APPLICATION OF A MODIFIED 5-DAY PROGESTERONE-BASED TIMED ARTIFICIAL INSEMINATION PROTOCOL FOR REPRODUCTIVE MANAGEMENT OF DAIRY HEIFERS

By

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To my nephew and niece, Bruno and Francesca Rabaglino, for all what it means for me to be far away from them during the early years of their lives

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

APPLICATION OF A MODIFIED 5-DAY PROGESTERONE-BASED TIMED ARTIFICIAL INSEMINATION PROTOCOL FOR REPRODUCTIVE MANAGEMENT OF DAIRY HEIFERS

By

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A total of 2,582 nulliparous Holstein heifers, between 13 to 14 months of age from two commercial dairy farms, were used in three studies to evaluate a modified ovulation synchronization protocol, the 5 day (d) Co-Synch + CIDR (controlled internal drug-release containing progesterone), for timed artificial insemination (TAI) of dairy heifers.

In the first study, the main objective was to synchronize ovulation of dairy heifers with the 5 d Co-Synch protocol as a platform to evaluate if flumixin meglumin (FM) injected 15.5 or 16 d after TAI could improve pregnancy per TAI (P/TAI). Overall P/TAI to first TAI in heifers synchronized with the 5 d Co-Synch protocol was 58.3% with 2.3% pregnancy loss. The application of FM did not improve P/TAI compared with controls.

In the second study, main objectives were to evaluate if one injection of prostaglandin F2alpha (PGF2a) at the time of CIDR withdrawal could be as effective as two injections in the 5 d Co-Synch + CIDR protocol, and to test this protocol with one PGF2a injection in a field verification trial for first TAI and second TAI of nonpregnant heifers. There were no differences in P/TAI or corpus luteum regression between heifers receiving one or two injections of PGF2a. In the field verification, overall P/TAI after two sequential synchronizations of ovulation with

the 5 d Co-synch + CIDR protocol and two TAI services was 81.0% with a pregnancy loss of 5.0%.

The third study had as the main objective to test the 5 d Co-Synch + CIDR protocol with one PGF2a injection for TAI with sexed semen. Overall P/TAI to first TAI was 40.9% with a pregnancy loss of 7.4%, the expected results when sexed semen is used for insemination.

In conclusion the 5 d Co-Synch + CIDR protocol with one injection of PGF2a is an efficient TAI management program to achieve acceptable P/TAI for first and second TAI in dairy heifers, either with sexed or conventional semen, without the need for estrous detection.

CHAPTER 1
INTRODUCTION

Replacement dairy heifers represent the future production of a dairy herd. In order to sustain genetic progress and maintain an economic advantage from higher milk production, heifer breeding programs should include artificial insemination (AI) and calving to occur around 24 months. A commonly used reproductive management is AI at detected estrus from a spontaneous displayed estrus (Stevenson et al., 2008a). Consequently, efficient detection of estrus is of paramount importance for effective reproductive management with AI (Ferguson and Galligan, 1993). However, in contrast to lactating dairy cows, time spent on detection of estrus in heifers is limited, which can delay time of first AI and therefore increase age at first calving, associated with additional costs (Caraviello et al., 2006).

Based upon an understanding of the factors controlling ovarian follicular growth, insemination programs that allow for fixed timed AI (TAI) of dairy cattle, without the need for estrous detection, have been developed (Thatcher et al., 2000). Therefore, application of ovulation synchronization protocols may enhance the use of AI in dairy heifers (Peeler et al., 2004). One program that has been successful for TAI of lactating dairy cows is the Ovsynch program, in which injections of gonadotropin-releasing hormone (GnRH) are given 7 days (d) before and 48 hours (h) after an injection of prostaglandin F2alpha (PGF2a), and cows are inseminated 16–20 h after the second injection of GnRH (Pursley et al., 1995; Burke et al., 1996). If TAI in the Ovsynch protocol is performed at the same time as the second GnRH injection, the protocol is referred to as Co-Synch (Geary and Whittier, 1998). Stage of the estrous cycle when Ovsynch is initiated influences pregnancy per TAI (P/TAI); the early luteal phase (i.e., between d 5 and 10) of the cycle results in greater P/TAI (Vasconcelos et al., 1999). Compared to lactating cows, heifers have a faster rate of follicular growth (Pursley et al., 1997)

and a higher frequency of 3-wave follicular cycles (Savio et al., 1988). Thus, heifers present a lower probability to ovulate a dominant follicle in response to the first GnRH in the Ovsynch protocol compared with cows (Pursley et al., 1995, 1997). If Ovsynch is initiated during the later stages of the estrous cycle, the orginal corpus luteum (CL) from the preceding spontaneous ovulation will regresses before PGF2a injection. The lack of ovulation to the first GnRH would result in no accessory CL formation and potentially, in an increased risk of premature regression of the original CL and expression of estrus, thereby causing asynchrony at TAI (Rivera et al., 2004, 2005). Inclusion of controlled internal drug-release (CIDR, Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) insert containing progesterone in the Ovsynch or Co-Synch protocols suppresses ovulation during the period of CIDR insertion thereby allowing 100% submission rate of heifers for TAI (Rivera et al., 2005). Bridges et al.(2008) deduced that an increase in P/TAI would be achieved if the Co-Synch + CIDR protocol were modified by reducing the interval to 5 d from the first GnRH treatment to the PGF2a injection and withdrawal of the CIDR insert and lengthening the proestrus interval from PGF2a to the second injection of GnRH and TAI to 3 d (5 d Co-Synch + CIDR). In beef cows, this approach resulted in greater P/TAI compared to a 7 d Co-Synch + CIDR with the second GnRH and TAI occurring concurrently at 60 h (80.0% versus 66.7%, respectively). Due to the shortened interval from the initial GnRH to PGF2a in the 5 d Co-Synch + CIDR protocol it is not known whether regression of an induced accessory CL occurs with one injection of PGF2a. Therefore, a second PGF2a injection was applied approximately 12 h after the first PGF2a injection.

One technology that has been implemented in the dairy industry in the last years is the use of sexed semen for AI of cattle. The goal of sexed semen use in AI of the dam is to obtain an offspring of a preferred gender. Despite the lower fertility compared with conventional semen,

the use of sexed semen in dairy farms is justified because it enhances the producer's ability to economically obtain replacement heifers, mitigating some of the effects of high culling rates and poor reproductive efficiency (Weigel, 2004). Considering the challenges faced by dairy producers to raise dairy heifers, an ideal reproductive management program should improve pregnancy results and increase the number of females born. The utility of TAI combined with the commercial availability of sexed semen, could prove to be an effective reproductive management program of dairy heifers if acceptable P/TAI are obtained, especially in herds with inefficient estrous detection (De Vries et al., 2008).

The main goals of the series of studies presented in this thesis were to evaluate P/TAI in dairy heifers synchronized with the 5 d Co-Synch + CIDR protocol proposed by Bridges et al.(2008); to modify this protocol applying one PGF2a injection instead of two at the time of CIDR insert withdrawal; to test the 5 d Co-Synch + CIDR protocol with one injection of PGF2a for synchronization of the first TAI and resynchronization of nonpregnant heifers for a second TAI; to compare conventional and sexed semen for insemination with the 5 d Co-Synch + CIDR protocol.

Results from these studies provide dairy producers an alternative reproductive management program for dairy replacement heifers, enhancing the use of TAI from proven sires without the need for estrous detection. Consequently, dairy producers could increase the profitability of this reproductive management system.

CHAPTER 2
LITERATURE REVIEW

The Bovine Estrous Cycle

General Characteristics

The duration of the bovine estrous cycle has a range of 17 to 24 d, with an average of 21 d. The cycle is divided into two distinct phases and four stages, according to the dominant structure present in the ovary. The phases are the follicular and the luteal phase. The follicular phase, where the primary structure present in the ovary is a dominant follicle which produces estradiol, is short (20% of the estrous cycle) and is characterized from the time of regression of the CL to ovulation of the dominant follicle. The luteal phase is much longer (80% of the estrous cycle), the dominant structure present in the ovary is the CL which produces progesterone and is characterized from the time of ovulation until CL regression.

Estrous cycle stages are subdivisions of the follicular and luteal phases. Proestrus and Estrus are part of the follicular phase and Metestrus and Diestrus comprise the luteal phase.

Proestrus begins when progesterone declines as a result of luteolysis (CL regression) and lasts about 3 d. Estrus is charactherized by behavioral symptoms such as sexual receptivity and mating due to high estradiol influence. It has a duration period that ranges from 6 to 24 h and ovulation occurs 24 to 32 h after onset of estrus. Metestrus is the period of CL formation after ovulation, and lasts 2 to 5 d. Diestrus is the longest stage of the estrous cycle (10 to 14 d) and is the period when the CL is fully functional and progesterone concentration is high and ends with luteolysis of the CL (Senger, 2003).

Hormonal Regulation of the Estrous Cycle

The estrous cycle is regulated by endocrine and neuroendocrine mechanisms, namely hormones from the hypothalamus and pituitary glands and steroids secreted by the ovary, known

as the hypothalamic-pituitary-ovarian axis (HPO). The hypothalamus secretes GnRH. The pituitary hormones are follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the hormones secreted from the ovary are the steroid hormones, estradiol and progesterone. Below is a description of the characteristic and functions of each of these hormones as part of the HPO axis.

Gonadotropin-releasing hormone

This hormone is a decapeptide produced by neurosecretory neurons whose terminal axons are located in the median eminence of the hypothalamus. It gains access to local blood circulation through fenestrations in small vessels and is carried to the anterior pituitary by the hypothalamic-pituitary portal system. Once in the pituitary it binds to specific receptors on gonadotropes cells (luteotropes and folliculotropes) to induce release of LH and FSH, respectively, into the bloodstream to reach and target cells (Norman and Litwack, 1998). The frequency of the GnRH stimulus may be the major regulator of the relative proportions of FSH and LH synthesized and secreted, with less frequent GnRH pulses leading to preferential FSH secretion and more frequent GnRH pulses to LH secretion (Hadley, 2000).

Follicle stimulating and luteinizing hormones:

These hormones are glycoproteins composed of two chains, alpha and beta subunits. The alpha subunit is identical within species, while the beta subunit is structurally distinct, providing hormonal specificity (Bousfield et al., 1994). The growth of periovulatory follicles in the ovary is dependent on both FSH and LH acting in concert. The differential secretion of the gonadotropins at various stages of follicular growth is associated with initiation and continued growth of follicles toward ovulation, or atresia, at different stages of growth. FSH is important for the initiation and early growth of antral follicles, and LH becomes more important with increasing development and maturation of follicles (Lucy et al., 1992). Follicular growth and

steroidogenesis are dependent on the coordinated actions of FSH and LH with their receptors on granulosal cells and thecal cells of ovarian follicles (two cells/two gonadotropin theory; see below) (Bao and Garverick, 1998).

Receptors for FSH are present on granulosa cells, but not thecal cells, with FSH receptor (FSHr) mRNA expressed in follicles with as few as two layers of granulosa cells (Tisdall et al., 1995). FSH induced expression of cytochrome P450 aromatase (P450arom) that converts androgen to estradiol in granulosa cells (Hillier et al., 1994). Granulosa cells also express estradiol receptors, which may mediate autocrine estradiol action within the granulosa cell layer. The effects of estradiol on granulosa cells are to amplify overall actions of FSH (Richards, 1994).

LH receptors (LHr) are present in theca cells to induce androgen synthesis; although LHr appears in the granulosa cells at later stages of follicle development. Androgens are synthesised in thecal cells through cell-specific expression of P450 17 alpha-hydroxylase (P450c17), which is under LH control (Smyth et al., 1993). During the preovulatory surge, LH acting directly on granulosa cells initiates luteinization, to induce progesterone synthesis and secretion of the granulosa cells that transform into small and large luteal cells of the CL after ovulation to become the CL (Hadley, 2000).

Follicular steroidogenesis is regulated by binding of FSH and LH to specific, high-affinity, G-protein-coupled plasma membrane receptors. The principal mediator (second messenger) of FSH and LH action is cAMP, however, other cellular effector systems could also be involved (Ginther et al., 1996).

Estradiol

This hormone is derived from cholesterol as are all steroid hormones. Its production is explained by the two-cell two-gonadotrophin theory, proposed by Fortune and Quirk (1988) and

revised by Hill et al., (1994) (Figure 2-1). This theory states that thecal cells with FSHr and granulosal cells with LHr have both the cytochrome P450 side-chain cleavage (P450scc), the enzyme necessary for conversion of cholesterol to C-21 steroids: pregnenolone or progesterone, precursors for synthesis of androstenedione in thecal cells. Binding of LH to its receptor on thecal cells stimulates activity of the cytochrome P450c17 inducing the conversion of C-21 steroids to androstenedione. This compound is then metabolized to estradiol in granulosa cells by the cytochrome P450arom, enzyme induced by the binding of FSH to receptors (Bao and Garverick, 1998). Estradiol and FSH induce synthesis of LH receptors on membranes of granulosal cells during later stages of follicular development, and, consequently, estradiol enhances its own secretion (Richards, 1980).

Estradiol production increases within the follicle during the preovulatory phase and is highest at the time of the GnRH surge. Principal effects of estradiol are development of female reproductive organs allowing for ovum fertilization in the oviduct and inducing sexual receptivity. In the central nervous system (CNS), it has a negative (most part of the cycle) effect by tonic stimulation of the tonic center in the hypothalamus (Hadley, 2000). In contrast, during the proestrus period, increasing estradiol concentrations with decreasing progesterone after luteolysis increase the LH pulse frequency, further culminating in a large prevulatory LH surge (Adams et al., 2008). Estradiol, acting synergistically with progesterone induces behavioral estrus (Hafez and Hafez, 2000).

Progesterone

This steroidal hormone is synthesized by luteals cells of the CL present in the ovary during the luteal phase. As steroidal hormone, the principal precursor for its synthesis is cholesterol. Inside the cell cholesterol is transported into the mitochondria. The enzyme responsible of the transport of cholesterol from outer to the inner mitochondrial membrane is Steroidogenic Acute

Regulatory Protein (StAR), and the process constitutes a rate limiting step in progesterone synthesis. In the inner mitochondrial membrane the enzyme P450scc is present, to cleave the side of chain from cholesterol to form pregnenolone, which is then converted to progesterone by the enzyme 3 beta hydroxysteroid dehydrogenase/isomerase (3b-HSD) (Rekawiecki et al., 2008). Progesterone is the hormone of pregnancy and is responsible for preparing the reproductive tract for zygote implantation and the subsequent maintenance of the pregnant state. In the CNS progesterone inhibits pituitary gonadotropin secretion by action on the tonic center of the hypothalamus; therefore it inhibits follicular development (Hadley, 2000).

The Follicular Phase: Ovarian Follicular Growth

Folliculogenesis is defined as the formation of the Graafian (mature, preovulatory) follicle from a pool of primordial (nongrowing) follicles (Spicer and Echternkamp, 1986).

There are two stages of ovarian antral follicle development. Firstly, a 'slow' growth phase which takes more than 30 d from antrum acquisition at 300 μm to the 'small' follicle stage of 3–5 mm in diameter. The second 'fast' growth phase may only take 5–7 d, and is usually described as a follicle wave (Lussier et al., 1987). In the bovine, the ovarian dynamic is characterized by two, three or four consecutive follicular waves per estrous cycle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989a). Each wave involves the recruitment and the sudden growth, within 2–3 days, of a cohort of small follicles. The growth rate is similar among follicles of the wave for approximately 2 days. Thereafter the selection of a dominant follicle occurs, which continues to grow and mature to the preovulatory stage while others in the wave (subordinate follicles; Austin et al., 2001) undergo atresia (Adams et al., 2008). Recruitment is the process whereby a cohort of follicles begins to mature in a milieu of sufficient pituitary gonadotrophin stimulation to permit progress towards ovulation; a cohort may be of 8 – 41 follicles. Selection is the process whereby a follicle avoids atresia and undergoes further

development and becomes competent to achieve a timely ovulation. Dominance is the process whereby a single follicle achieves and maintains its dominance over the other recruited follicles, which undergo atresia (Hodgen, 1982) (Figure 2-2).

