and reassignment after failure to conceive or loss of pregnancy. The primary effect of treatment on milk production with a single dose of Posilac[®] is expected to dissipate over 14 days.

Dairy Heifers. Sixty-eight first service dairy heifers of breeding age and weight from three farms (Mark Teets, Eglon, WV, n = 31; Wade Harsh, Eglon, WV, n = 23; and West Virginia University, Morgantown, WV, n = 14) were assigned randomly to receive either 500 mg rbST (n = 35) or no treatment (n = 33) at insemination. Heifers were either maintained in a free stall barn (WVU) or on pasture with access to a barn (private producers). Estrus was synchronized in 18 control and 18 rbST-treated heifers using the same treatment as in dairy cows. This study was completed on two farms between September and December 2001 and on the third farm from March 2001 to November 2002. Artificial insemination was performed by a single experienced technician at each of the three locations.

Beef Cows. In May to July, 2001 and 2002, 267 lactating beef cows of mixed breeding that were at least 45 days postpartum were studied at first service on three farms owned by West Virginia University (n = 107, 57 and 103, respectively). Cows were

assigned randomly within age classification (2, 3 to 5 and ≥ 6 yrs), and body condition score (≤ 5 , 6, or ≥ 7 ; scale 1 to 9; Whitman, 1975) within farm, to receive either 500 mg rbST (n = 137) or no treatment (n = 130) at artificial insemination, 12 hours after detection of either spontaneous (n = 76; 36 rbST, 40 control) or synchronized (n = 191; 101 rbST, 90 control) estrus. Cows were observed twice daily for signs of estrus for ten days. On day 5, any cow that had not displayed spontaneous estrus was treated to synchronize estrus, with the same procedure used in dairy cows.

Data Collected. Pregnancy was diagnosed by transrectal ultrasonography (Aloka 500, Corometrics Medical Systems, Wallingford, CT, fitted with a 7.5 MHz probe) at 28 to 36 days post breeding in those animals that did not return to estrus. Area ($\frac{1}{2}$ width x $\frac{1}{2}$ height x π [minus the area of any cavity within the CL]) of any CL, diameters of follicles ≥ 5 mm and crown-rump length of the embryo in pregnant cows were recorded as potential indicators of later loss of pregnancy between initial diagnosis of pregnancy and re-evaluation. A 5-mL venous blood sample was collected from the pregnant animals at pregnancy diagnosis for determination of concentrations of progesterone (Sheffel et al., 1982) and estradiol (dairy only; Rozell and Keisler, 1990; Tortonese et al., 1990). Dairy animals were re-examined by rectal palpation or ultrasonography beyond 45 days, and beef cows were re-examined by ultrasonography beyond 60 days to evaluate retention of pregnancy. Days in milk (DIM) at each insemination were recorded for dairy cows. Birth and weaning weights were recorded for the progeny of the beef cows. Adjusted weaning weights were calculated by the equation ($([\text{weaning weight} - \text{birth weight}] / \text{weaning age}) \times 205 + \text{birth weight} + \text{age of dam and gender of calf adjustment}$), using the

adjustment factors published in the guidelines of the Beef Improvement Federation (2002).

Statistical Analyses. The main effects of treatment, age (2, 3 to 5 and ≥ 6 years of age for dairy and beef cows), body condition (≤ 2.75 and ≥ 3.00 for dairy cows and heifers or ≤ 5 , 6 and ≥ 7 for beef cows), service during which treatment assignment was made (1 or ≥ 2 , dairy cows only) and DIM (≤ 100 or > 100 days) and their interactions (where indicated) on conception rates were determined using the LOGISTIC procedure of SAS (1997), with nonsignificant ($P > 0.05$) effects and interactions removed by backward selection. Because estrous synchronization does not affect conception rates or retention of pregnancy (Starbuck et al., 2004; Chebel et al., 2004), whether or not animals had been synchronized was not included in the model statement. Lactating, dairy cows were divided into two groups based on DIM (≤ 100 or > 100 DIM). The desire was to group the animals into normal breeders and problem-breeders, described loosely as those inseminated after reaching peak milk production. The least-squares analysis of variance of the general linear model (GLM) procedure of SAS (1997) was used to look for the independent effects of treatment on area of the CL, diameter of the largest follicle ≥ 5 mm, crown/rump length of the embryo, concentrations of progesterone or estradiol at pregnancy diagnosis, and birth and weaning weights.

Table 1. Mean values for service number, age, days in milk (DIM) and body condition score (BCS; scale 1 to 5) for dairy cows either treated with rbST (n = 48) or left untreated (n = 62) at insemination ($P > 0.05$ for all traits; mean \pm SEM).

Treatment	Service #	Age (yr)	DIM	BCS
Control	1.6 \pm 0.8	3.7 \pm 1.8	120 \pm 65	2.9 \pm 0.4
rbST	1.5 \pm 0.8	3.8 \pm 1.9	114 \pm 57	2.9 \pm 0.4

Results

Dairy Cows. Overall, treatment with rbST improved conception rates (60.4% vs. 40.3%; $P < 0.05$), but did not affect retention of pregnancy (87.0%). The ability of treatment with rbST to affect conception rates varied with stage of lactation ($P < 0.05$; Figure 5). Conception rates in treated and untreated groups were similar in cows ≤ 100 DIM, but were improved when cows were treated at > 100 DIM. Furthermore, there was a tendency for an interaction of stage of lactation and age of the cow, independent of treatment (Figure 6; $P = 0.07$). Conception rates declined as stage of lactation increased in cows aged 2 years and ≥ 6 years, but improved as stage of lactation increased in cows aged 3 to 5 years. Body condition score at rbST treatment did not affect conception rates. Treatment with rbST did not affect diameter of the largest follicle (9.3 ± 4.1 mm) or concentrations of estradiol (1.2 ± 0.3 pg/mL) at initial pregnancy diagnosis. Likewise, rbST treatment did not alter size of the CL (443.2 ± 102.8 mm²), concentration of progesterone (2.4 ± 1.0 ng/mL) or crown-rump length of the embryo (12.0 ± 3.1 mm) at initial pregnancy diagnosis.

Dairy Heifers. Treatment with rbST did not increase conception rates (55.9%) or rates of pregnancy retention (94.7%). Treatment with rbST did not affect size of the CL (351.9 ± 78.8 mm²), concentrations of progesterone (3.0 ± 1.0 ng/mL) or crown-rump length of the embryo (13.1 ± 4.6 mm). There was a tendency ($P = 0.06$) for estradiol to be lower at pregnancy diagnosis in treated heifers than in controls (1.1 ± 0.35 vs. 1.4 ± 0.55 pg/mL), but diameter of the largest follicle (9.8 ± 2.5 mm) at that time was not affected.