Ovulation occurs due to a surge in GnRH which induces an LH surge, and also, a peri-ovulatory FSH rise which stimulates the cohort of antral follicles to grow beyond 4 mm in diameter. The cohort grows over the next 36 to 48 h, after which the dominant follicle of 8 to 9 mm in diameter is selected (Adams et al., 1992; 2008), while the subordinate follicles undergo atresia via apoptosis. The dominant follicle is responsible for the high ovarian estradiol and inhibin-A secretion, that maintains low FSH concentrations to prevent any further follicle cohort growth (Ireland et al. 1984; Ginther et al. 1999, 2000a,b; Austin et al., 2001). Subordinate follicles are dependent on elevated FSH concentrations for their continued growth and ability to synthesize estradiol. This is quite evident in that with FSH withdraw more and more follicles initiate apoptotic changes as FSH drops further below their requirement threshold (Mihn et al., 2002). The selected dominant follicle is relatively FSH independent and at the last stage of dominant follicle selection it prevents its closest competitor from continued growth by a final reduction in FSH (Ginther et al. 1999, 2000a, b). Time of deviation is defined the greatest difference in growth rates (diameter changes between adjacent examinations) between the two largest follicles at or before the examination when the second-largest follicle reached its maximum diameter. Deviation and selection are considered synonymous. The mean day at the beginning of deviation in growth rates of the two largest follicles is d 2.8, when the future dominant follicle is 8.5 mm of diameter mean and the largest subordinate follicle is 7.2 mm (Ginther et al., 1996).

Compared to subordinate follicles, the dominant follicle has higher levels of mRNAs for gonadotropin receptors and enzymes involved in steroid synthesis (17alpha-hydroxilase, P450scc, 3b-HSD, and StAR) and also it acquires LHr on granulosa cells. However, it is not clear whether the identical increase in LHr expression in the dominant follicle is cause or consequence of the selection process (Mihm and Evans, 2008). Development of LHr on granulosa cells could allow a shift from FSH to LH dependence and prepare the dominant follicle for further differentiation in response to the LH surge (Fortune, et al., 2001). It has been proposed that the acquisition of LHr by granulosa cells of the dominant follicle stimulates an abrupt increase in estradiol production, which would suppress circulating FSH concentrations, and increases intracellular cAMP, which would protect the selected follicle from the FSH decrease (Ginther et al., 1996).

If the animal is in the luteal phase of the estrous cycle (diestrus) then the dominant follicle reaches its maximum size (12-20 mm) and is maintained for 3 to 6 d before undergoing atresia (Ginther et al., 1989b; Knopf et al., 1989), allowing another FSH rise and emergence of a new follicular wave. The dominant follicle of the first wave in two-wave cycles and of the first and second waves in three-wave cycles, undergo atresia. If luteal regression occurs during the growing phase of the dominant follicle from the final (second or third) wave (beginning of proestrus) then the dominant follicle responds to the decrease in circulating progesterone, and the subsequent increase in LH pulse frequency. The follicle secretes sufficient estradiol which in turn elicits a LH/FSH surge and ovulates after estrus (Fortune, et al., 2001) (Figure 2-3). However, the dominant follicle of any follicular wave, including the first, can ovulate if the appropriate endocrine conditions are provided by induction of luteolysis during its tenure of dominance (Kastelic et al., 1990).

The Luteal Phase: Regulation of CL Function

The CL is a transitory endocrine gland that mainly secretes progesterone but also, other products of luteal origin are oxytocin, noradrenaline, prostaglandin I2 and E2 (Rekawiecki et al., 2008), prolactin (Shibaya et al., 2007) and estradiol (Okuda et al., 2001); and has a key role in establishment and maintenance of pregnancy in domestic mammals (Niswender et al., 1994; Fields and Fields, 1996). In cattle and other domestic animals, lifespan of the CL is mainly controlled by the uterine luteolysin, prostaglandin F2alpha (PGF2a), which affects the length of the estrous cycle (Milvae, 2000). The CL is one of the few tissues that exhibits regular periods of growth (CL formation), function and luteolysis (Schams and Berisha, 2004).

Corpus luteum formation

The preovulatory LH surge induces a series of morphological and biochemical changes in cells of the theca interna and granulosa cells of the preovulatory follicle that further differentiate into small and large luteal cells after ovulation. This process is termed luteinization (Berisha and Schams, 2005). Luteinization is characterized by increased progesterone production as a consequence of a switch from producing estradiol to progesterone. This is due to a reduction or abolition of enzymes responsible for transforming progesterone into estradiol, like P450c17 and P450arom (Juengel and Niswender, 1999). Tissue growth depends upon growth of new blood vessels (angiogenesis) and establishment of a functional blood supply (Berisha and Schams, 2005). Ovarian blood flow decreases shortly after ovulation but increases afterwards gradually in parallel with the increases in CL volume and plasma progesterone concentrations from d 2–5 with angiogenesis (Acosta et al., 2003) that reflexes normal CL development.

Regulation of progesterone synthesis

The primary hormones supporting the development and function of the CL are LH and growth hormone (GH) (Schams and Berisha, 2004). Membrane receptors for LH are located

mainly in small luteal cells, and binding of LH induces progesterone production (Niswender and Nett, 1988). Receptors for GH are located mainly on large luteal cells (Lucy et al., 1993), which are responsible for 80% of total progesterone production by the CL (Niswender et al., 1985). Progesterone also has been shown to regulate its own synthesis in the CL (Kotwica et al., 2004). Progesterone increases its own synthesis by stimulating expression of enzymes in the pathway that transform cholesterol to progesterone, like StAR protein, cytochrome P450scc and 3β-HSD (Kotwica et al., 2004). Another factor that enhances the expression of the same enzymes is PGE2 that leads also to an increase in progesterone synthesis (Rekawiecki et al., 2005). Increased progesterone concentrations in luteal cells protect them from apoptosis, while disruption of steroidogenesis and reduced ability of luteal cells to produce progesterone induces apoptosis (Liszewska et al., 2005).

Luteolysis

If conception does not occur, functional and structural regression of the CL begins to occur around d 16 in heifers (Ginther et al., 2007) and is caused by episodic release of PGF2a from the uterus by reaching the CL through a counter current system between the uterine vein and the ovarian artery (Thorburn et al., 1973). Progesterone is the hormone that regulates the lifespan of the CL. It exerts an inhibitory action on PGF2a secretion due to a down-regulation of receptors for estradiol and oxytocin in the endometrium until d 12. This is followed by desensitization to progesterone and an up-regulation of estradiol and oxytocin receptors after this period. This is essential for initiation of endometrial-CL luteolytic mechanisms (Mayer et al., 1988; Schams and Berisha, 2004). Therefore, there is an increase in estradiol receptor activation by circulating estradiol that stimulate the synthesis of endometrial oxytocin receptors with subsequent oxytocin induced secretion of PGF2a from the uterus, which stimulates secretion of oxytocin from the CL to produce a positive feedback loop to increase PGF2a to luteolytic concentrations (Silvia et al.,

1991). The source of estradiol during luteolysis is the ovarian follicles, especially the largest or future dominant follicle that would become the ovulatory follicle (Beg and Ginther, 2006). Follicular estradiol is an important regulator of the timing of uterine PGF2a secretion in cattle (Araujo et al., 2009).

One of the luteolytic actions of PGF2a released from uterus is to stimulate nitric oxid (NO) production and release in the arterioles of the peripheral vasculature of the mature CL, inducing vasodilation of the arterioles, increasing the blood flow to the CL. The acute increase in blood flow triggers the cascade of luteolysis (Miyamoto et al., 2005). Uterine or exogenous PGF2a directly increases endothelin-1 and angiotensin II secretion from microcapillary vessels within the CL without blood flow mediation. These vasoactive peptides suppress progesterone secretion from neighboring luteal cells (Girsh et al., 1996; Miyamoto et al., 1997) and may induce long-term vasoconstriction of luteal arterioles as plasma progesterone decreases. Other luteolytic actions of PGF2a are to stimulate intracellular Ca2+ influx (Davis et al., 1987) and protein kinase C (Wiltbank et al., 1989). Also, it has been suggested the invasion of immune cells during relatively early stages of luteolysis, and physiological roles of immune cell–luteal cell interactions, chemokines, and major histocompatibility complex expression as part of the luteolytic process (Townson et al., 2003; Cannon et al., 2003).

Differences between the Estrous Cycles of Holstein Heifers and Lactating Cows

In reference to the number of follicular waves, it has been reported that in the Nelore breed *(Bos Indicus)* most heifers exhibit a three follicular wave pattern whereas the majority of cows have a two wave pattern (Figuereido et al., 1997). In the Holstein breed *(Bos Taurus)* it has been reported that heifers have a faster rate of follicular growth (Pursley et al., 1997), and that three follicular waves is the most common pattern (Sirois and Fortune, 1988), although others did not

find a significant difference between cows and heifers, in the frequency of cycles with two or three waves (Wolfenson et al., 2004).

There are no difference between cows and heifers in the maximal size achieved by the largest dominant follicle for the non-ovulatory waves but, for the ovulatory wave, the dominant (preovulatory) follicle is larger in size in lactating cows than heifers (Sartori et al., 2004) with a tendency for longer duration of dominance (Wolfenson et al., 2004) (Figure 2-4).

Regardless of having larger ovulatory follicles, lactating cows have lower maximal serum estradiol concentrations around estrus than heifers and nonlactating cows, which have an earlier estradiol peak after luteolysis than cows (Ahmad et al., 1996; Inbar et al., 2001; Sartori et al., 2004; De La Sota et al., 1993). The low estradiol concentrations in cows has been related either to a lower steroidogenic capacity of the preovulatory follicle or to higher metabolism of estradiol in cows than in heifers, since lactating cows have a much greater steroid metabolism than nonlactating cows (Sangsritavong et al., 2002). Nevertheless, in the lactating cow the dominant preovulatory follicle has to grow to a larger size and be present for a longer time to reach a sufficient estradiol concentration to initiate the luteolytic cascade and subsequently induce the GnRH surge for ovulation (Sartori et al., 2004). Reduced preovulatory circulating estradiol concentrations could be one of the main reasons for the altered reproductive physiology in lactating cows, which results in a reduced length and/or intensity of behavioral estrus (Nebel et al., 1997). Also, lower estradiol concentration around ovulation could cause poor fertilization and poor early embryonic development (King et al., 1994). If serum estradiol levels are low, and there is a decline in inhibin production, there could be a decline in the inhibitory influence of estradiol over FSH release. Precisely, a small but significantly higher concentration of FSH is present during the cycle in cows than in heifers. A higher FSH concentration would induce

growth of more than one large follicle and a higher rate of double ovulations (Wolfenson et al., 2004). Lactating cows present a higher incidence of multiple ovulations (Fricke and Wiltbank, 1999). This fact also explains the higher twinning rate in multiparous cows compared to primiparous cows (Kinsel et al., 1998).

Lactating cows develop more luteal tissue volume than heifers that can be detected by d 4 of the cycle (Sartori et al., 2004). There is a positive correlation between size of the ovulatory follicle and luteal tissue volume (Vasconcelos et al., 2001). If lactating cows have a larger ovulatory follicle, CL volume would be larger. Despite the larger luteal tissue, serum progesterone concentration is lower in cows than in heifers, detected from d 6 of the cycle to ovulation (Sartori et al., 2004; Wolfenson et al., 2004). The higher steroid metabolism in lactating cows could explain this difference. Lower serum progesterone would allow increased pulse frequency of LH, causing a premature maturation of the oocyte (Revah and Butler, 1996), resulting in ovulation of an aged oocyte after the estradiol-induced GnRH surge. This could contribute also to lower fertility observed in lactating cows compared with heifers.

Reproductive Management of the Estrous Cycle of Cattle

Artificial Insemination

Artificial insemination is considered to be the most important technique developed for the genetic improvement of farm animals, because a few selected males produce enough sperm to inseminate thousands of females per year (Hafez and Hafez, 2000). Artificial insemination continues to be the method of choice for dairy producers to increase the genetic quality of their cattle (Vishwanath, 2003). Other advantages of AI over breeding by natural service include control of venereal diseases, availability of accurate breeding records, an economic service and safety through elimination of dangerous males (Hafez and Hafez, 2000).

Application of AI in farm animals began in Russia and Japan in the early 20th century (Hafez and Hafez, 2000) and became popular in the United States in the 1950s, with the development of frozen bovine semen. However, the necessity for daily estrous detection is a detriment to the successful implementation of AI, especially in beef cattle and virgin dairy heifers. For this reason, the development of protocols that control estrus and ovulation is of major importance to cattle producers (Lauderdale, 2009).

Development of Synthetic Reproductive Hormones to Manage the Estrous Cycle

Progesterone

Progesterone is a 21 carbon steroid derived from cholesterol produced principally by the CL. Progesterone belongs to a class of hormones called progestogens, and it should not be confused with progestins, which are synthetically produced progestogens. Progesterone was co-discovered by Willard Myron Allen with his anatomy professor George Washington Corner at the University of Rochester Medical School in 1933. Allen first determined its melting point, molecular weight, and partial molecular structure. He also gave it the name Progesterone derived from **Proge**stational **Ster**oidal ket**one** (Allen, 1970).

The first description of the use of progesterone in cattle was published by Ulberg et al., (1951). They reported that 25 mg of progesterone in corn oil injected subcutaneously daily in cattle prevented estrus and CL formation. If smaller doses were used (3.125 mg) follicular development was not inhibited but, it was minimal at 50 mg. They concluded that this finding supported the theory that progesterone inhibits LH release from the pituitary.

Early studies done by Nellor and Cole (1956) used crystalline progesterone in a starch emulsion injected in beef heifers once subcutaneously, with the intention to prevent estrus and CL formation. They performed three studies. In one study, TAI was used for the first time in beef heifers, by injecting subcutaneously 540 mg of the progesterone emulsion followed 15 d later by

an injection of 750 IU subcutaneously of equine gonadotropin. Timed AI was performed 48 hrs after the equine gonadotropin injection. However, P/TAI was not reported.

In the early 60's medroxyprogesterone acetate (MAP), an orally synthetic progestin, was used for estrous synchronization of cattle (Hansel et al., 1961; Zimbelman, 1963). One hundred and eighty mg of MAP was fed for 18 d, followed by visual estrous detection 1 to 6 d after the last MAP feeding. An estrous detection rate of 90% was reported. During 1965 to 1967, MAP was commercially available for estrous synchronization of cattle, under the name of Repromix (The Upjohn Company) However, due to product cost it was removed from the market.

Melengestrol acetate (MGA Premix, Pfizer Animal Health, NY), was another oral progestin product used to suppress estrus and prevent ovulation (Zimbelman and Smith, 1966a,b).

Although the intention of the first studies was to apply MGA for estrous control of cattle, increases in body weight were observed when heifers were fed 0.25 to 0.75 mg of MGA (Bloss et al., 1966; Zimbelman and Smith 1966b). This finding led to a number of studies for its use in the feedlot industry. Finally, MGA was approved by the Food and Drug Administration (FDA) in 1968, "for increased rate of weight gain, improved feed efficiency, and suppression of estrus". However, it was not until 1997 that the FDA approved the use of MGA for estrous synchronization of cattle, due to regulatory decisions. Recommendations for MGA are to feed 0.5 mg daily for up to 24 d to suppress estrus in heifers intended for breeding. The duration of the feeding may vary between protocols. As MGA was commercially available through the feedlot approval, MGA was used for beef cattle estrous synchronization from about 1970.