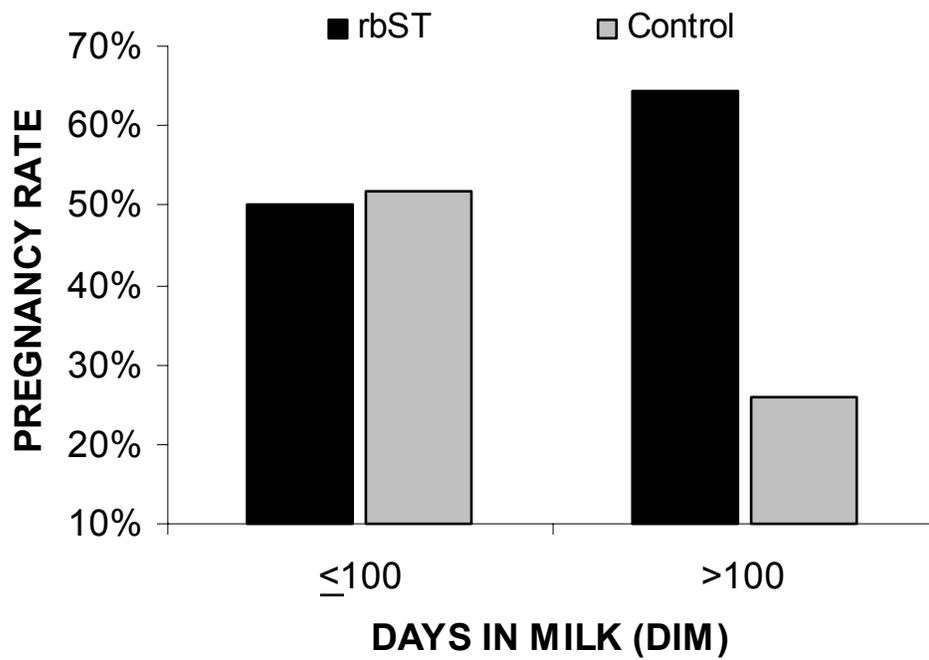


Figure 5. Conception rates in rbST treated and untreated groups were similar in cows \leq 100 DIM, but were improved when cows were treated at $>$ 100 DIM ($P < 0.05$; $n = 22, 29, 28$ and 31 , respectively).

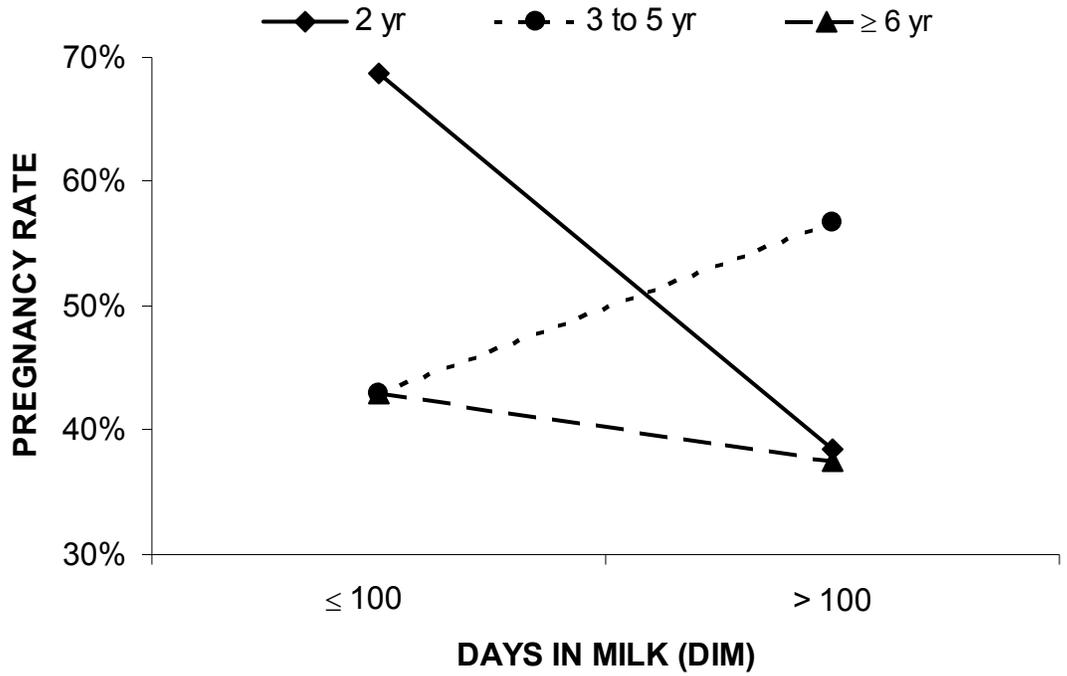


Figure 6. Effect of age on conception rate at two stages of lactation ($P = 0.07$; $n = 16, 28$ and 7 and $13, 30$ and 16 for $2, 3$ to 5 and ≥ 6 yrs, at ≤ 100 DIM and > 100 DIM, respectively).

Beef Cows. Treatment with rbST had no effect on conception rates (61.4%) or pregnancy retention (97.6%). Body condition score or age at treatment did not affect conception rates. Size of the CL ($415.0 \pm 104.0 \text{ mm}^2$), concentration of progesterone at diagnosis of pregnancy ($3.7 \pm 1.8 \text{ ng/mL}$), diameter of the largest follicle ($9.1 \pm 2.2 \text{ mm}$), crown-rump length of the embryo ($15.4 \pm 6.5 \text{ mm}$), birth weight ($87.8 \pm 10.8 \text{ lbs.}$) and adjusted weaning weight ($576.7 \pm 59.2 \text{ lbs.}$) were not affected by treatment.

Table 2 displays the means of the main variables evaluated for dairy cows, dairy heifers and beef cows. Generally, conception rates and retention rates were lowest in dairy cows and highest in beef cows, with intermediate values for dairy heifers. Crown-rump length of the embryo and concentration of progesterone were greater in dairy heifers than in dairy cows, while being greater still in beef cows.

Table 2. Conception rates, pregnancy retention rates and characteristics of embryonic development, ovarian follicles and concentrations of steroid hormones in dairy cows, dairy heifers and beef cows (mean \pm SEM).

Characteristic	DAIRY COWS		DAIRY HEIFERS		BEEF COWS	
	Value	n	Value	n	Value	n
Conception Rate (%)	49.1%	110	55.9%	68	61.4%	267
Retention Rate (%)	87.0%	57	94.7%	38	97.6%	164
Size of CL (mm^2)	443 ± 103	47	352 ± 79	35	415 ± 104	147
Largest Follicle (mm)	9.3 ± 4.1	47	9.8 ± 2.5	35	9.1 ± 2.2	147
Crown Rump (mm)	12.0 ± 3.1	43	13.1 ± 4.6	35	15.4 ± 6.5	137
Progesterone (ng/mL)	2.4 ± 1.0	44	3.0 ± 1.0	30	3.7 ± 1.8	146
Estradiol (pg/mL)	1.2 ± 0.3	44	1.2 ± 0.5	32	NA	

Discussion

A single treatment with rbST at insemination improved fertility in dairy cows, but not in dairy heifers or beef cows. Conception rates did not improve when treatment with rbST was given at ≤ 100 DIM, a time when pregnancy rates were increased by rbST in previous studies (Moreira et al., 2000a; Moreira et al., 2000b). The majority of animals used on those studies were high-producing dairy cattle from Florida, an area known to have heat-stress-related declines in reproduction. Unlike those animals, the cows on this study did not experience prolonged heat-stress. At > 100 DIM, the cows were beyond peak lactation and in a state of positive energy balance, factors that might have contributed to the improved conception rates in response to treatment with rbST. Cows open or not cycling regularly beyond 100 DIM and dairy cows under heat-stress represent two populations of problem-breeder cows. Under these conditions, conception rates and pregnancy rates have been improved by the addition of rbST at or near insemination. Whereas, when fertility is generally high (as in both first-service dairy heifers and beef cows), conception rates might not be influenced strongly by treatment with rbST at insemination. While some producers might not be willing to initiate long-term treatment with rbST due to costs or perceived negative effects of extended use, those individuals might be willing to use a single treatment to increase conception rates in problem cows.

Loss of pregnancy between days 30 and 60 of gestation is an area of heightened concern, particularly to dairy cattle producers (Dailey et al., 2002). As stated earlier, loss rates of 7 to 33% have been reported recently for lactating dairy cattle (Smith and Stevenson, 1995; Warnick, 1999; Inskeep, 2002; Silke et al., 2002; Nation et al., 2003). Present results affirm that beef cows and dairy heifers had greater rates of pregnancy

retention between approximately days 30 and 60 than dairy cows (97%, 94% and 87%, respectively). Concentration of progesterone at week 5 of gestation affected retention of pregnancy in dairy cattle; cows with concentrations of progesterone in the low quartile for a population of animals had lower rates of pregnancy retention than cows with greater concentrations of progesterone (Starbuck et al., 2004). While other researchers reported rbST-induced increases in concentrations of progesterone during the subsequent cycle (Schemm et al., 1990; Lucy et al., 1994; Morales-Roura et al., 2001) and increases in day-17 luteal weights (Lucy et al., 1995), in this study neither concentration of progesterone nor size of the CL was affected at week 5 post-treatment with rbST in any of the three types of animals.

Likewise, concentrations of estradiol during week 5 of gestation might affect retention of pregnancy. In beef cows with pregnancy maintained by exogenous progestogen and in which a new CL had been induced, more pregnancies were retained when concentrations of estradiol were lower at 31 to 35 days of gestation (Bridges et al., 2002). Pritchard et al. (1994) found a linear negative association of pregnancy with estradiol on days 14 to 17 post estrus in beef cows. In contrast, retention of pregnancy between weeks 5 and 9 was increased in dairy cows with greater concentrations of estradiol at week 5 of gestation (Starbuck et al., 2004). Because concentrations of estradiol in rbST-treated and untreated dairy animals did not differ, and conception and retention rates did not differ with treatment in beef cows, the concentration of estradiol was not determined for samples available from beef cows.

The improvement in conception rates with rbST treatment was most likely due to increased concentrations of progesterone during the post-insemination period, decreased

production of PGF_{2α} during maternal recognition of pregnancy (Badinga et al., 2002), and (or) enhanced early embryonic development. However, the stimulatory effects of treatment with rbST that resulted in increased conception rates did not improve retention of pregnancy. Retention of pregnancy may be associated with characteristics of the cow or factors within the embryo or early fetus. McMillan (1998) evaluated previous studies involving embryo transfer of two embryos to a single recipient by a statistical model to predict embryo survival and reported that maternal and embryonic factors contributed approximately equally to embryonic survival or loss.

Administration of rbST can stimulate embryonic development directly (Moriera et al., 2000b) or indirectly *in vitro* through IGF-I (Palma et al., 1997). Bilby et al. (1999) reported that when a single treatment of rbST was given at insemination, concentrations of IGF-I were increased between days 6 and 14, a critical period for bovine embryonic elongation. Despite these aforementioned reports of stimulated embryonic development at earlier stages of development, in this study neither increases in embryonic size at approximately 30 days in any type of animal nor increases in birth or weaning weights of the resulting offspring of the beef cows were observed. Lucy et al. (1995) reported no difference in day-17 conceptus length following rbST treatment from day 0 through 16 of gestation. Treatment of gilts with pST between days 28 and 40 of gestation increased fetal survival and crown-rump length of the neonate without increasing fetal or birth weights, while the market-weight offspring had longer carcass sides (Kelley et al., 1995). Rehfeldt et al. (2001) found increased concentrations of muscle protein in market-weight animals following treatment of gilts with pST from day 10 to 27 of gestation.

In the present study, there was an interaction between treatment and DIM (Figure 5) and a tendency for an interaction between age and DIM affecting conception rates (Figure 6). Chebel et al. (2004) reported that the conception rates to first-service was greater in primiparous than multiparous cows and that multiparous cows were 13% less likely to conceive during a lactation than primiparous cows. In a study similar to this present one, Morales-Roura et al. (2001) evaluated the effect of two treatments with rbST, given at estrus and repeated 10 days later, on conception rates in repeat-breeder dairy cows that were approximately 220 DIM. They reported that conception rates were higher in rbST-treated cows than untreated cows, specifically in cows in second to fourth lactation, but not in cows in first, or in fifth or greater lactation. Therefore, it is suggested that cows aged 3 to 5 years appear to remain more responsive to rbST treatment later in lactation than primiparous or cows ≥ 6 years. The practice of breeding later in lactation for the purpose of extending duration of lactation would appear feasible in cows of this age group and conception rates in this group could be improved by treatment with rbST.

Interestingly, beef cows had greater concentrations of progesterone, smaller diameters of the largest follicle and greater crown rump lengths of the embryo at 28 to 36 days of pregnancy than dairy cows; factors which are expected to contribute to increased retention of pregnancy. While beef cows had smaller CL than dairy cows, previous work reported that size of the CL at time of embryo transfer did not influence pregnancy rates in recipient cows (Spell et al., 2001). While size of the largest follicle on either ovary during week five of pregnancy might not directly affect retention of pregnancy, the presence of an estrogenic follicle at the time of maternal recognition of pregnancy is

thought to reduce CL maintenance by altering the uterine environment, potentially enhancing secretion of $\text{PGF}_{2\alpha}$ (Robinson et al, 2001).

Conclusions

Based on the results of this study, treatment of a portion of the overall dairy cow population with a single dose of rbST at insemination would be beneficial. In conclusion, a single treatment with rbST at insemination enhanced conception rates in lactating dairy cows, specifically those > 100 DIM, but did not increase conception rates to first service in either dairy heifers or suckled beef cows.