In one study by O'Brien and Zimbelman (1970), the occurrence of estrus was evaluated after a short or long term feeding period of MGA. Heifers were fed for a short period (21 d) or

long period (63 or 84 d) and detected in estrus for 32 d after completion of feeding. There were no differences in percentages of total group detected in estrus in 32 d (82 to 91% range), concluding that there was no detrimental influence of long-term MGA on reproductive performance of feedlot heifers. Different protocols have been developed in beef cattle with the use of MGA combined with other reproductive hormones, like the MGA Select Program (Wood et al., 2001), in which MGA is fed for 14 d, followed by an injection of GnRH on d 26 and an injection of PGF2a on d 33. This protocol gives a higher estrous response (87%) in postpartum suckled beef cows compared with the use of this protocol without the addition of GnRH (76%) (Patterson et al., 2001).

Combination of progesterone with estradiol

Curl et al. (1968) reported that a subcutaneous implant of a progestogen controlled estrus and ovulation in cattle. Based in this finding and other earlier studies, Wiltbank et al., (1971) developed a subcutaneous implant consisting of northandrolone, a synthetic progestin, implanted subcutaneously the flank region in beef heifers for 9 d and an injection of 5mg of estradiol valerate (EV) intramuscularly, at the time of implantation to regress the CL. Ninety three percent of the 43 heifers receiving this treatment were in estrus in a 96 hr period. Injection of 2 mg of estradiol-17beta to heifers 24 hr after removal of the implant resulted in 98% to 100% of cycling heifers to show estrus in a 48 h period and 100% to ovulate in a 36 h period.

Other studies followed with a poly-hydroxy polymer subcutaneous implant containing norgestomet instead of northandrolone, in combination with EV (Spitzer et al., 1976, 1978; Miksch et al., 1978). The final product, known commercially as Syncro-Mate-B, was approved by the FDA in 1982, “for synchronization of estrus/ovulation in cycling beef cattle and non-lactating dairy heifers”. Syncro-Mate-B consists of a 6-mg norgestomet poly-hydroxy polymer

implant inserted subcutaneous for 9 d plus an intramuscular injection of 3 mg of norgestomet and 5 mg of EV at the time of implantation.

Prostaglandin F2alpha

Prostaglandin F2a is one of three types of prostaglandins. These hormones are members of a group of lipid compounds that are derived enzymatically from fatty acids. Every prostaglandin contains 20 carbon atoms, including a 5-carbon ring. Prostaglandins were isolated for the first time from the seminal fluid in 1935 by the Swedish physiologist Von Euler (1935), and independently by Goldblatt (1935). As it was believed to be part of the prostatic secretions, they received the name of "prostaglandin" from the prostate gland. Although, secretions from the prostate gland contain prostaglandin, it was found in later research that many other tissues secrete prostaglandins for various functions.

Prostaglandin F2a can be use for luteolysis or regression of the CL in cattle. The luteolytic action of PGF2a was reported by Lauderdale (1972), Liehr et al. (1972), and Rowson et al. (1972). The effective dose to produce luteolysis in cattle is 25 mg of PGF2a given intramuscularly (Lauderdale et al., 1977), and can be injected at 11-14 d interval (Lauderdale et al., 1981). In this study, Lauderdale et al., (1981) evaluated the single or double PGF2a injection management. In the double PGF2a injection cattle were injected intramuscularly in the gluteal muscles twice at 11 d interval and detected in estrus after the last treatment. Percent of beef heifers detected in estrus during the first 5 d was significantly greater than the control (64% versus 17% respectively). In dairy heifers, a similar result (73% of heifers in estrus for the treatment group versus 12% for the controls) was observed.

The use of PGF2a was approved by the FDA using two injections given 11 to 14 d apart (1979) or by a single injection (1981) for estrous synchronization programs in cattle. Dinoprost tromethamine (25 mg intramuscular) is the salt of the naturally occurring PGF2a (Lutealyse®,

Pfizer Animal Health, New York, NY). Synthetic analogs of PGF2a that also are approved by the FDA for cattle are: Closprostenol sodium (500 µg intramuscular, Estrumate®, Schering-Plough, Animal Health Corp, Summit, NJ) and Fentprostalene (1 mg subcutaneous, Bovilene®, Fort Dodge Animal Health, Division of Wyeth, Madison, NJ).

Combination of progesterone and PGF2a

Progesterone in combination with PGF2a for estrous synchronization of cattle was in the form of silastic coils impregnated with progesterone wrapped around a stainless steel core and inserted into the vagina. This product was known as a PRID (progesterone releasing intravaginal device) and was used alone for the first time by Roche (1976). The PRID was inserted vaginally for 6 or 7 d combined with a PGF2a injection at d 6. This protocol enhanced P/AI compared with the use of PGF2a alone (Smith et al., 1984).

Another intravaginal progesterone insert developed for estrous synchronization is the CIDR (controlled internal drug release) insert that contains 1.38 g of progesterone. In 1997, the application of the CIDR (Eazi-Breed CIDR®, Pfizer Animal Health, New York, NY) insert in combination with PGF2a was approved by the FDA for estrous synchronization of beef cattle and dairy heifers. Later, it was approved for the same purpose in lactating dairy cattle.

In an extensive field study conducted with beef cows, beef heifers and dairy heifers, the CIDR was inserted during 7 d and 25 mg of PGF2a was injected on d 6. The incidence of estrus during the first 3 d after the CIDR- PGF2a protocol was: in postpartum beef cows 59% versus 12% for control; in beef heifers 65% versus 13% for control; and in dairy heifers 84% versus PGF2a alone treated heifers. The concurrent treatment of CIDR insert and PGF2a improved synchronization rates relative to PGF2a alone or the control (Lucy et al., 2001).

Gonadotrophin releasing hormone

Gonadotrophin releasing hormone, also known as gonadorelin, is a decapeptide hormone produced by the hypothalamus, obtained either from natural sources or is synthetically produced. The commercially available products in the United States are the gonadorelin hydrochloride and the gonadorelin diacetate tetrahydrate.

The first study reporting the effect of GnRH on both FSH and LH in cattle was conducted by Kaltenbach et al., (1974). They injected 250 µg intramuscularly or 1 mg of GnRH as a single intracarotid injection to beef heifers; they measured pituitary hormones in blood samples collected every 15 minutes during 3 hours after GnRH injection. They found that GnRH can stimulate release of both LH and FSH in heifers, and that 250 µg of GnRH given intramuscularly is as effective as 1 mg given intracarotid. The quantity of LH and FSH released appears to be affected by the endogenous milieu of steroid hormones. They noted a small response to GnRH given during the luteal phase of the estrous cycle but an increase in serum LH if GnRH was given at or near estrus when peripheral levels of estradiol are relatively high, suggesting that LH and FSH release appears to be affected by the endogenous milieu of steroid hormones. This study gave an important approach to the bases for understanding the bovine estrous cycle. Another important study in this aspect was done by Thatcher and Chenault, (1976), testing differences in LH responsiveness to GnRH at different intervals after PGF2a treatment. On this study, 25 heifers were assigned randomly to receive 100 µg GnRH i.m. at 0, 12, 24, 48, and 60 h after PGF2a, and blood samples were collected after GnRH injections to measure progesterone, estradiol and LH in plasma. It was found that at 48 and 60 h after PGF2a injection, plasma LH responses to GnRH were maximum, related with a decline in plasma progesterone and an increase in estradiol levels. The authors concluded that GnRH after PGF2a treatment will

synchronize the preovulatory surge of LH, given an important contribution to the knowledge for the development of ovulation synchronization programs.

Subsequent studies on the utilization of GnRH reported that the larger follicle in the ovary could ovulate or undergo atressia in response to exogenous GnRH leading to the concept that ovulation would be achieved or not, according the stage of follicle dominance when GnRH is injected (Thatcher et al., 1989; Twagiramungu et al., 1992a,b; Schmitt et al., 1994). Understanding of follicular dynamics that occur in the ovary during the estrous cycle (see below) gave the link to the use of GnRH for controlling the estrous cycle in cattle.

The commercial available products containing GnRH are in two chemical forms. In the form of diacetate tetrahydrate (100 μg/cow, intramuscular or intravenous) the product is Cystorelin® (Merial Ltd, Duluth, GA), this company has also pioneered: Fertelin™ and OvaCyst™ (IVX Animal Health, Inc., St. Joseph, MO) and Fertagyl® (Intervet, Inc. Millsboro, DE). In the form of diacetate hydrochloride (100 μg/cow, intramuscular) the product is Factrel® (Fort Dodge Animal Health, Division of Wyeth; Fort Dodge, IA).

The first GnRH approved by the FDA in 1986 for its use in cattle was Cystorelin, indicated for the treatment of ovarian cysts. The other generics of Cystorelin were approved subsequently.

Identification of the Ovarian Follicular Waves of Cattle: Ultrasonography

The first study postulating that 2 waves of follicular activity occur during the bovine estrous cycle was done by Rajakoski (1960) following histological examinations of ovaries recovered on known d of the cycle.

A precise method for sequential in vivo monitoring of the ovarian structures was not available until the development of an ultrasound-based diagnostic imaging technique, referred as ultrasonography (US). The first compound contact B-mode scanner (a linear array of transducers that simultaneously scans a plane through the body that can be viewed as a two-dimensional

image on a screen) was developed in 1962, after about two years of work, by Joseph Holmes, William Wright, and Ralph Meyerdirk, supported by U.S. Public Health Services and the University of Colorado (Woo, 2002).

Ultrasonography was used by Pierson and Ginther (1984) to study ovarian dynamics in the bovine for the first time. They examined the reproductive tracts of five heifers by US to monitor follicular and luteal dynamics. The ultrasound used was a Fischer Vetscan (Fischer Imaging Corporation, Schiller Park, Illinois). Ovulation was detected by the disappearance of the larger follicle (>13 mm) in an ovary. Principal points that they concluded after this study were: 1) growth of a large follicle to an ostensibly ovulatory size followed by regression at approximately mid-cycle, 2) selective accelerated growth of the follicle destined to ovulate approximately three d prior to ovulation, and 3) regression a few d before ovulation of the larger follicles that were not destined to ovulate. This study supported the hypothesis that 2 waves of follicular activity occur during the bovine estrous cycle. In subsequent studies by the same authors, they determined that some animals could present 3 follicular waves instead of 2 (Pierson and Ginther, 1986; 1987). This theory was sustained by studies done by Savio et al. (1988) and Sirois and Fortune (1988). Ginther et al., (1989) studied the temporal associations of follicular events in 15 Holstein heifers, with 2 or 3 follicular wave cycles. Basically, they found that the follicular waves appear around d 0.2 and 9.6 in the 2-waves cycles; and around d 0.5, 9 and 16 for the 3-waves cycles. There were no differences in the characteristics of the first wave in both types of cycles. Interval from detection of the dominant follicle to cessation of growth of the associated subordinate follicles was around 3 d, and did not differ among types of waves in both types of cycles. Nevertheless, for the ovulatory wave the length of the interval from emergence of the follicle to ovulation was 10.9 d for the 2-wave cycle, with a diameter of the dominant follicle on

the d before ovulation of 16.5 mm. For the 3-wave cycle, the length of the interval from emergence of the follicle to ovulation in the third wave was 6.8 d and the diameter of the ovulatory follicle was 13.5 mm. The mean length of 2-wave cycle was shorter than for 3-wave cycle (20.4 d versus 22.8 d, respectively). The mean d of the luteal regression was 16.5 d for the 2-wave cycle and 19.2 d for the 3 wave cycle (Figure 2-5).

From these results, Ginther et al. (1998) concluded the following: 1) the dominant follicle that emerges in each wave produces regression of subordinate follicles of the wave and also, suppresses the emergence of another wave, 2) the periodic development of waves continues until CL regression and 3) the dominant follicle present at the time of luteolysis becomes the preovulatory follicle. It was also defined that "selection" of the dominant follicle occurs when diameter deviation in growth rate between the dominant and largest subordinate follicle (Ginther et al., 1996; Ginther, 2000). The average diameter of the largest follicle of the wave at the onset of diameter deviation is 8.5 mm (Ginther et al., 1996). At this time, the dominant follicle acquires the ovulatory capacity and will respond to exogenous GnRH. This response increases with the growing in diameter of the dominant follicle (Sartori et al., 2001).

Recognition of follicular dynamics that occurs in each estrous cycle was the key for the understanding of the response to GnRH injection according to the stage of follicular development. With this knowledge, the use of hormone combinations not only allows control and synchronization of estrus and synchronization of ovulation in cattle. In ovulation synchronization protocols it is important to manage the time when GnRH is given in relation with the stage of the follicle dominance, in order to successfully achieve ovulation of the dominant follicle at a predicted time. If the approximate time that ovulation occurs is known, AI can then be performed at a fixed time, avoiding the necessity for estrous detection.

Estrous Cycle Synchronization Protocols

The purpose for regulating the estrous cycle in cattle is to control the time of estrus in order to reduce the labor required for daily estrous detection, concentrating observation efforts during periods of expected manifestation of estrus (Larson and Ball, 1992).

There has been numerous estrous synchronization protocols developed in cattle using PGF2a alone or in combination with progestogens. An estrous synchronization protocol widely used worldwide is two injections of PGF2a; the first injection is given at a random d of the cycle followed by the second injection 11-14 d later (Lauderdale et al., 1981). This interval is recommended based on the fact that sufficient time must pass to allow those animals with a CL that responded to the first PGF2a injection to have a new CL mature enough to respond to the second injection. As PGF2a is not effective to regress a developing CL that are present on the ovary during the first 5 d of the estrous cycle (Lauderdale, 1972), those animals that did not respond to the first injection, should have a mature CL capable to undergo regression at the second PGF2a injection 11 or 14 d later (Lucy et al., 1986). Therefore, all animals become synchronized after the second injection.

A limitation of the PGF2a use for synchronization of estrus is that its effectiveness depends on the presence of a CL. Consequently, it will not be successful if anestrous cattle or prepuberal heifers are part of the group to be synchronized (Short et al., 1990; Patterson et al., 1992). One method that could improve synchrony of estrus after a single injection of PGF2a is to treat cattle with a progestogen for 7 d before PGF2a (Macmillan and Peterson, 1993). An effective protocol to improve synchronization rate consists of the application of a CIDR insert for 7 d with an intramuscular injection of PGF2a (25 mg, Lutealyse®) on d 6 (Lucy et al., 2001), as shown in figure 2-6. This program has the advantage that includes a progesterone source, which could initiate estrus and ovulation in prepubertal heifers and anestrous cows (Anderson et

al., 1996; Fike et al., 1997). Therefore, it is effective in both non-cyclic and cyclic cattle (Lucy et al., 2001).

Ovulation Synchronization Protocols

In the protocols mentioned above, detection of estrus is facilitated by the fact that estrual activity occurs during a specified period of time after protocol implementation. Nevertheless, the effectiveness of the estrous synchronization strategies must rely on visual detection of estrus (Rivera et al., 2004). Thus, research has focused on the development of synchronization programs that could overcome the problems and limitations associated with visual detection of estrus (Fricke, 2004). Such programs are based on the understanding of factors that control ovarian follicular growth to synchronization of follicular development, luteal regression and ovulation; therefore, AI can be conducted at a fixed-time without the need for estrous detection (Thatcher et al., 2000)

The Ovsynch protocol

One of the first ovulation synchronization protocols successfully developed for lactating dairy cows was the Ovsynch (Pursley et al., 1995). Subsequent studies modified the original Ovsynch protocol in order to improve synchrony and fertility to the protocol. Such modifications include presynchronization with PGF2a (Moreira et al., 2001), altering the timing of AI in relation to ovulation, and testing the various injection intervals of the original protocol (Fricke, 2004). However, these protocols not only have to be successful in achieving acceptable P/TAI but they must be practical to implement within the daily operation of the farm or the protocol will fail due to lack of compliance (Fricke, 2003).

The Ovsynch protocol consists of administering GnRH, followed 7 d later with an injection of PGF2a, 48 h later a second administration of GnRH and TAI 16 to 24 h later (Pursley et al., 1995; Burke et al., 1996) (Fig 2-7). The physiological principles for the Ovsynch

protocol have been reviewed by Pursley et al. (1995). The intention for the first injection of GnRH is to cause an ovulation of a large functional follicle, inducing a new follicular wave and increasing the likelihood for a large growing follicle at the time of PGF2a injection. Another function for this first GnRH injection is to increase the percentage of animals synchronized to a single injection of PGF2a, because a higher synchronization rate was achieved when GnRH was injected 6 or 7 d prior to PGF2a administration (Thatcher et al., 1989). The 7 d period between the first GnRH injection and the PGF2a injection was based on the fact that lactating dairy cows have a responsive CL by 7 d after estrus. The purpose of the second GnRH injection in the Ovsynch protocol is to ovulate the preovulatory follicle from the induced follicular wave after the first GnRH at a precise time, in order to increase synchrony of ovulation.

In the study by Pursley et al. (1995), a high percentage of lactating cows (90%) at a random stage of the estrous cycle ovulated a follicle after this first injection of GnRH. In contrast, only approximately 50% of heifers ovulated a follicle to this injection. With the second GnRH injection, ovulation was synchronized within an 8-h period in all lactating cows and in all heifers in which a CL was regressed.

Several studies have shown Ovsynch to be a highly effective and an economical strategy for improving reproductive performance in high-producing lactating dairy cows (Burke et al., 1996; Pursley et al., 1997; Risco et al., 1998). Further modifications of the Ovsynch protocol, like presynchronization with double injection of PGF2a by 14 d apart before the initiation of the Ovsynch protocol 12 d after the second PGF2a (Moreira et al., 2001) or the use of the double Ovsynch program (Souza et al., 2008) have shown to improve fertility to the Osynch protocol.

The Co-Synch protocol

The Co-Synch protocol is based on the same physiological principles of the Ovsynch protocol, with the difference that TAI is performed at the same time of the second GnRH

injection (Geary and Whittier, 1998) (Figure 2-9). Thus, it requires one less handling of cows, resulting in a more labor efficient synchronization program. This is especially important when the protocol is applied to beef cattle. An early study comparing the Ovsynch and the Co-Synch protocol in beef cows found a decrease in P/TAI for the Co-Synch (49% versus Ovsynch, 57%), (Geary and Whittier, 1998). Nevertheless, subsequent data from the same authors have shown no difference in P/TAI between beef cows receiving Ovsynch or Co-Synch protocols (Ovsynch: 57%; Co-Synch: 58%) (Geary et al., 2001). In an ovulation synchronization protocol, the expected time of ovulation is between 24 to 32 h after the second injection of GnRH (Pursley et al., 1995). Thus, TAI could be performed at the same time of the induced LH surge (0 h) or 24 to 32 h before ovulation, just prior to ovulation (24 h), or between these two times (8 and 16 h). A decrease in P/TAI is obtained if TAI is performed after the time of ovulation (36 h) and the ideal time to perform TAI is around 16 h after GnRH (Pursley et al., 1998). As insemination occurs closer to the time of ovulation, P/TAI for cows TAI 24 h after GnRH might be greater than those TAI together with GnRH. However, no difference in P/TAI were found between both treatments (Portaluppi and Stevenson, 2005); or for cows TAI together with GnRH, or at 8, 16, and 24 h after GnRH (Pursley et al., 1998).

The 5 d Co-Synch + CIDR protocol

This protocol was developed by Bridges et al. (2008), and consists in a modification of the Co-Synch program. The interval from the first GnRH treatment to the PGF2a injection is reduced to 5 d and the proestrus interval from PGF2a to the second injection of GnRH/TAI is lengthened to 3 d. As a CIDR insert was applied between the first GnRH and the PGF2a injections the protocol is named as 5 d Co-Synch + CIDR (Figure 2-9).

This modification of the Co-Synch program was based on the hypothesis that reducing the interval from the initial GnRH treatment to PGF2a and lengthening the interval until the second

GnRH injection (proestrus period) would increase estradiol secretion by the pre-ovulatory follicle and increase fertility. The hypothesis was based on the results of a series of experiments performed by Mussard et al., (2003, 2007), summarized on Table 2-1 and explained on page 60. These experiments showed that the longer the proestrus period the higher the P/TAI. In addition, a greater P/TAI was observed in cows with increased estradiol at induced ovulation (Perry et al., 2005). Younger dominant follicles, 4 d after emergence, have increased intra-follicular estradiol concentrations and a greater capacity to produce estradiol than dominant follicles evaluated later in the follicular wave (Valdez et al., 2005). Therefore, increased estradiol production by a younger follicle can be achieved if luteal regression and withdrawal of the CIDR insert occur earlier relative to the follicular wave emergence, and the interval between PGF2a and the second GnRH administration is longer. Furthermore, if follicles fail to respond to the initial GnRH then the reduced period between GnRH and PGF2a and CIDR insert exposure reduces the probability of these follicles from becoming aged or to have a prolonged dominance. In beef cows, this approach resulted in higher P/TAI compared to a 7 d Co-Synch + CIDR with the second GnRH and TAI concurrently at 60 h (80.0% versus 66.7%, respectively). Due to the shortened interval from the initial GnRH to PGF2a in the 5 d Co-Synch + CIDR protocol it is not known whether regression of an induced accessory CL occurs with one injection of PGF2a. Therefore, a second PGF2a injection was applied approximately 12 h after the first PGF2a injection, which results in additional animal handling and cost. However, in beef cows, the use of one injection of PGF2a in the 5 d Co-Synch + CIDR protocol resulted in a 17% reduction in P/TAI compared with the use of two injections of PGF2a (Kasimanickam et al., 2009). In lactating dairy cows, one injection of PGF2a in the 5 d Co-Synch 72 h protocol was not effective as two injections of PGF2a to regress accessory CL, since CL regression was 58.7% and 95.8% for one or two injections of PGF2a,

respectively (Chebel et al., 2008). Two injections of PGF2a appear to be necessary when this protocol is applied to beef or lactating dairy cows.

Reproductive Management of Replacement Dairy Heifers

Artificial Insemination

Replacement heifers represent the future production of a dairy herd. In order to sustain genetic progress and maintain an economic advantage from higher milk production, dairy producers should breed replacement heifers to proven sires used in AI. The main advantage of the use of AI for breeding heifers is the genetic progress that the dairy producer gains in herd replacement. This genetic progress is directly related to an economic advantage for the dairy farm. For example, it has been reported that milk yield in daughters of AI-proven bulls was 366-444 kg greater compared to daughters bred to natural service (NS) sires (Overton and Sischo, 2005). Lifetime profit of dairy replacement heifers is maximized when heifers calve between 23 and 25 months of age (Head, 1992). However, actual calving ages of first calf heifers are greater than these on many herds. Thus, to maintain genetic progress and maximize profitability, heifer breeding programs should include AI and calving to occur around 24 months.

A commonly used reproductive management is AI at detected estrus from a spontaneous displayed estrus (Stevenson et al., 2008a). Obviously, detection of estrus is crucial for effective reproductive management with AI (Ferguson and Galligan, 1993). Estrous detection efficiency is greater for dairy heifers because heifers express estrus more frequently and longer than lactating dairy cows (Nebel et al., 1997).However, in contrast to lactating dairy cows, time spent on estrous detection in heifers is limited, which can delay time of first AI and therefore increase age at first calving, associated with additional costs (Caraviello et al., 2006).

Estrous Synchronization Programs

The use of estrous synchronization programs optimizes the use of AI (Xu and Burton, 1999). Nevertheless, dairy producers must still rely on visual detection of estrus (Rivera et al., 2004).

Estrus could be synchronized in heifers through the use of a double injection of PGF2a 11 to 14 d apart. Almost 100% of heifers should be at the right stage of the cycle to have a CL that respond to PGF2a and therefore, most heifer display estrus within 7 d after the last injection (Jochle et al., 1982). However, this program requires the presence of a CL in the ovary and it would not be effective in prepuberal heifers that are not cycling (Short et al., 1990; Patterson et al., 1992).

Even though heifers display estrus during the 7 d after the double PGF2a injections, the use of other hormones to increase estrous activity after PGF2a administration and thus increase the efficiency of estrous detection, have been evaluated. In a study by Cavalieri et al., (2005) conducted in Australia, estradiol benzoate was used to increase estrous activity and synchronization after the two PGF2a injections. They found that estradiol benzoate increased the percentage of heifers detected in estrus by 80 h, 96 h and 120 h after the two PGF2a injections, by an estimated 29%, 11% and 8%, respectively. However, it decreased P/AI by an estimated 17%.

Other alternative to improve synchrony of estrus is the treatment with an exogenous progestogen for 7 d before PGF2a (Macmillan and Peterson, 1993). This ensures that CL will regress in response to PGF2a because all cattle will have a CL that has developed for at least 7 d. The efficacy of an intravaginal insert (CIDR insert) during 7 d and an injection of PGF2a on d 6 for synchronizing estrus was evaluated in postpartum beef cattle, peripubertal beef heifers, and dairy heifers (Lucy et al., 2001). In dairy heifers, a greater incidence (84%) of estrus was

detected during the first 3 d of the breeding period compared with the PGF2a treated heifers (57%), but P/AI during the first 3 d or during the 31 d breeding period were not improved for CIDR+ PGF2a compared with PGF2a treated dairy heifers, although it was higher for beef cows and heifers. They concluded that the inclusion of the CIDR insert in combination with PGF2a improved estrous synchronization rates relative to PGF2a alone or control. Improved estrous synchrony led to greater P/AI for beef cows and beef heifers but failed to improve P/AI for dairy heifers.

Ovulation Synchronization Protocols

Importance of ovulation synchronization protocols compared with estrous synchronization protocols.

The use of AI to breed heifers is underutilized on many dairy farms, and the use of NS alone or in combination with AI is preferred (Hogeland and Wadsworth, 1995). A major limitation for use of AI in dairy replacement heifers is time and effort related with daily estrous detection (Erven and Arbaugh, 1987; Caraviello et al., 2006), especially if heifers are located at remote locations. Ovulation synchronization protocols that include TAI, could allow for increased use of AI in heifers, avoiding the need for detection of estrus (Peeler et al., 2004).

In addition to the advantage that represents the increase use of AI for genetic improvement of the dairy, the implementation of an ovulation synchronization protocol also represents an economical advantage for dairy producers. Detection of estrus requires more labor per animal and therefore increases costs compared to a synchronization program on a dairy farm (Olynk and Wolff, 2008). In an overall economical analysis of reproductive management strategies used in the United States commercial dairy farms, synchronization programs had a greater expected net present value (NPV) than visual estrous detection programs (Olynk and Wolff, 2008). With a TAI management program, the time from puberty to conception is reduced, representing a lower

negative cash flow due a decrease in feeding cost, and higher positive cash flow from an increase in the lifetime profit of heifers (Moreira, 2009).

The Ovsynch protocol in dairy heifers

When the Ovsynch protocol was developed for use in dairy cattle, it was evaluated either in lactating cows or heifers (Pursley et al., 1995). The authors found that 90% (18/20) of cows, but only 54% (13/24) of heifers responded to the first injection of GnRH by ovulating a dominant follicle. Due to the lower percentage of heifers ovulating to the first injection of GnRH, which resulted in only 75% of heifers being synchronized, this protocol did not appear to be as effective for synchronizing heifers as lactating dairy cows.

The Ovsynch protocol was compared to a detection of estrus program consisting of an injection of GnRH agonist followed by a PGF2a injection 7 d later and AI at detected estrus (Schmitt et al., 1996). The Ovsynch protocol had greater P/TAI if the GnRH agonist injection was administered 48 h after PGF2a injection (d 9) instead of 24 h (d 8) (45.5% versus 25.8% respectively). Nevertheless, P/TAI were always reduced in the TAI protocol.

These studies lead to the conclusion that dairy heifers respond poorly to the Ovsynch protocol, consequently the application of this program has not been recommended for dairy heifers.

A modification of the Ovsynch protocol was tested by Rivera et al. (2004). Heifers were synchronized for TAI in the following manner: GnRH at d 0, PGF2a at d 6 and GnRH at d 8 together with TAI (6 d Co-Synch 48 h protocol). Pregnancy per AI was compared between these heifers and heifers that were detected in estrus and AI. Pregnancy per AI at 30 d after first AI tended to be greater for heifers detected in estrus (46.5%) than for heifers bred to the 6 d Co-Synch protocol (38.3%), although there was a technician effect. Because the 17.7% of the heifers on the TAI protocol displayed estrus before the second GnRH injection, the authors concluded

that the TAI protocol can result in an acceptable P/AI if detection of estrus is performed between the GnRH and PGF2a treatment and heifers are AI at detected estrus.

The "problem" with ovulation synchronization protocols in dairy heifers

The pattern of follicular development in heifers is different from that of lactating dairy cows (Sartori et al., 2004). Heifers have a faster rate of follicular growth (Pursley et al., 1997) and a higher frequency of thee wave follicular cycles (Savio et al., 1988). Therefore, if an ovulation synchronization protocol is started with GnRH at a random stage of the estrous cycle, failure to synchronize ovulation for TAI occurs. When the first GnRH injection is given at the beginning of a follicular wave, i.e. at d 2 or 10 of the cycle, a dominant follicle is not present, resulting in an low ovulation frequency to the first GnRH injection (Moreira et al; 2000). If a new follicular wave emerges, LH receptors are not expressed in the granulosa cells of growing follicles during the first d of the follicular wave (Xu et al., 1995). This is prior to the time of deviation in follicular growth rates between eventual dominant and subordinate follicles that occur on average at d 2.8 d after follicular wave emergence, when dominant follicle diameter is 8.5 mm and 7.2 mm for the largest subordinate follicle (Ginther et al., 1996).

In heifers with three follicular waves, approximately 57% of the estrous cycle is at a stage of follicular development that is not responsive to the first GnRH injection to cause an ovulation and initiate a new follicular wave. As a result of this lack of ovulation to the first GnRH injection (e.g., given at day 16 of cycle with a heifer having a 3-wave follicular cycle), the CL originating from the preceding spontaneous ovulation regresses before PGF2a treatment. Thus, heifers would express estrus prematurely close to the time of the PGF2a injection (Rivera et al., 2004, 2005).

It has been shown effectively that stage of the estrous cycle at which synchronization is initiated influences reproductive responses to the TAI protocol. If the protocol is initiated during

an environment of high progesterone concentration (d 5 to 10 of the estrous cycle) then heifers had improved synchrony and fertility after TAI compared with heifers initiating the protocol during other stages of the cycle (Moreira et al., 2000).

Strategies to avoid expression of estrus during TAI protocol

Strategies have been developed to overcome expression of estrus during TAI protocols by synchronizing heifers in the early luteal phase, to avoid detection of estrus during the synchronization period. These strategies mainly focus on two components: 1) presynchronization prior to initiation of the TAI protocol and 2) supplementation of exogenous progesterone during the synchronization period to avoid premature ovulation and asynchrony of insemination.