EXPERIMENT IV:

**ASSOCIATION OF FERTILITY WITH NUMBERS OF ANTRAL FOLLICLES
THAT DEVELOP WITHIN A FOLLICULAR WAVE DURING THE ESTROUS
CYCLE IN CATTLE**

Abstract

The relationship of conception rate at first service to numbers of follicles developed during a follicular wave was examined in 102 suckled, beef cows and 14 heifers. Follicular development was monitored using ultrasonography for either two (Trial 1) or three (Trial 2) consecutive estrous cycles (pre-breeding, breeding, and post-breeding equivalent). Animals were examined on alternate days from day 6 after first estrus (day 0) until ovulation and from day 6 after insemination until next ovulation or day 24 of pregnancy and were observed for estrus twice daily and inseminated artificially at either the second (Trial 1) or third estrus (Trial 2). Cows were classified as having two (2W) or three (3W) waves of follicular development for each estrous cycle. Numbers of follicles ≥ 4 mm per wave were determined, and based on the maximum diameter they attained, were classified as small (4 to 6 mm), medium (7 to 10 mm) or large (≥ 11 mm) follicles. Total numbers of follicles, and primarily numbers of small and medium follicles, were affected by trial and within trial by cow, estrous cycle and follicular wave. Heifers had more small and total numbers of follicles, but fewer large follicles than cows in 1995 ($P < 0.05$). The average number of antral follicles per wave in the breeding cycle or post-breeding period did not affect conception rates, which averaged 84%. Repeatability of the total numbers of antral follicles between and among estrous cycles and follicular waves ranged from 0.01 to 0.97. In conclusion, fertility was not affected by the numbers of antral follicles ≥ 4 mm in diameter in a single follicular wave.

Keywords: follicle, follicular wave, fertility, cattle,

Introduction

Growth and atresia of ovarian follicles occurs in a wave-like manner throughout the bovine estrous cycle (Rajakoski, 1960), including the period after breeding (Ginther et al., 1989b). A follicular wave begins with the emergence of a cohort of follicles ≥ 4 mm in diameter (Knopf et al., 1989) and is often characterized by development of one large follicle and regression, or atresia, of the subordinate follicles (Ginther et al., 1989a). The largest follicle is anovulatory during the luteal phase and ovulatory during the follicular phase, once the corpus luteum has regressed. The occurrence of two (2W) or three (3W) follicular waves within a single estrous cycle is most common (Ginther et al., 1989a; Knopf et al., 1989; Fortune, 1993; Ahmad et al., 1997). Conception rate was greater in cows with three rather than two follicular waves during the cycle immediately before breeding in dairy cows (Townson et al., 2002), or the equivalent of a cycle immediately after breeding in beef cows (Ahmad et al., 1997), while others have shown no effect (Bleach et al., 2004; Celik et al., 2005).

The number of follicles that developed within a follicular wave was variable among dairy cows, but highly repeatable (0.95) within cow (Burns et al., 2005). Additionally, Singh et al. (2004) reported for beef cows high values (0.77 and 0.71) for the correlation of numbers of follicles ≥ 2 mm in diameter in successive waves within cow before and during superstimulation. The response to superstimulation was related to the number of follicles per follicular wave and could be predicted by a single ultrasound examination at wave emergence (Singh et al., 2004). Additionally, the response to superstimulation varied with age of the cow and was either positively or negatively affected by increasing concentrations of follicle stimulating hormone (FSH) depending

upon breed of the cow (Lerner et al., 1986; Breuel et al., 1991). Increasing maternal age was associated with decreased fertility (Erickson et al., 1976). Thirteen- and 14-year-old cows had fewer 4- to 5-mm follicles recruited into a wave than their one-to four-year-old daughters, despite having greater circulating concentrations of follicle stimulating hormone (FSH; Malhi et al. 2005). Similarly, concentrations of FSH were greater in dairy cows that averaged fewer than 15 antral follicles per wave than those that produced greater numbers of follicles per wave (21 to 25; Burns et al., 2005). Thus, a relationship might exist between fertility and the number of antral follicles that develop within a wave.

The objectives of this study were to 1) determine if there is a direct association between fertility of the oocyte ovulated from the dominant follicle of the wave immediately prior to insemination and numbers of antral follicles ≥ 4 mm in diameter that develop within a wave, and 2) evaluate the repeatability of total numbers of antral follicles ≥ 4 mm in diameter that develop during consecutive estrous cycles, within waves of consecutive estrous cycles, and within cows between estrous cycles two years apart in beef cattle.

Materials and Methods

Data available for this study were collected in two earlier studies in beef cattle; the first was completed between April and July, 1995 (Ahmad et al., 1997) and the second between April and August, 1997 (H. Hernandez-Fonseca, unpublished), hereafter designated as Trial 1 and Trial 2, respectively. Ahmad et al. (1997) observed a trend for greater conception rate in cows with three follicular waves in the breeding estrous cycle

and a significantly greater conception rate in cows with three waves during the equivalent period after insemination. In addition, a greater proportion of the cows had a three wave pattern of follicular development in the period after insemination than during the breeding estrous cycle. The primary objective of the study by Hernandez-Fonseca was to confirm whether the occurrence of two or three waves of ovarian follicular development during the estrous cycle before and after the equivalent of one breeding cycle affected fertility and the secondary objective was to evaluate changes in the number of follicular waves from one estrous cycle to another before breeding and from the cycle before breeding to the equivalent period after breeding. However, the number of cows with three follicular waves was insufficient to allow a test of either objective. Because the original studies were focused on wave patterns rather than the numbers of follicles, the recording of smaller follicles was not as precise as in the work by Singh et al. (2004) or Burns et al. (2005), in which the goal was to examine numbers of follicles and thus, all follicles ≥ 2 mm or ≥ 3 mm, respectively, were recorded. However, these data afforded an opportunity to examine whether fertility was related to the numbers of follicles ≥ 4 mm during a follicular wave in cattle, which was not evaluated in the aforementioned studies.

Animals, detection of estrus and breeding. Beef cows and heifers of mixed breeding (primarily Angus, Hereford, and Simmental crosses from the herd at Reymann Memorial Farm, Wardensville, WV) were utilized. Data were available from 38 cows and 14 heifers from Trial 1, and 64 cows from Trial 2. Eight cows studied in Trial 1 were utilized again during Trial 2. Similar methods were followed for both years. Animals were observed for estrus twice daily for 30 min each time. Twelve hours after detection

of a second or third estrus (see description below) each animal was inseminated artificially with frozen-thawed semen, from one of three bulls of known high fertility.