Presynchronization programs: It has been shown that the use of a double injection of PGF2a administered 14 d apart for presynchronization before the initiation of the Ovsynch protocol 12 d after the second PGF2a, increase fertility in lactating dairy cows (Moreira et al., 2001). The program is known as the Presynch/Ovsynch. In dairy cows, this program increased P/TAI by 17.3 percentage units at 72 d compared with cows receiving Ovsynch without presynchronization (Moreira et al., 2001). The Presynch/Ovsynch program with the second GnRH injection at 56 h and TAI at 72 h had greater P/TAI (38.6%) compare to Presynch/Co-Synch at 48 h (29.2%) or 72 h (25.4%) (Brusveen et al., 2008). In other study it was evaluated P/TAI and incidence of ovulation to the first GnRH obtained when the interval from presynchronization to initiation of the TAI protocol was reduced from 14 to 11 d in cows (Galvao et al., 2007). Compared with 14 d, lactating dairy cows presynchronized 11 d before initiation of the TAI protocol had increased P/TAI (40.5% at 38 d versus 33.5% for cows with 14 d of interval). This increase in P/TAI observed with the 11 d interval was likely the result of improved ovulatory response to the first GnRH because cows that ovulated had increased P/TAI.

In dairy heifers, the application of a GnRH injection 7 d before the onset of the 6 d Co-Synch 48 h TAI protocol has been evaluated (Rivera et al., 2006). These authors hypothesized that with the GnRH injection before the TAI protocol, an environment of high progesterone concentration due to ovulation of a dominant follicle and production of CL would occur. Nevertheless, proportion of heifers displaying estrus before the scheduled TAI and mean d of estrous expression during the protocol did not differ between the application of GnRH for presynchronization or not. This fact was attributed precisely to the higher frequency of three follicular waves and more rapid turnover of follicles in dairy heifers. Pregnancy per TAI at 30 d did not differ between treatments (44% for no presynchronization versus 49% for presynchronization). The authors concluded that presynchronization with an injection of GnRH 7 d before the onset of a 6 d Co-Synch 48 h TAI protocol failed to improve synchronization response in randomly cycling dairy heifers.

Supplementation of exogenous progesterone during the synchronization period: In a study by Rivera et al. (2005) it was evaluated the proportion of heifers submitted to TAI in heifers synchronized with the 6 d Co-Synch 48 h protocol with the inclusion of a CIDR insert (6 d Co-Synch 48 h + CIDR) compared with the 6 d Co-Synch 48 h protocol without a CIDR insert. Pregnancy per TAI did not differ between both groups (29% for the group without the CIDR insert and 32% for the group with a CIDR insert). In this experiment, P/ TAI was profoundly affected by variation among herd AI technicians. However, as an interaction between treatment and technician was not detected, the main effects of treatment on experimental endpoints were valid. One of the main objectives of this study was to evaluate if the inclusion of the CIDR insert between the first GnRH injection and the PGF2a injection would suppress estrus without affecting fertility. Results showed that none of the heifers receiving the CIDR insert displayed

estrus during the protocol before scheduled TAI, whereas 24% of heifers when the CIDR insert was not included expressed estrus 4.5 ± 0.4 d after the first GnRH injection.

In conclusion, the inclusion of a CIDR insert in a TAI protocol may be successfully implemented when detection of estrus is a limiting factor for AI programs in dairy heifers.

Resynchronization of Nonpregnant Heifers after First Service

When inadequate protocols are used, such as the regular Ovsynch, a majority of heifers submitted to the first TAI fail to conceive. To avoid detection of estrus after first TAI for a second breeding of nonpregnant heifers, a useful alternative is to resynchronize ovulation after the first TAI protocol.

Different synchronization strategies have been evaluated. In an early study by Van Cleeff et al., (1996) dairy heifers were synchronized for estrous detection with a CIDR insert during 9 d and injected with PGF2a on d 7. From all the heifers treated, 85% of them were detected in estrus and inseminated between 48 h to 72 h after CIDR insert removal. Those heifers were assigned to no further treatment or resynchronization of return to service with a used CIDR insert for d 17 to 22 after AI. They showed that the inclusion of the CIDR insert from d 17 to 22 after AI was effective to resynchronize return to estrus of nonpregnant heifers. In this study, although detection of estrus was performed either for first and second service it contributed to the concept of the utility of a resynchronization program to facilitate management of nonpregnant heifers after the first service.

In the study mentioned above by Rivera et al. (2005), besides evaluating the inclusion of a CIDR insert in the TAI protocol for the first TAI service, they also determined the effect of a CIDR insert to resynchronize return to estrus for dairy heifers failing to conceive to first TAI. Heifers were assigned to receive no further treatment or to receive a new CIDR insert during d 14 to 20 after TAI. CIDR resynchronization resulted in a tighter synchrony of estrus for heifers

failing to conceive to first TAI, since 78% of the heifers came in estrus during 72 h after the CIDR removal compared with 50% of controls detected in estrus during that time. Heifers were AI at detected estrus for the second service. The use of the CIDR insert for resynchronization increased P/AI to second service, since P/AI at 40 to 60 d was 47% for the group receiving the CIDR insert between from 14 to 21 d after TAI at first service compared with 26% for the control group, re-inseminated for a second AI service after rubbed tail chalk.

In a study conducted in lactating dairy cows by Fricke et al. (2003), cows were synchronized with a Presynch plus Ovsynch protocol for the first TAI. For resynchronization of a possible second TAI, the Co-Synch schedule was used (GnRH d 0, PGF2a at d 7, and GnRH + TAI at d 9). Cows were assigned to start the first GnRH injection of the resynchronization protocol at d 19, 26 and 33 after the first TAI. Nonpregnancy diagnosis by ultrasound after first TAI was performed at d 26 in the 19 and 26 d group and at 33 d in the 33 d group. GnRH was administered to all cows in the 19 d group but only to nonpregnant cows in the 26 and 33 d groups. Although, administration of GnRH to pregnant cows at 19 d after first TAI service did not result in embryonic losses, initiation of resynchronization protocol 19 d after first TAI service resulted in a lower P/TAI (23%) to the resynchronized TAI (second service) compared with initiation of resynchronization at 26 (34%) or 33 (38%) d after the first TAI service. The authors concluded that a good management strategy to implement a resynchronization protocol is at the time of the pregnancy diagonosis by ultrasound at 26 d or 33 d (or by transrectal palpation at 33 d).

The effects of a second resynchronization protocol starting on d 26 after TAI on ovarian profile and d open in dairy cows diagnosed nonpregnant were evaluated by Osawa et al. (2009). Holstein cows received an Ovsynch protocol for a first TAI. At d 26 after TAI, pregnancy

diagnosis by ultrasound was performed. Nonpregnant cows were assigned to be resynchronized with the Ovsynch protocol initiated at the same d of the ultrasound diagnosis or insemination at detected estrus. The ovarian and hormonal profiles were compared between the first and second Ovsynch protocol periods in the resynchronized cows. The authors found that during the resynchronization period at 26 d , the diameter of the dominant follicle and plasma estradiol concentration were significantly greater and the plasma progesterone concentration significantly lower at the time of the second GnRH injection compared with the time of the PGF2a injection. These differences were not detected during the first synchronization period when animals were at different stages of the estrous cycle, concluding that the stage of the estrous cycle could be more consistent among animals during the second synchronization period than during the first. Pregnancy per AI was 30.4% and 14.3% for the resynchronization or control groups respectively; concluding that resynchronization at 26 d of nonpregnant cows could be successfully applied for a second TAI.

Similar reproductive responses could be expected in dairy heifers if resynchronization is initiated at the time of pregnancy diagnosis. Heifers exhibit higher P/AI than lactating cows (Pursley et al., 1997). Consequently, the application of a TAI protocol either for the first and second TAI could result in a high proportion of heifers pregnant after two ovulation synchronization managements, avoiding the need for estrous detection.

Factors Affecting Pregnancy in Dairy Cattle

Definitions

There are two main reasons why dairy cattle do not become pregnant after AI, fertilization failure and pregnancy loss. Pregnancy loss can occur during the embryonic period (before d 42 of gestation) or during the fetal period (after d 42 of gestation). These terms were standardized by the Committee on Bovine Reproductive Nomenclature (1972) (Santos et al., 2004).

Furthermore, embryonic losses could be further classified as early embryonic loss if it is prior to d 16 of gestation with luteolysis around d 17 and a return to estrus around d 21 to 24. In late embryonic loss after d 16 of gestation, luteolysis does not occur immediately and the CL lifespan is slightly extended and return to estrus is delayed beyond d 24 (Humblot, 2001).

Embryo Survival in Dairy Heifers and Lactating Dairy Cows

Fertilization failure and early embryonic loss

Sartori et al., (2002) evaluated fertilization rate and embryonic development in dairy cows and heifers during the warm season and in dairy cows during the cool season. In heifers, they reported a 100% fertilization rate with 71.9% of viable embryos at d 6 after ovulation. In lactating cows, fertilization rate and percentage of viable embryos was 55.3% and 33.3%, respectively for the warm season and 87.8% and 52.8%, respectively for the cool season. This finding supports the detrimental effect of heat stress on embryo quality and survival in lactating cows.

Late embryonic and fetal loses

Results from several studies have shown that in dairy heifers, embryonic and fetal losses are lower than in lactating dairy cows. In nulliparous dairy heifers, late embryonic and fetal losses average around 2.5% (ranging from 1.5% to 10.2%) compared to an average of 10.7% (ranging from 8.3% to 24.0%) in lactating dairy cows (Santos et al., 2004).

Differential Factors Affecting Embryo Survival between Dairy Heifers and Lactating Dairy Cows

Several factors contribute to the difference in embryo survival between heifers and cows. Although, there is a lack of research on factors that affect pregnancy in dairy heifers, described below are some distinct characteristics between lactating cows and heifers than may contribute to

the difference in embryo survival. These factors are mainly related with physiological characteristics of cows due to lactation.

Milk production and metabolism

One obvious difference between cows and heifers is that cows have to become pregnant when they are lactating, whereas heifers do not. Moreover, lactating cows have been genetically selected through the years for increased milk yield. Analysis of several data sets clearly shows an antagonistic relationship between milk production and reproduction in dairy cattle (Dematawewa and Berger, 1998; Hansen, 2000). In one study, Harrison et al., (1990) investigated during the first 75 d postpartum the primary metabolic adjustments that relate to the reproductive status of two groups of cows with high and average milk production. The main differences that they found for cows with high milk production were a longer period of suppressed estrous behavior and an increased gross efficiency, suggesting that high milk production cows use more of their digestible energy for milk production or that they mobilized more body reserves. They also maintained a higher feed intake compared with the average group. The higher feed intake could increase liver blood flow which could increase metabolism of steroid hormones, like progesterone and estradiol. This hypothesis was tested by Sangsritavong et al., (2002). They measured steroid metabolism by continuous infusion of progesterone and estradiol and calculated the metabolic clearance rate for these hormones under various physiological conditions. They also estimated the liver blood flow using the metabolic clearance rate of bromosulphthalein. Their principal findings were that in response to increased feeding of lactating cows (cows under a continuous high plane of nutrition) there is chronically an elevation of liver blood flow and metabolic clearance rate of progesterone and estradiol. In consequence, a similar production level of progesterone or estradiol may result in much lower circulating steroid concentrations in lactating dairy cows. Lower estradiol concentrations could be related with

lower expression of observed estrus, as was noted in the study by Harrison et al. (1990). Lower progesterone concentration may affect embryo survival, since progesterone plays a major role in stimulating the production of several endometrial proteins and growth factors (Geisert et al., 1992), orchestrating the histotrophic environment for nourishment of the conceptus (Santos et al., 2004). It has been shown that during the estrous cycle and despite the larger luteal tissue, serum progesterone concentration is lower in cows than in heifers when examined from d 6 of the cycle to ovulation (Sartori et al., 2004; Wolfenson et al., 2004).

In summary, lower progesterone concentration related with higher milk yield could be an important factor determining lower embryo survival in lactating cows compared with no-lactating, nulliparous heifers.

Body condition score

Body condition score (BCS) is an accepted, noninvasive, subjective, quick, and inexpensive method to estimate the degree of fatness (Waltner et al. 1993). The most prevalent method of BCS estimation is based on visual and tactile appraisal of subcutaneous fat in the caudal, dorsal regions, in which cows are assigned a BCS based on a five-point scale (1= emaciated, 3 = average, and 5 = obese) (Wildman et al., 1985). In a study by Waltner et al. (1993) in a herd producing >9500 kg of milk per cow in 305 d, the amount of body fat at calving and the use of body fat, as estimated by BCS, was related to milk production, with an excessive BCS loss during lactation due to negative energy balance. Waltner et al. (1993) also found that BCS at calving and the loss of BCS were related quadratically to milk production. In a study by Domecq et al. (1996), the relationship between BCS and conception at first service in a herd of high yielding Holstein dairy cows (n=720 cows) was investigated. Cows that lost one point of BCS in the first month of lactation were 1.5 times less likely to conceive than were cows that did not lose one point of BCS. In support of this study, it was found that 1 unit drop in BCS from

calving to 30 d postpartum increased the odds ratio (OR) for pregnancy loss by 2.41 fold (López-Gatius et al. 2002) or, if the 1 unit loss was from d 28 to 56 of gestation, cows had a 3.2-fold increase in OR for pregnancy loss in the same period (Silke et al. 2002).

In summary, it is known that the loss of BCS is associated strongly with the negative energy balance as a consequence of high milk yield and inadequate dry matter intake, and that this is also associated with lower probability of conception or higher pregnancy loss after conception. As heifers are not lactating, their BCS is more uniform than the changes observed in lactating cows.

Uterine diseases

Another evident difference between heifers and cows is that heifers have not experienced parturition, in other words, they do not have to become pregnant during a postpartum period as lactating cows.

Parturition predisposes cows to microbial contamination of the uterine lumen which has an important impact on health and productivity (Sheldon et al., 2008). The expression of clinical uterine infection (puerperal metritis, clinical endometritis) depends on the balance between factors such as the animal, immunity, the number and pathogenicity of the microbes, and the uterine environment. Typically, 25–40% of animals have metritis in the first 2 weeks after calving, and the disease persists in up to 20% of animals as clinical endometritis (Sheldon et al., 2008). Uterine diseases are associated with sub-fertility and infertility and are key risk factors for the occurrence of abnormal progesterone profiles indicating delayed ovulation, cystic ovarian disease or long luteal phases (Opsomer et al., 2000; Royal et al., 2000). Furthermore, it is assumed that a healthy endometrium is necessary for successful establishment of pregnancy so, uterine diseases could reduce conception (Sheldon et al., 2008) and also, induce embryo mortality if uterine infection occurs after conception (Semambo et al., 1991).

In dairy heifers, the immunity of the uterus is not compromised because they have not undergone parturition and do not experience uterine involution. Therefore, the uterus of heifers would be "intact" and in a much better conditions at the time of insemination compared with the uterus of a postpartum dairy cow.

Mastitis

Mastitis is an inflamation of the mammary gland caused in cattle primarily by bacteria, and it has been associated with either anovulation at estrus, fertilization failure, or embryonic mortality (Barker et al., 1998; Schrick et al., 2001). Hansen et al., (2004) suggested that activation of inflammatory or immune responses external to the reproductive tract, as occurs in mastitis, can lead to embryonic mortality, and this disrupts the reproductive axis at several points including the hypothalamic–pituitary axis, ovary, oocyte and the embryo. Clinical mastitis is very rare to occur in dairy heifers that have not calved, and in the few cases of mastitis in heifers, the role of the fly in transmission of S. aureus mastitis has been implicated (Fox, 2009). Instead, incidence of mastitis in dairy cows is much more common. In a study that compared the annual prevalence of common diseases on dairy operations in the United States (Hill et al., 2009), it was found that the most common diseases in cows were mastitis and lameness, affecting 16% and 11% of the population, respectively.

Lameness could also be another differential predisposing factor affecting reproduction in cows but probably less in heifers. In the dairy farm heifers are generally located on pastures, and it has been demonstrated that a lower incidence of lameness occurs in a grazing system (Haskell et al., 2006). In Holstein cows, lameness has been related with delayed ovarian activity during the early postpartum period associated (Garbarino et al., 2004) with longer calving to conception interval (Hernandez et al., 2005).

In summary, the factors listed above represent some of the major differences between lactating cows and heifers influencing reproduction. Nevertheless, many other factors (either for the type of management or the animal physiology) could determine the lower fertility observed in lactating cows compared with heifers.