Ovarian ultrasonography. Ovaries in each animal were evaluated by transrectal ultrasonography (Aloka 500 fitted with a 7.5 MHz probe; Corometrics Medical Systems, Wallingford, CT). The locations of the corpus luteum and each antral follicle ≥ 4 mm in diameter on each ovary were drawn on an ovarian map and the diameters of these structures were recorded. Ultrasonographic examinations were completed by a single technician during Trial 1 and by four technicians during Trial 2. For Trial 1, ovaries were examined from day 6 after first estrus until second estrus (breeding cycle) and then daily until apparent ovulation (disappearance of the largest follicle). Ultrasonography recommenced on day 6 after second estrus and continued on alternate days until third estrus or until day 24 of pregnancy. Because the dominant follicle of the final wave did not ovulate in pregnant cows, animals were followed for the equivalent of one cycle after insemination, hereafter designated the post-breeding cycle. For Trial 2, examinations by ultrasonography were completed in an identical manner, except that two estrous cycles per cow were evaluated before breeding (designated pre-breeding and breeding cycles).

Day 6 was utilized as the first day of observation in each estrous cycle because no animal would be expected to complete the first wave of follicular development before day 8 (Sirois and Fortune, 1988; Knopf et al., 1989; Ginther et al., 1989c). Thus, estimates of number of follicles in the first wave would not include follicles that became atretic before day 6 and either did not reach or had regressed below 4 mm. Each animal not detected in estrus after insemination was examined by transrectal ultrasonography on approximately day 25 and again 10 days later to confirm pregnancy status.

Follicular waves. The day of first detection of a 4- or 5-mm follicle that subsequently grew to at least 10 mm was taken as the first day of a second or third wave (day of emergence). Each estrous cycle (pre-breeding, breeding or post-breeding) was classified as having either two or three follicular waves, according to whether the second or third sequentially largest follicle became the ovulatory follicle. Pregnant cows were considered to have three waves if the third consecutive follicle arose on (or before) day 16, because that was the average day of detection of the third wave follicle in cycles before breeding (Ahmad et al., 1997). Additional information regarding animals, ultrasonography, and numbers of waves was described by Ahmad et al. (1997). Conception rates were 74% (n = 27) and 100% (n = 25) for Trial 1, and 84% (n = 57) and 100% (n = 7) during Trial 2 for cows with two or three waves during the post-breeding period, respectively.

Numbers of follicles per wave. Numbers of follicles ≥ 4 mm per wave were determined, and based on the maximum diameter they attained, were classified by diameter as small (4 to 6 mm), medium (7 to 10 mm) or large (≥ 11 mm) according to Pierson and Ginther (1984). Numbers of small, medium and large follicles from each wave were combined to generate the total number of follicles per cycle, or cycle equivalent in the post-breeding period in pregnant cows. The total number of follicles per cycle was divided by the appropriate number of waves (either 2 or 3) in the breeding and post-breeding cycles to generate the average number of follicles per wave for each cycle.

Statistical analyses. Factors affecting the numbers of small, medium, large or total follicles were evaluated using the GLM procedure of SAS (1997). Trial, cow,

estrous cycle, follicular wave, and the interaction between estrous cycle and follicular wave were included in the model. There was a significant difference in the total number of follicles recorded between the two trials that was attributed to differences in the accuracy of recording small follicles, particularly those approximately 4 mm in diameter. Thus, it was necessary to evaluate data from each trial independently. When the wave effect was significant, contrasts were performed to determine if the relationship between numbers of follicles of each class size and wave could be described by a line with linear or quadratic properties. To evaluate potential differences between cows and heifers during Trial 1 in the total numbers of follicles of small, medium, large or all sizes combined, age of cow and the interactions between age and wave and age and cycle were included in the model.

The effects on conception rates of the average number of antral follicles ≥ 4 mm in diameter per wave in both the breeding and post-breeding cycles and the total number of antral follicles ≥ 4 mm in diameter in the ovulatory wave of the breeding cycle and the second wave of the post-breeding cycle were determined using the LOGISTIC procedure of SAS. Pritchard and co-workers (1994) found a negative linear association of pregnancy rate with concentrations of estradiol in peripheral blood on days 14 to 17 post estrus. This timing is coincident with the second follicular wave and the start of the third follicular wave (if it occurred). Thus, the second wave of the post-breeding cycle was examined because the duration of dominance of the largest follicle would affect concentrations of estradiol (Ireland et al., 1984).

Repeatability, defined as the proportion of the total variance that could be attributed to animal variance, was calculated using the variance components estimated by

the MIXED procedure of SAS. Repeatability of the total number of follicles ≥ 4 mm in diameter per wave was examined between or among consecutive estrous cycles, between or among consecutive follicular waves within an estrous cycle and between or among corresponding follicular waves in consecutive estrous cycles. Repeatability from Trial 1 to Trial 2 of the total number of follicles in the breeding and post-breeding estrous cycles and between follicular waves within those two estrous cycles per cow over time was evaluated in eight cows that were examined during both Trials 1 and 2.

Results

The numbers of small, medium, and total follicles during both years and the numbers of large follicles during Trial 2, but not Trial 1, varied among cows ($P = 0.01$). During Trial 2, total numbers of follicles varied among cycles ($P = 0.01$) due to the tendency of cycle to affect the numbers of small and medium follicles ($P = 0.07$ for each; Figure 7A). Total numbers of follicles per estrous cycle appeared to decline slightly from the pre-breeding cycle to the breeding cycle and the post-breeding period. Wave affected the numbers of small and total follicles during both years ($P < 0.01$) and medium follicles in Trial 1 ($P = 0.03$; Figure 7B). The relationships among total numbers of follicles in consecutive waves during each trial, and among numbers of small follicles in consecutive waves during Trial 1, could be described by a quadratic pattern ($P < 0.05$). In each case, the number of follicles in wave 2 was greater than the average numbers of follicles from waves 1 and 3 combined.

Heifers had more small and total antral follicles, but fewer large follicles than cows (Table 3). The range for the total number of antral follicles recorded per wave was

2 to 25 (n = 66 waves) and 1 to 28 (n = 171 waves) for heifers and cows, respectively. The interaction between wave and age affected the numbers of small and total follicles ($P < 0.05$). In general, more small follicles were recorded for each wave in heifers than cows; more specifically in wave 3, the number of small follicles averaged 13.3 ± 2.3 and 7.4 ± 0.9 , for heifers (n = 14) and cows (n = 38), respectively.

Conception rates (means were 87% and 86% for Trials 1 and 2, respectively) were not affected by the average number of antral follicles ≥ 4 mm in diameter per wave during either the breeding or post-breeding cycles or by the total number of antral follicles ≥ 4 mm in diameter in the ovulatory wave of the breeding cycle or in the second wave of the post-breeding cycle. The average numbers per wave of total follicles in the breeding and post-breeding cycles and in the ovulatory wave of the breeding cycle and second wave of the post-breeding cycle for Trials 1 and 2 are reported in Table 4.