Common Factors Affecting Fertility in Dairy Cows and Heifers

Described below are the main factors that affect similarly cows and heifers that can have an impact on reproduction. These factors are environmental and are related with AI management.

Heat stress

Heat stress can potentially alter the reproductive function not only from a direct effect but also from an indirect consequence of physiological changes for regulation of body temperature (Hansen, 2007). Thermal stress negatively influences bovine fertility in several aspects, and most components of the reproductive system have been found to be susceptible to heat stress including: the oocyte, granulosa and, particularly, theca cells within the preovulatory follicle, the developing embryo during early stages of development, the CL, the uterine endometrium, and the anterior pituitary (Wolfenson et al., 2000).

Heat stress effects are more pronounced in lactating dairy cows, since genetic selection for milk production has produced an animal with high internal heat production, and therefore, with increasing susceptibility to hyperthermia (Hansen, 2007). Lactating cows manifested greater increases in body temperature than heifers exposed to similar environment (Sartori et al., 2002). Nevertheless, it has been shown that heat stress is a factor that also can negatively influence P/AI after first service. A study by Wolfenson et al.(1993) examined the effects of acute heat stress and oxytocin injection on plasma concentrations of PGF2a in cyclic and pregnant dairy heifers, finding a greater proportion of pregnant heifers responsive to oxytocin injection if they were under heat stress. They concluded that under heat stress conditions, the conceptus may not be

fully capable of inducing normal endogenous inhibition of PGF2a synthesis in the endometrium of pregnant heifers, which could lead to embryonic loss. In another study conducted in tropical Australia (Orr et al., 1993), P/AI in heifers was reduced from 80 to 55% as mean daily maximum temperature increased from 26 ºC to 27.5 ºC. At mean maximum temperatures above 27.6 ºC for heifers, P/AI to 3 inseminations were consistently below 60%. Also, Donovan et al. (2003) examined factors associated with conception to first AI in Holstein heifers in Florida. Season of first AI was evaluated as an environmental factor. They found that heifers inseminated during the summer season were 76% less likely to become pregnant to first AI than those inseminated during winter. These studies show that heat stress is an issue factor that can affect fertility in heifers.

Technician effect

Many factors associated to a successful AI have been related to technical expertise, semen handling, and correct semen placement in the uterus during AI (Barth, 1993).

One of the most critical parts of the insemination technique is depositing semen in the uterine body, anterior to the cervix (Nebel, 2007).

In a study done by Peters et al.(1984), radiography was used to locate inseminating syringe tip and the inseminate distribution in excised bovine reproductive tracts. They found that the proportion of syringe tip placements were only 40% in the uterine body and among all inseminators, 82% were unable to place the syringe in the uterine body greater than 60% of the time, with a wide variation among individual inseminators found.

It has been reported that a common mistake of some technicians is to deposit semen in the cervix while withdrawing the pipette or straw during AI (Zavy and Geisert, 1994). Sperm retention by the female reproductive tract is not different if sperm if deposited in the uterine

horns or uterine body. But, if sperm is deposited in the cervix, there is a retrograde sperm loss that is almost twice than the loss observed after corneal deposition (Gallagher and Senger, 1989).

Obviously, not only semen deposition in the animal could affect the successful use of AI, but also all the process related to the insemination technique. In a study by DeJarnette and Marshall (2005), the interactions of straw-thawing method with sire and extender type (milk or egg yolk-based) on post thaw sperm motility were examined. Straw-thawing methods were 35°C water for 45 seconds or after air-thawing by wiping straws with a paper towel upon removal from the storage vessel and placing directly into the insemination gun. They found that among all significant interactions of thaw method with sire and (or) extender type, air-thaw was consistently associated with a lower measure of post-thaw sperm motility or conception.

In another study (Dalton et al., 2004), factors associated with the insemination technique and subsequent P/AI in dairy cows were compared between professional AI technicians and herdsman-inseminators. For the eight commercial herds participating in the study, mean P/AI were 45% and 27% for herds AI with professional or herdsman inseminators, respectively. They stated that technicians could maximize P/AI if the procedures related with the insemination technique are conducted carefully: follow appropriate thawing semen process, use appropriate hygienic procedures, maintain thermal protection of straws during AI syringe assembly and transport to the cow, and deposit semen in the uterus of the cow within approximately 15 min after thawing. As suggested by Nebel (2007), the highest quality semen placed in the most fertile cow at just the right time will not result in pregnancy if the breeding technique is not performed properly. This statement summarizes the importance of technician performance during the AI process.

Sire effect

Quality of semen used for AI is clearly associated with AI success. Differences in post-thaw sperm viability, progression of spermatozoa in the female internal genital tract, capacitation, acrosome reaction and fertilizing capacity are all factors that influence pregnancy results (Januskauskas et al., 1999; Hunter, 2003).Seminal differences that decrease P/AI can be categorized as compensable or uncompensable. Males with compensable deficiencies require higher number of sperm to increase pregnancy, since these defects affect sperm transport and function in the female reproductive tract, including initiation of the fertilization process and the block of polyspermy. When differences in fertility among males or inseminates are independent of sperm dosage then the seminal deficiencies are known as uncompensable. Such deficiencies are important to the maintenance of the fertilization event and subsequent embryogenesis (Saacke et al., 2000; Saacke, 2008a,b). In compensable deficiencies, there are differences among bulls in response to time of insemination (Dalton et al., 2001). If the bull has little to no compensable deficiencies it would easily meet the threshold numbers of sperm to the cow by AI, performing as well at low sperm dosages as at normal, and be less vulnerable than other bulls to inseminator error in semen placement and handling. Therefore, sires that have low compensable deficiencies would perform well over a broader time span relative to when ovulation occurs. In contrast, if seminal compensable deficiencies are high, bulls would be more vulnerable to dilution rates, inseminator competence, and timing of insemination in relation to ovulation, requiring a later breeding, closer to ovulation, to optimize their efficiency in sperm access to the ovum (Saacke, 2008b).

In a study by Kasimanickam et al., (2008) the effect of three sires on pregnancy outcome was evaluated in beef cows synchronized with the Ovsynch + CIDR and Co-Synch + CIDR protocols for TAI. There was no difference in the P/TAI between Ovsynch + CIDR (54.4%) and

CO-Synch + CIDR (52.2%) protocols but, differences in the P/TAI between sires were detected 53.2%, 48.1% and 58.7% for sires 1, 2 and 3 respectively. Sire 2 had poorer P/TAI compared to Sire 3. The authors concluded that there are differences in sire fertility regardless the TAI protocol, possibly due to the sire differences in sperm capacitation process.

Strategies to Improve Fertility and Embryo Survival

Pregnancy result is multifactorial and a diversity of animal management and environmental factors affect fertilization and embryo survival. Aspects from the physiology of reproduction in the animal are difficult to control but several strategies have been developed in order to improve fertility and embryo survival. The following factors have been employed to improve fertility and related to the TAI studies conducted in heifers in this thesis.

Length of proestrus and follicle maturation

It was found that GnRH-induced ovulation of follicles less than 11 mm in diameter resulted in decreased P/AI and increased late embryonic mortality in beef cows (Perry et al., 2005). In addition, it has been reported that at a given diameter, the fertility of the ovulatory follicle is closely related with the length of proestrus and the capacity of the ovulatory follicle to produce elevated concentrations of estradiol preceding TAI, and reducing the proestrus length induces subsequent short luteal cycles reducing P/TAI (Mussard et al., 2003; 2007).

Mussard et al. (2003, 2007) tested the hypothesis that luteal function and P/AI would be reduced in beef cows when follicles that had not reached full maturity were induced to ovulate, compared with cows that spontaneously exhibited estrus and had ovulated. After synchronization of the follicular wave by follicle aspiration, and injections of PGF2a latter at d 1.5 and 2 to induce luteolysis, follicular growth was monitored by ultrasound, and when the dominant follicle reached 10 mm in diameter, cows received 100 μg of GnRH to induce ovulation or no treatment so that cows could ovulate spontaneously. Artificial insemination was performed 12 h after the

onset of estrus in the cows with spontaneous ovulation and 12 h after the GnRH injection in the GnRH treatment. Diameter of follicles ovulating spontaneously was around 12 mm, and luteal tissue and plasma concentrations of progesterone during the subsequent midluteal phase were also greater. They concluded that premature induction of the LH surge reduced the diameter of the ovulatory follicle, luteal function, and P/AI, since P/AI at 30 d was 75.9% for cows with induced ovulation and 100% for cows with spontaneous ovulation. In a second study, Mussard et al. (2003) compared P/AI and luteal function between beef cows induced to ovulate with GnRH a large and mature follicle, or a small and immature follicle. Follicular wave was also synchronized by follicular aspiration but, in this case, a single dose of PGF2a was administered when the newly emerged dominant follicle attained a diameter of 8 mm. Ovulation was induced with 100 µg of GnRH when the diameter of the dominant follicle reached 10 mm in one group of cows (small follicle) or 13 mm in other group of cows (large follicle).AI was performed 12 h after GnRH injections. Cows that were induced to ovulate a smaller follicle had lower concentrations of progesterone and a decrease in P/AI at 30 d (4.4%) compared to cows ovulating a follicle of 13 mm in diameter, which presented a P/AI of 57.4%.

In attempt to determine if such a reduction in P/AI, observed when animals are induced to ovulate smaller follicle, was due to defects in fertilization, embryonic development, early embryonic mortality, or combination of these factors, a third experiment was performed. Yearling beef heifers received a similar scheme of treatment as above (second experiment) but, instead of being inseminated after GnRH induced ovulation, heifers received an embryo at d 7 after the LH surge. Heifers induced to ovulate a large follicle (13 mm of diameter) had 66.7% of pregnancy at 30 d compared to only 8.3% if heifers ovulated a smaller follicle (10 mm), which also presented lower progesterone concentrations. This difference highlighted the fact that

ovulation of smaller follicle could affect the capacity to maintain the pregnancy, rather than cause a failure in fertilization.

With these results, the authors concluded that ovulation of immature follicles not only affects oocyte competence but, also it influences uterine environment necessary to maintain pregnancy. The authors also noted the significant difference in P/AI in cows that were induced to ovulate a 10 mm diameter follicle in these experiments, since in the first experiment P/AI was 75.9% but only 4.4% in the second experiment. They attributed this fact to the difference in times when PGF2a injection was applied, since in the first experiment follicles emerged coincidentally with luteal regression but, in the second experiment, luteal regression was not induced until the dominant follicle reached the 8 mm in diameter. Thus, the longer proestrus period achieved in the first experiment could induce a higher gonadotropic stimulation and more development of the dominant follicle, compared with a dominant follicle of the same diameter developed with a shorter proestrus period. This lead to the hypothesis that length of proestrus is as important as follicle diameter in determining follicle competence, as measured by P/AI. In Table 2-1 is summarized the studies by Mussard et al., (2003, 2007) depicting the relationships between duration of proestrus, follicle diameter and age at ovulation, and P/AI. This Table effectively demonstrates that the longer the proestrus period the higher the P/AI, and this fact is not precisely related with follicle diameter or age (i.e., the dominant follicle has reached at least 10 mm in diameter).

The hypothesis was reconfirmed with a recent study by Bridges et al. (2009), which compared fertility and concentrations of estradiol during proestrus with characteristics of the subsequent estrous cycle in beef cows with long and short proestrus intervals. Following synchronization of the follicular wave through follicular aspiration, the dominant follicle was

induced to ovulate with a GnRH injection after either a 2.25 d (long proestrus) or 1.25 d (short proestrus) proestrus period.. Diameter of the ovulatory follicle was similar between treatments (around 13 mm in one experiment and 12 mm in other), and proestrus was defined as the period from PGF2a injection to GnRH-induced LH surge. They found that cows with a longer proestrus had grater P/TAI (50.0% versus 2.6% for cows with short proestrus), higher concentration of progesterone,less occurrence of short luteal phase in the subsequent luteal phase, and higher concentration of estradiol during the proestrus period (from d -1.9 to d 0, the d of GnRH administration). With these results they highlighted that follicular characteristics beyond follicle diameter are critical in determining follicle maturity, the likelihood of a normal length luteal phase, and fertility.

Therefore, TAI synchronization programs could be modified in a manner to ensure that physiologically mature follicles are present in the ovary. One modification was to shorten the interval between the first GnRH and PGF2a injections and lengthen the interval from the PGF2a injection to the second GnRH injection (Bridges et al., 2008) as previously discussed for the 5 d Co-Synch + CIDR protocol

Maintenance of the CL

The maintenance of the CL as a source of progesterone secretion is essential for pregnancy. Around d 12 after conception, the mononuclear cells of the conceptus trophectoderm secrete interferon τ (Thatcher et al., 2001), reaching a concentration peak in the uterine lumen around d 15 to 17 of pregnancy. This protein inhibits the expression of oxytocin receptors in the luminal epithelium and consequently, inhibits the episodic release of PGF2a from the endometrium (Farin et al., 1990; Demmers et al., 2001). Some of the embryonic losses in cattle could occur because the interferon τ secreted by the conceptus is not able to inhibit the luteolytic cascade of PGF2a (Thatcher et al., 2001). Therefore, a strategy to increase embryo survival could

be to inhibit secretion of PGF2a in order to inhibit the luteolytic process, especially for conceptuses that are slightly behind in development (Guzeloglu et al., 2007). Flunixin meglumine (FM) is a potent nonsteroidal, antiinflammatory agent that inhibits cyclooxygenase, thus preventing conversion of arachidonic acid to PGF2a (Odensvik, 1995). Treatment with FM given to cows by intramuscular injections twice daily during the first 6 days postpartum inhibited the secretion of PGF2a, as indicated by a decrease in the concentration of 13, 14-dihydro-15-keto-PGF2α (PGFM) in the peripheral blood (Guilbault et al., 1987). In a study by Guzeloglu et al. (2007), Holstein heifers were injected with 1.1 mg/kg of FM as a double injection 12 h apart on d 15.5 and 16 of pregnancy (after TAI) and were compared with controls (heifers not receiving FM). Heifers that received FM had a P/TAI of 76.9% at 29 d and 69.2% at d 65, while P/TAI in controls was 50% and 46.2% respectively. The application of FM increased embryo survival in this study. Merrill et al. (2007) studied the effect of FM in transported and non-transported beef cows on P/AI. Transportation included 4 to 6 h by semi-tractor trailer on unpaved and paved roadways. Transported or non transported cows received 1.1 mg/kg of FM approximately 14 d after AI, soon after transportation in the transported group. In both groups, some cows were not injected with FM (control cows). Transportation of cows did not affect P/AI. However, treatment of cows with FM increased P/AI irrespective of whether they were transported or not. This finding resulted in the speculation that stress associated with handling of cows for sample collection, independent of transport stress, could produce a release of PGF2a that FM treatment would lower.

Sexed Semen Technology

Development of the X and Y Sperm Sorting Procedure

In sexed semen, the fractions of X and Y chromosome bearing sperm have been modified from the natural mix through sorting and selection. In an ejaculate, semen contains

approximately 50% of X- or Y bearing sperms. In an attempt to separate these fractions, it was necessary to know the characteristics of each spermatozoon carrying the X or Y chromosome.

Different characteristics between the X and Y chromosome bearing sperm include: size, weight density, swimming speed, electrical surface charges, surface macromolecular proteins, differential effects of pH, and differing effects of atmospheric pressure (Summer and Robinson, 1976; Ericsson and Glass, 1982). However, due to minor difference in these characteristics between the X and Y sperm, it was impossible to use them as a base for sorting purposes. The most important difference that allowed the development of the sorting procedure is the difference in DNA content between X and Y-bearing chromosomes. This was suggested for the first time by Morruzi (1979) who reported that in the bovine, this difference was 4.2% more DNA for the X bearing sperm. Different attempts were performed to separate the sperm based in their DNA content. The best approach was developed by Garner et al. (1983) who demonstrated that the flow cytometric method was capable of precisely determining DNA content differences between X- and Y-sperm. Nevertheless, the sperm were killed in the process of making them permeable to the stain for analysis. Using flow cytometry methodology, it was detected that the actual difference in DNA content between X and Y bearing sperm is 3.7%.