Estimates of repeatability of the total number of antral follicles ≥ 4 mm in diameter between and among estrous cycles and follicular waves are shown in Table 5. In general, repeatability among waves and cycles was similar in Trial 1, when more small follicles were recorded, but lower in Trial 2 and lower among waves than among cycles in Trial 2. The highest repeatability was for total numbers of follicles from the third wave of the breeding cycle to the third wave of the post-breeding period in Trial 1 (0.97). The second highest repeatability was for wave 1 to wave 3 within cycles in Trial 1 (0.57), the average per wave from the breeding to the post-breeding cycle in Trial 1 (0.56), and the total number of follicles per ovulatory wave between the breeding cycle and post-breeding period (0.56). A greater value (0.44) was recorded for repeatability over all three waves within estrous cycles in Trial 1 and for wave 2 among estrous cycles in

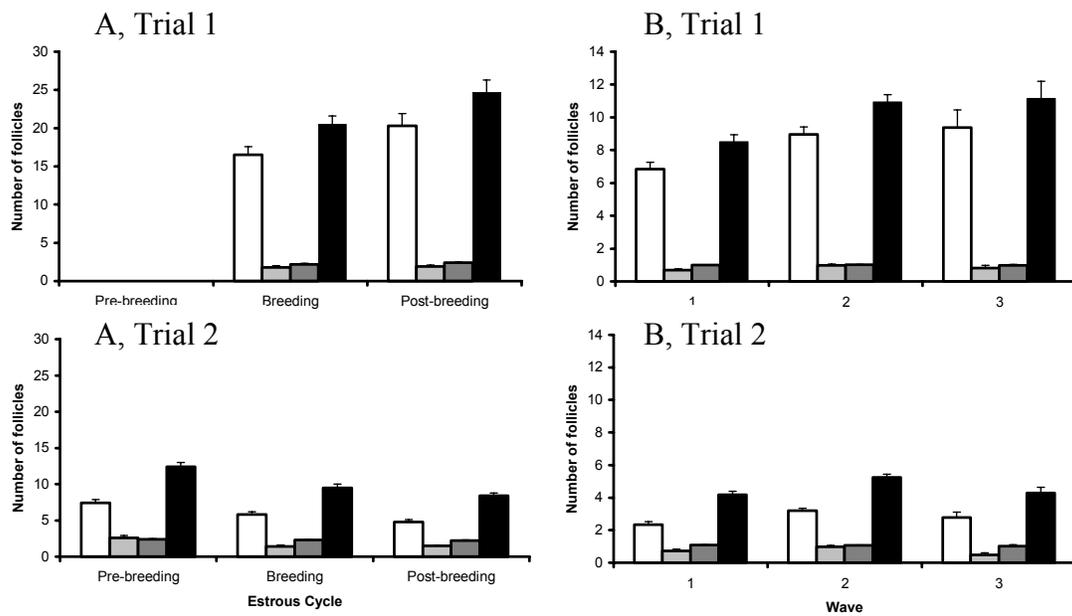


Figure 7. Numbers of small, medium, large, or total follicles (designated by white, light gray, dark gray, and black columns, respectively) during the pre-breeding, breeding, or post-breeding estrous cycle (A) and within follicular waves (B) in beef cows during two years (Mean \pm SEM). Ultrasonographic examinations began on day 6 of each estrous cycle. The total number of antral follicles differed with estrous cycle in Trial 2 ($P = 0.01$) and the numbers of small and total follicles in both Trials 1 and 2 and the numbers of medium follicles in Trial 1, differed with follicular wave ($P = 0.01$).

Table 3. Mean numbers of small, medium and large follicles per wave in beef heifers and cows during Trial 1^a.

	n	Small	Medium	Large	Total
Heifers	14	9.6 ^x	0.7	0.9 ^x	11.3 ^x
Cows	38	7.5 ^z	0.9	1.0 ^y	9.4 ^y

^a Overall SEM = 0.29

^{x,y} values with different subscripts differ by $P < 0.01$.

^{x,z} values with different subscripts differ by $P < 0.001$.

Table 4. Average numbers per wave and ranges in numbers of total follicles in the breeding and post-breeding cycles and in the ovulatory wave of the breeding cycle and second wave of the post-breeding cycle^a.

	Cycle				Wave		
	Breeding		Post-breeding		Ovulatory	2nd Post-breeding	
	Mean	Range	Mean	Range	Mean	Mean	Range
Year I	9.9	9 to 48	10.0	8 to 61	11.1	10.5	3 to 21
Year II	4.4	4 to 19	4.0	2 to 17	4.9	4.9	1 to 14

^a Overall SEM = 0.12

Table 5. Repeatability of total number of follicles ≥ 4 mm in diameter from one follicular wave to another or one estrous cycle to another in each of two years or between two non-consecutive years (n = 52, 64, and 8, respectively).

	Trial 1	Trial 2	Trial 1 to Trial 2
Waves Within Cycles			
1 to 2	0.50	0.14	
2 to 3	0.29	0.13	
1 to 3	0.57	0.05	
1 to 2 to 3	0.44	0.14	
Cycle			
Breeding to Post-Breeding	0.56	0.24	
Pre-breeding to Breeding		0.19	
Pre- to Post-breeding		0.25	
Pre- to breeding to Post-breeding		0.22	
Wave 1 Among Cycles			
Breeding to Post-Breeding	0.07	0.19	
Pre-breeding to Breeding		0.09	
Pre- to Post-breeding		0.02	
Pre- to breeding to Post-breeding		0.08	
Wave 2 Among Cycles			
Breeding to Post-Breeding	0.44	0.33	
Pre-breeding to Breeding		0.01	
Pre- to Post-breeding		0.22	
Pre- to breeding to Post-breeding		0.18	
Wave 3 Among Cycles			
Breeding to Post-Breeding	0.97		
Pre-breeding to Breeding		0.18	
Pre- to Post-breeding			
Pre- to breeding to Post-breeding			
Ovulatory Wave			
Breeding to Post-Breeding	0.56	0.17	
Pre-breeding to Breeding		0.14	
Pre- to Post-breeding		0.07	
Pre- to breeding to Post-breeding		0.16	
Cycle			
Post-breeding			0.28
Wave			
1			0.47

Trial 1. The latter comparison, wave 2 among cycles, had the greatest repeatability (0.33) in Trial 2. For estrous cycles two years apart, the total number of follicles per cycle and per wave ranged from 5 to 28 (n = 16 cycles) and from 2 to 14 (n = 73 waves), respectively, among animals. Repeatability of the total number of follicles from Trial 1 to Trial 2 was 0.28 for the post-breeding period and 0.47 for wave 1. The repeatability of total numbers of antral follicles in the breeding cycle or in waves 2 and 3 could not be estimated due to the small sample size.

Discussion

The cow is a monovulatory species and the number of large follicles (≥ 11 mm) in a follicular wave is most often one or two. In this study, it was as great as three for one cow on two occasions. Thus, the number of large antral follicles that developed within a wave did not differ greatly among individuals, as expected based on the law of follicular constancy (Lipschutz, 1928). However, numbers of smaller antral follicles were more variable among cows, while being fairly repeatable within cow. The numbers of follicles 2 to 6 mm in diameter at the emergence of two consecutive follicular waves within one estrous cycle in beef cows were highly and positively correlated within cow (0.77; Singh et al., 2004). Burns et al. (2005) reported a high repeatability (0.95) of numbers of follicles ≥ 3 mm in diameter during consecutive follicular waves in dairy cattle, while Boni et al. (1997) reported slightly lower values of repeatability (0.58) for numbers of antral follicles that developed after ultrasound-guided aspiration of antral follicles every 7 to 10 days. The values in the current study for repeatability of the numbers of antral

follicles ≥ 4 mm in diameter among two or three consecutive follicular waves ranged between 0.29 and 0.50 in Trial 1 and 0.05 and 0.14 in Trial 2.

The high repeatability reported by Burns et al. (2005) was achieved most likely by the inclusion of follicles that were smaller in diameter (≥ 3 mm) and consistency among operators in recording such follicles. Singh et al. (2004) reported that 45% of the total number of follicles ≥ 2 mm following synchronization of estrus was comprised of follicles 2 to 3 mm in diameter in beef cows. Differences in repeatability between Trials 1 and 2 in the current study are most likely due to differences in technicians in their diligence of recording follicles that were near the minimum diameter (4 mm). The original intent of each of the experiments from which data for this study were collected was to evaluate development of follicular waves, with particular interest in the dominant follicle of each wave and was not focused on evaluating total numbers of follicles. Thus, repeatability of total numbers of follicles between and among waves and follicles was lower than that reported by Burns et al. (2005). Generally, repeatability of the total numbers of antral follicles increased as the minimum size of antral follicles recorded decreased among studies. Thus, small follicles bear the larger responsibility for the repeatability.

Because ultrasonography commenced on day 6 of each estrous cycle, estimates of number of follicles in the first wave did not include follicles that became atretic before day 6 and either did not reach or had regressed below 4 mm. The effect of wave on total numbers of follicles in each year, and on the number of small follicles in Trial 1 might be attributed to this fact. Generally, in both Trials 1 and 2, the repeatability of the total number of follicles from cycle to cycle was higher for the second follicular wave than for

the first wave. In addition, the lowest repeatability of total number of follicles per wave in Trial 1 (0.07) was recorded for wave 1 among cycles. All follicles that developed to a diameter ≥ 4 mm within the second follicular wave should have been recorded.

Driancourt (2001) speculated that the occurrence of two or three follicular waves might differ between beef and dairy cows. Estrous cycles with two waves were more frequent than those with three waves in lactating dairy cows (Taylor and Rajamahendran, 1991), dairy heifers (Ginther et al., 1989c), and beef cows and heifers (Ahmad et al., 1997). However, more estrous cycles with three rather than two waves of follicular development were recorded in Freisan x Hereford beef heifers (Savio et al., 1988) and in Brown Swiss cows (Celik et al., 2005). Freisan x Hereford beef heifers on low dietary intake (0.7% of body weight) tended to have a greater proportion of estrous cycles with three waves of follicular development compared to heifers with greater dietary intake (Murphy et al., 1991). That result could have been due to reduced clearance of progesterone in the underfed heifers (Murphy et al., 1991), which would be expected to lead to a reduced frequency of LH pulses (Adams et al., 1992), thus promoting more frequent atresia of dominant follicles.

All the factors affecting the number of follicular waves in cattle are not known. Interestingly, when dairy cows were segregated into those that produced greater (21 to 25) or fewer (≤ 15) average numbers of follicles per wave, concentrations of FSH were greater in cows with fewer follicles, while concentrations of IGF-I, estradiol and inhibin did not differ (Burns et al., 2005). However, because most estradiol is produced by the dominant follicle (Ireland et al., 1984), this finding might be expected. Similar results

were reported by Singh et al. (2004) for beef cows divided in half based on the numbers of follicles present on the day of first wave emergence after synchronization of estrus.

Some factors transiently reduced the numbers of follicles that developed during a follicular wave: lactation and nutrition (Lucy et al., 1991), heat stress (Badinga et al., 1993; Wolfenson et al., 1995), and stage of pregnancy (Ginther et al., 1996), which might explain the reported effects and tendencies for effects on numbers of primarily small follicles, and in some cases medium follicles, due to follicular wave or estrous cycle. In agreement with Burns et al. (2005), heifers in the present study had approximately 22% more small antral follicles than cows. The difference in numbers of small follicles recorded between heifers and cows might be explained by differences in physiological state, as the cows were suckling calves (Lucy et al., 1991). Differences between heifers and cows in the numbers of large follicles might simply reflect the fact that heifers have somewhat smaller dominant follicles (Sirois and Fortune, 1988).

In the current study, conception rate was not affected by the average number of follicles ≥ 4 mm in diameter that developed per wave within the entire breeding or post-breeding estrous cycle, or the total number of follicles during either the ovulatory follicular wave or the second follicular wave of the post-breeding estrous cycle. However, with the numbers of animals studied, conception rates ($>80\%$) were so high that differences would be difficult to detect. Conception rates were increased (Townson et al., 2002) or did not differ (Celik et al., 2005) in dairy cows with three rather than two waves during the breeding estrous cycle. Ahmad et al. (1997) reported increased conception rates in beef cows with three rather than two waves during the post-breeding equivalent of an estrous cycle. Cows with two follicular waves would be expected to

have greater concentrations of estradiol around the time of maternal recognition of pregnancy, which might decrease pregnancy rate (Pritchard et al., 1994), but Ahmad et al. (1997) found no difference in concentrations of estradiol during that period between cows with two or three follicular waves.

As noted earlier, Ahmad et al. (1997) reported that the proportion of cows having three waves increased from 10 % during the breeding cycle to 48% during the post-breeding period in Trial 1. They speculated that there might be an effect of pregnancy on the number of follicular waves during the post-breeding estrous cycle. In Trial 2, there was no significant change in the proportion of cows having three waves during the post-breeding period (11%) compared to the breeding (17%) or pre-breeding (22%) estrous cycle.

In conclusion, the numbers of small, medium, large and total antral follicles ≥ 4 mm in diameter per follicular wave varied among beef cows, and was relatively highly repeatable when small follicles were recorded most carefully. However, these numbers did not affect the fertility of the oocyte that was ovulated from the dominant follicle of the second or third wave at the time of insemination.