The proven method to separate live sperms was developed by Johnson (1987a,b). The vital stain used was the Hoechst 33342, which stains the DNA in intact sperms without killing them. The method consists of fluorescent activated cell sorting (Figure 2-10), known as the "Beltsville Sperm Sexing Technology" patented by the USDA (US Patent #692958, 04/26/1991), with Dr. Lawrence Johnson as the inventor.

The "Beltsville Sperm Sexing Technology"

The flow cytometry technique for sperm sorting consists of the following steps: (these have been reviewed by Garner and Seidel, 2008):

- Diluted sperm is incubated with Hoechst 33342, that stains the DNA content of both X and Y chromosome bearing sperms.
- After staining, sperms are pumped in a stream in front of a laser beam at specific wavelengths.
- The Hoechst 33342 emits fluorescence for excitation with the UV light. Because of higher DNA concentration, the X-bearing sperm emits slightly more light than the Y-bearing sperm.
- The emitted fluorescence is measured by a photomultiplier tube as sperm flow in a single-file in front of the tube, which is recorded by a computer.
- A crystal vibrator breaks the stream into individual droplets, with the purpose that each one contains a single sperm.
- The droplets are charged positively or negatively according to the DNA content.
- The droplets fall past positive and negative electrical fields, separating them into two streams for collection.
- Uncharged droplets are discarded into a third stream, which contains sperm that could not be accurately sexed (over half), does not contain a sperm, two sperms (rarely), as well as a dead sperm.

With the older class of sorters or 'standard-speed' systems, samples were sorted with a velocity of about 400,000 sperms per h (Johnson et al., 1989). If a standard insemination dose has 20 million sperms it would take 25 h to sort one insemination dose of 10 million sperm of each chromosome (X or Y) making extremely difficult the commercial application of sexed semen. Fortunately, the sperm sorter system was further developed (Johnson and Welch, 1999), sorting up to 6000 or more sperm/second each of X- and Y-sperm with more than 90% accuracy. The improvement in technique for the routine operation of the flow cytometer sorter and sexing sperm enables technicians to sort about 10 million sperms per h of each chromosome bearing sperm. In consequence, the procedure is still impractical and still very slow for the use of sexed semen at the typical insemination doses of sperm, around 20 million per dose (Seidel and Garner,

2002). To use the sexed sperm efficiently, a lower dose was established as satisfactory fertility for bulls is 2 million of frozen sperm per dose (Den Daas et al., 1998; Seidel et al., 1999).

Commercial application of sexed semen in United States began in 2003 with the granting of a sorting license to Sexing Technologies Inc. (Navasota, TX). Select Sires Inc. (Plain City, OH) was the first major AI organization in United States to offer the technology commercially

Fertility from Sexed Semen

Results from studies using sexed semen show that this technology skews the sex ratio of an offspring toward 90% of the desire sex, but results in a lower fertility. Lower sperm numbers per dose and reduced sperm viability have been implicated in the lower P/AI from sexed compared with conventional semen (Seidel et al., 1999). Sexed semen viability is reduced due to physical and chemical stresses that occur during the sorting process. These stresses include high dilution of gametes, staining with the DNA binding dye Hoechst 33342, mechanical forces during sorting, light from the UV laser beam, and projection into the collection tube under high pressure and centrifugation (Garner, 2006).

Thus, it can be expected that P/AI would be 70 – 80% of conventional semen with excellent management of cattle i.e., virgin heifers of excellent BCS and adequate age, inseminated at standing estrus and by professional inseminators compared to 50-60% of conventional with average to marginal management (Seidel et al., 1999).

In a study by DeJarnette et al. (2009), that analyzed farm records from 211 herds that used AI with sexed semen, P/AI was 80% of that obtained with conventional semen. However, the authors caution that results could have been biased by the preferential use of sexed semen to first service in heifers that displayed definitive signs of estrus.

Field Trials with Sexed Semen in Dairy Heifers

One early study with sexed semen before its commercial application was done by Seidel et al. (1999). In this study, a total of 11 trials in beef and Holstein heifers from different locations were conducted. They compared P/AI by AI with sexed semen deposited in the body or uterine horns with 1.5 or 3 million sperm dosage to conventional semen deposited in the uterine body with the regular 20 million sperm dosage. In the 4 trials done in Holstein heifers, estrous synchronization of heifers was performed with a single or double injection of PGF2a 14 days apart followed by AI at detected estrus. Overall P/AI at ~60 days, for the 1.5 million sperm dosage was 46.1% and 41.0% when deposited in the uterine body and uterine horns, respectively. Using the 3 million sperm dosage, 46.5% and 33 .0% in the uterine body and uterine horns, respectively. Total P/AI for sexed semen at ~60 days in the Holstein heifer's trials was 42.7% (157/368). The P/AI was the 61.4% (69.5%, 82/118) of that obtained with conventional semen at ~60 days.

From 1999 to 2004 several field trials conducted with virgin heifers were reviewed by Weigel (2004). Pregnancy per AI in these field trials typically ranged from 35 to 40% with sexed semen, compared with 55 to 60% with conventional semen. In a more recent study by Seidel and Schenk (2008), six field trials with sexed semen were performed: 3 in beef heifers, 2 in Holstein heifers and one in lactating dairy cows. They also compared sperm dosage and site of sperm deposition. The Holstein heifer trials were carried out in two locations. In one location, (a multi-year study including 611 heifers), the objective was to determine P/AI in heifers with either 1 million or 3 million total sexed semen per inseminate, deposited into either the uterine body or the uterine horns, with 20 million conventional sperm dosage. Estrus was synchronized in heifers by using two doses of PGF2a 14 days apart and inseminating at detected estrus. Pregnancy per AI at 58 days for the 1 million per sperm dosage was 39.0% in the uterine body and 28% in the

uterine horns, For the 3 million per sperm dosage, 44.0% in the uterine body and 43.0% in uterine horns. The 1 million sperm dosage was inferior compared to 3 the million sperm dosage when sexed sperm were deposited into the uterine horns. However, this difference was not observed when sexed sperm was inseminated into the uterine body. Overall P/AI at d 58 was 38.5% (187/486) with a 13% of pregnancy loss between days 28 and 58 of gestation. Pregnancy per AI with sexed semen was 70% of conventional (55%, 68/124).

In the other location, data were collected from 719 inseminations in four herds. The objective was to determine if increasing the sperm dosage could enhance P/AI with sexed sperm in Holstein heifers. Pregnancy per AI obtained with 1.5 million and 6 million total sexed sperm dosage was compared to that obtained with conventional semen. Pregnancy per AI ranged from 21% to 48%, with 38.2% average, for the 1.5 million sperm dosage. For the 6 million sperm dosage, P/AI ranged from 24% to 57%, with an average of 39.2%. There were no differences between insemination dosages. The overall P/AI obtained with sexed semen was 70.8% of conventional semen. In this last trial there was a notable variability in the P/AI by sire used for inseminations with sexed or conventional semen. One sire had 21% and 32% of P/AI with sexed (1.5 million dosage) and conventional semen respectively, while the most fertile sire obtained P/AI of 48% with sexed semen (1.5 million dosage) and 67% with conventional semen. It has been reported that the importance for careful selection of bulls used for AI should be based on accurate analysis of fertility under field conditions (Bodmer et al., 2005; Cerchiaro et al., 2007). Monitoring fertility results of sexed semen and using bulls with the highest fertility optimizes the overall fertility of sexed semen (Frijters et al., 2009).

Use of Sexed Semen in Dairy Farms

Studies using sexed semen for AI in dairy heifers show that a smaller P/AI can be expected compared with the use of conventional semen for AI. Despite the lower fertility, the use of sexed

semen in dairy farms is justified because it enhances producer's ability to economically obtain replacement heifers, mitigating some of the effects of high involuntary culling rates and poor reproductive efficiency. Furthermore, AI with X-bearing sperm to mate virgin dairy heifers would decrease the incidence of calving difficulty, because female calves are smaller than males (Weigel, 2004).

The return on investment for sexed semen for dairy producers depends on many factors, such as percent of reduction in conception, the cost of sexed semen, percent of females born and the differential cost between males and females calves. Many of these factors can change considerably from herd to herd and year to year (Thorban, 2008).

To increase the probability of acceptable pregnancy results, Select Sires recommends restricting the use of sexed semen to first and second service of virgin heifers in standing estrus. Use of a TAI program in absence of observed estrus is discouraged (Thorban, 2008; DeJarnette et al., 2009). Nevertheless, there is a lack of research on the application of TAI protocols using sexed semen in dairy heifers. The utility of TAI combined with the commercial availability of sexed semen could prove to be an effective reproductive management program of dairy heifers if acceptable pregnancy per TAI is obtained, especially in herds with inefficient estrous detection (De Vries et al., 2008).

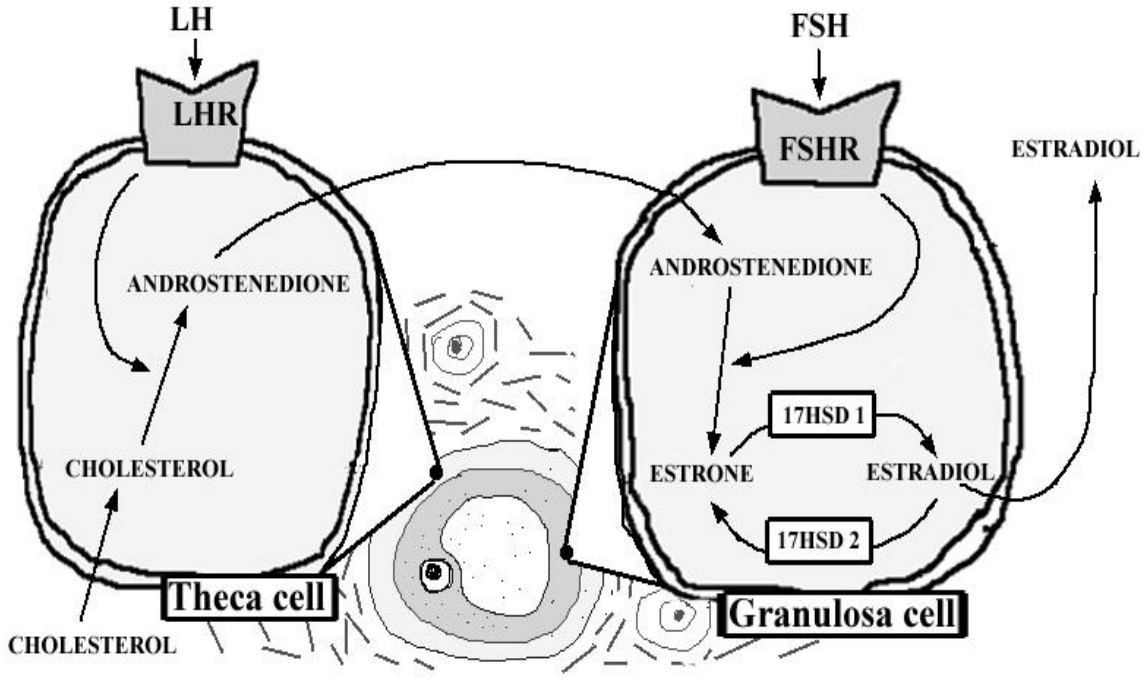


Figure 2-1. Two-cell-two-gonadotrophin theory. Thecal cells produce androgens (androstenedione) in response to LH. Androstenedione is delivered to the granulosa cells wherein they are aromatized to estrone which is further converted into estradiol, stimulated by FSH. (Source: http://herkules.oulu.fi/isbn9514266676/html/i267605.html. Last accessed: June, 2009).

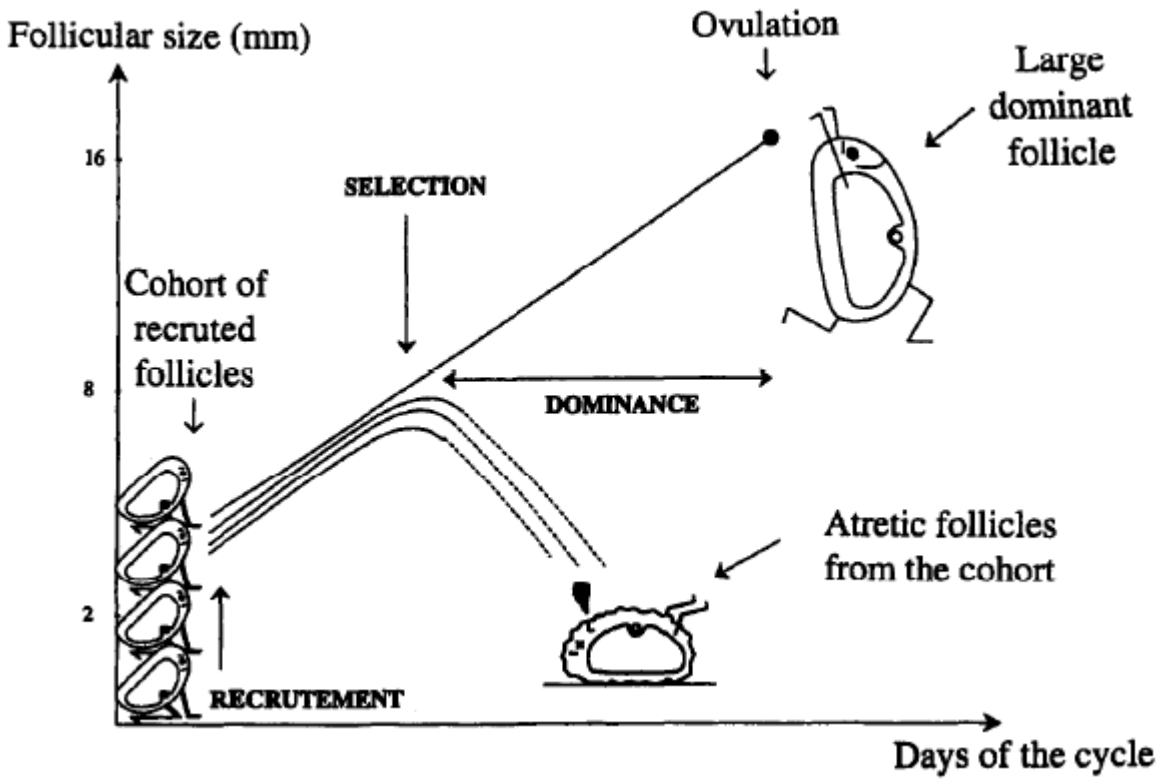
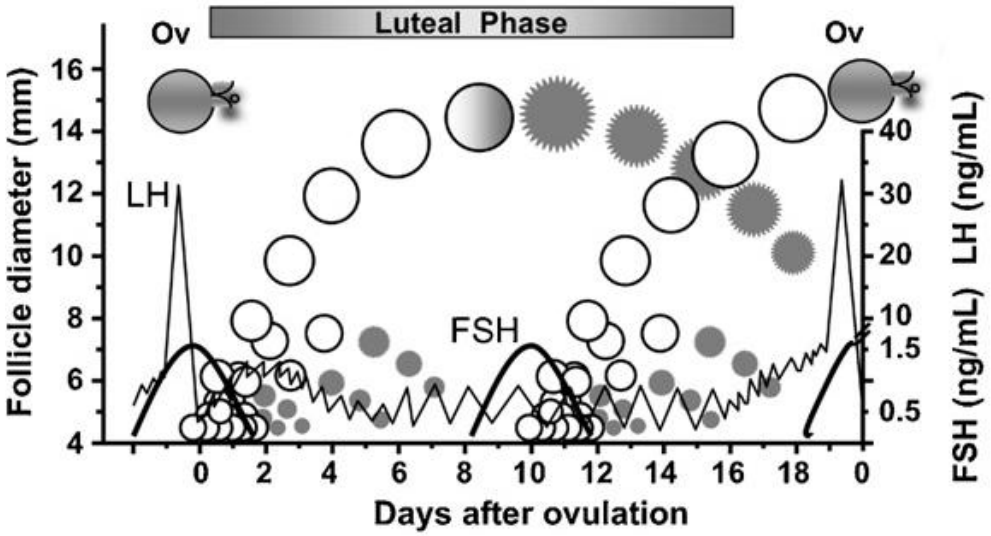
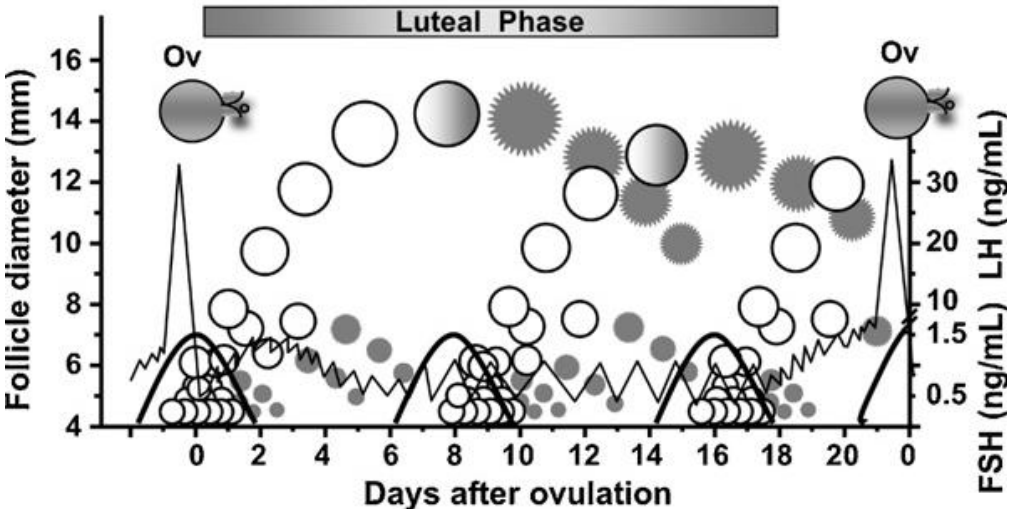