GENERAL DISCUSSION

While a multitude of factors influence both conception rate and loss of pregnancy, this series of studies has contributed specific information about individual factors that influence each variable. While progesterone is essential for maintenance of pregnancy, the overall concentration does not appear to be a main factor involved with loss of pregnancy. Conception rates were increased after treatment with rbST, which in part, could be due to increased concentrations of progesterone in the subsequent estrous cycle after treatment with rbST (Schemm et al., 1990; Lucy et al., 1994; Morales-Roura et al., 2001). However, retention of pregnancy was not affected by treatment with rbST at insemination, although concentrations of progesterone were not affected around day 30 of pregnancy. There appears to be some minimum concentration that is needed to maintain pregnancy at what might be considered 'normal' rates. For instance, pregnancy was maintained in ovariectomized gilts with one third the peripheral concentration of progesterone found in intact pregnant gilts (4 vs. 15 ng/mL; Ellicott and Dziuk, 1973). Thus, production of progesterone by the CL was three times the concentration of progesterone necessary for maintenance of pregnancy. In the lutectomized dairy cow, the minimum requirement of progesterone to maintain pregnancy to term was 0.75, 0.88 or 0.50 mg/lb of body weight at day 30, days 45 to 60 or day 75 of gestation, respectively (Tanabe, 1966). Tanabe (1970) reported that the amount of exogenous progesterone required to maintain pregnancy in lutectomized cows decreased as pregnancy continued, most likely due to increased production of progesterone by the placenta. Even early production of steroids from the placenta just after attachment might reduce the requirement for production of progesterone by the CL within the local environment. A

local, unilateral relationship between the CL and conceptus is required for maintenance of pregnancy during maternal recognition of pregnancy (Thatcher et al., 1986; Bazer, 1992). It continues to exist early in the second month of gestation (Lulai et al., 1994; Bridges et al., 2000), but in the current study, appeared to dissipate early in the third month. While the placenta would begin to produce greater concentrations of progesterone and other steroids after attachment, these concentrations are not adequate to maintain pregnancy without production of progesterone by the CL until approximately day 160 (McDonald et al., 1953; Estergreen et al., 1967). There is no direct evidence available that maintenance of the cows on lower-than-normal concentrations of progesterone prior to induction of a new resulted in a compensation by the fetus that would have resulted in its ability to survive under these specific conditions when the CL was contralateral to the uterine horn of pregnancy.

Although beef and dairy cattle are of the same species, their differences go beyond their segregated primary roles of production. Beef cows had greater conception rates (61 and 84%) than dairy cows (49%) after similar insemination procedures. Treatment with rbST at insemination might not be able to improve upon the greater conception rates reported for beef cows, but influenced positively the relatively poor conception rates reported for dairy cattle after traditional insemination or timed artificial insemination (Moriera et al., 2000a,b). In addition to higher conception rates, estimates of loss of pregnancy during the late embryonic and early fetal period are only 2 to 6% for beef cows (reviewed by Inskeep et al., 2002) compared to 12% for dairy cows (Santos et al., 2004). This could be attributed to possible differences in patterns of follicular development (Driancourt, 2001) or to differences in concentration of progesterone around

the time of placental attachment in beef and dairy cows (3.6 ng/mL vs. 2.4 ng/mL, respectively). Although loss of pregnancy during the late embryonic and early fetal period is not commonly high in beef cows, retention of pregnancy was only 76% to day 53 of gestation when normal concentrations of progesterone were reduced by half. The mean concentration of progesterone that maintained pregnancy in 76% of the cows was lower than the concentration at or below which only 50% of pregnancies were maintained in dairy cattle (Starbuck et al., 2004). Thus, beef cattle might require a lower threshold concentration of progesterone than dairy cattle to maintain pregnancy.

Concentrations of progesterone in experiment I were not as great as desired and did not differ between treatments with new and used CIDRs (1.8 ng/mL). The original intent was to utilize the new and used CIDRs to provide concentrations of progesterone ranging from 6 ng/mL (high end of normal for a pregnant cow) to approximately 2.8 ng/mL (the concentration at which only 50% of dairy cows maintained pregnancy; Starbuck et al., 2004). The original studies that evaluated concentrations of progesterone released from a CIDR insert were performed in ovariectomized, dry dairy cows. Seven days after insertion, just over 0.5 g of the 1.38 g of progesterone contained originally in a CIDR device was eluted and over that time, peripheral concentrations averaged 3 ng/mL (Rathbone et al., 2002). The mean concentrations of progesterone generated from the two CIDR treatments were similar to concentrations reported for cows with two CIDRs and no CL (Shaham-Albalancy et al., 1997) and for cows with two CIDRs and treated with deslorelin, an GnRH agonist, with ad libitum access to feed (Rabiee et al., 2002), but did not equal those reported for ovariectomized, non-lactating dairy cows with one new CIDR (Rathbone et al., 2002). Cerri et al. (2005) reported that approximately 17% of

cows with no CL and a single CIDR (new or used) had concentrations of progesterone >1 ng/mL during the seven days of CIDR treatment. Thus, the amount of evidence reporting that concentrations of progesterone in a lactating, beef cow with no CL on CIDR treatment are well below those reported previously for the dry, ovariectomized dairy cow is growing.

The rate of metabolism of progesterone is related directly to liver blood flow and physiological condition (e.g., lactating, non-lactating, heifer; Sartori et al., 2002). Sustained rates of elevated liver blood flow were seen in cows consuming more feed, which correlated inversely with peripheral concentrations of progesterone (Sangsrivong et al., 2002). Thus, the lower than expected concentrations of progesterone in experiment I were most likely due to greater rates of metabolism of progesterone resulting from high feed intake. In addition, Rabiee and co-workers (1999) reported greater rates of release of progesterone from CIDRs in cows that were grazing rather than under confined conditions. Thus, release of progesterone from CIDRs was associated with housing and diet (which are often varied among beef and dairy operations) in addition to other factors that alter the rate of blood flow to the anterior vagina.

Age commonly was associated with differences in fertility among cows in the four studies, even more so than concentrations of progesterone. Twenty-two percent more antral follicles per wave were recorded for heifers than cows in the study by Burns et al. (2005) and in the current studies. However, it should be noted that the significance of this effect has yet to be determined. Dairy heifers in general do not have high rates of pregnancy loss such as have been reported for the lactating dairy cow. Differences were not limited to those between cows and heifers, but also among cows of different age

classifications. Loss of pregnancy was greater following lutectomy and replacement therapy with exogenous progesterone in cows ≥ 4 years old than in younger cows. Some factors that might result in differences in fertility among cows of various ages are: differences in partitioning of nutrients, variation in rates of metabolic clearance or production of hormones, altered endocrine environment, or the effects of repeated pregnancies on reproductive tissues.

In summary, this series of studies identified age and concentration of progesterone, but not prior treatment with rbST at insemination as specific factors affecting retention of pregnancy. Likewise, treatment with rbST increased conception rate in dairy cattle, especially those at > 100 DIM, but fertility, as measured by conception rate to first service, did not appear to be associated with the average number of all antral follicles ≥ 4 mm in diameter that developed during a wave within a estrous cycle. Additional studies are needed to determine the specific mechanisms by which age of cow and reduced concentrations of progesterone affect reproductive tissues or the conceptus and thus affect the likelihood of the pregnancy being maintained to term. Further research is needed to evaluate differences between beef and dairy cows that account for reduced retention of pregnancy in the dairy cow. Concentrations of progesterone in beef animals with CIDR inserts should be re-evaluated. Limited research has been focused on the grazing dairy cow, an animal which when compared to the ‘traditional’ dairy cow might provide additional information about the metabolic factors that influence retention of pregnancy.

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