Figure 2-2. Mains events during a follicular wave. Each wave could be represented by a number (cohort) of follicles that begin a running together (recruitment). In one moment one of the follicles (the dominant follicle) will take obvious advantage (selection) over the others, that exhausted, leave the running and regress (atresia). If at the end of the running the dominant follicle is in a low-progesterone environment, it will successfully achieve the goal and ovulate, as occur in this figure. Reprinted with permission from Dr. M. A. Driancourt. (Source: Driancourt, 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology. 55:1211-1239. Figure 1, page 1213).



A.



B.

Figure 2-3. Dynamics of ovarian follicular development during the estrous cycles in cattle. A) Two-wave estrous cycle. B). Three-wave estrous cycle. FSH surge (thick line) precedes emergence of each wave. LH surge (thin line) precedes ovulation. LH pulse of high frequency that induce ovulation is result of low-circulating progesterone concentrations. Reprinted with permission from Dr. G. Adams. (Source: Adams et al., 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69: 72-80. Figure 1, page 74.)

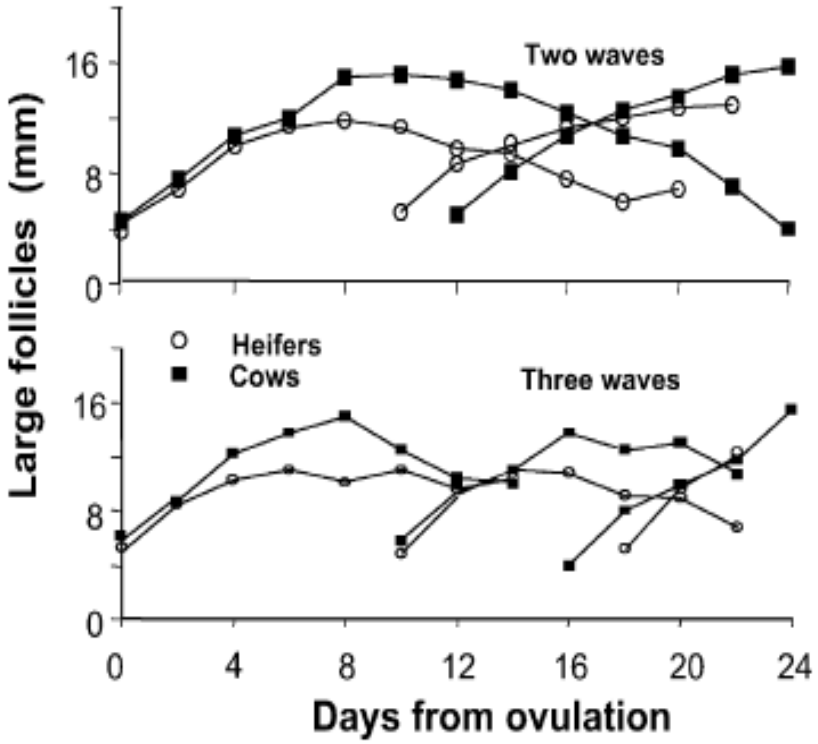


Figure 2-4. Diameters of the dominant follicles during the estrous cycle in cows and heifers exhibiting two-wave cycles and three-wave cycles Reprinted with permission from Dr. D. Wolfenson. (Source: Wolfenson et al., 2004. Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. Theriogenology 62:1042–1055. Figure 1, page 1047).

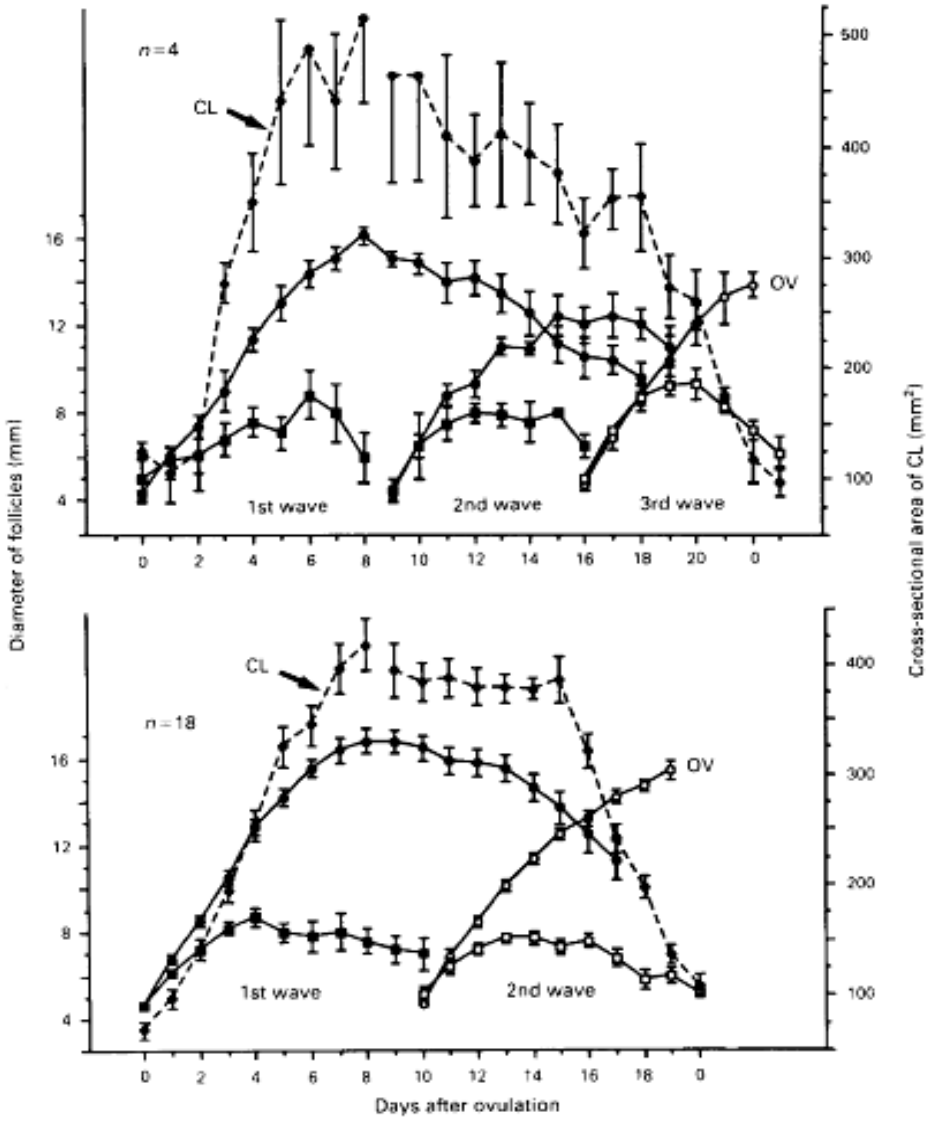


Figure 2-5. Mean profiles of diameters of dominant and largest subordinate follicles and the cross-sectional luteinized area of the CL for estrous cycles with 3 and 2 follicular waves. Regression of the CL begins between days 18 and 20 for 3-wave and 15 and 16 days for 2-wave cycles. OV = ovulation. Reprinted with permission from Dr. O. J. Ginther. (Source: Ginther et al., 1989b. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J. Reprod. Fertil. 87:223-230. Figure 1. Page 227).

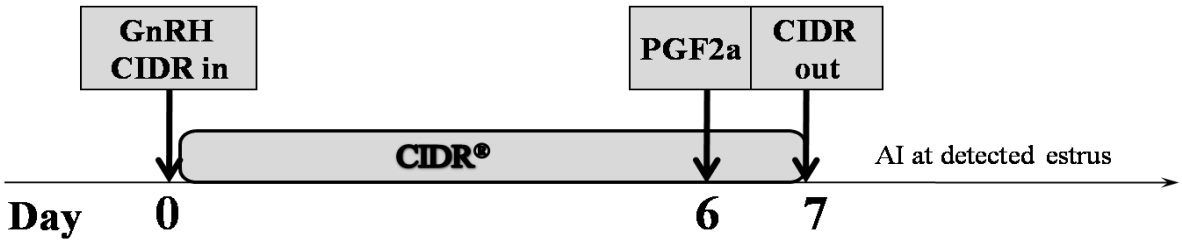


Figure 2-6. Implementation of the CIDR insert to control estrous behavior in heifers.

Figure 2-7. The Ovsynch protocol.

Figure 2-8. The CoSynch protocol.

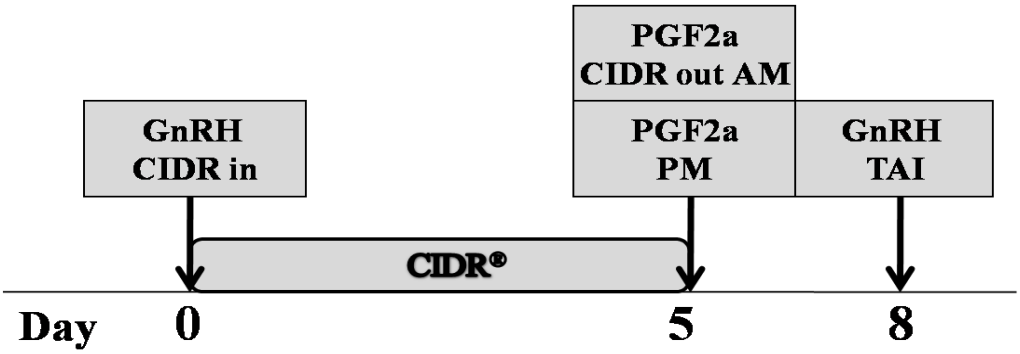


Figure 2-9. The 5 d Co-Synch +CIDR protocol

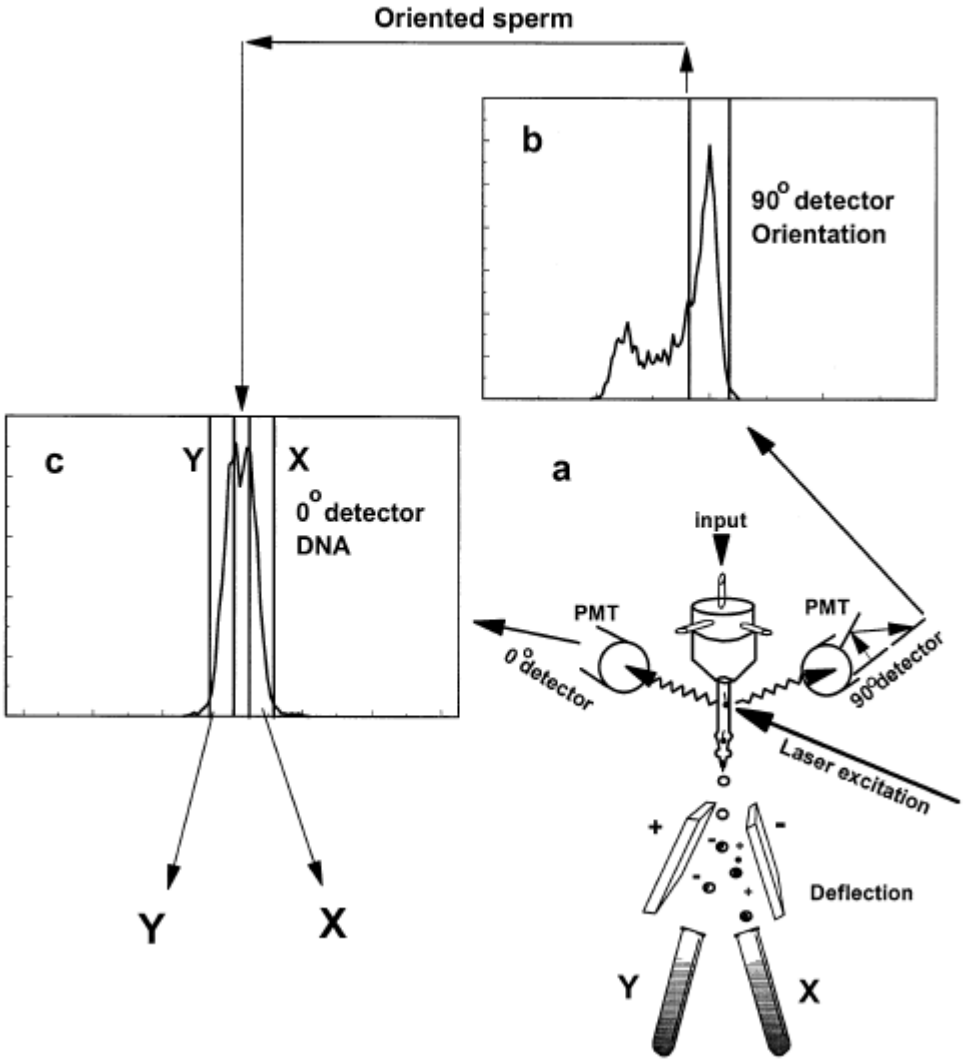


Figure 2-10. Schematic diagram of the flow cytometer - cell sorter modified for sorting sperm. Represents the current technology in high-speed sorting for resolution of X- from Y-chromosome-bearing sperm based on their difference in DNA. Reprinted with permission from Dr. L. A. Johnson. (Source: Johnson, 2000. Sexing mammalian sperm for production of offspring: the state-of-the-art. Anim. Reprod. Sci. 60–61:93–107. Figure 1. Page 97.

Table 2-1. Conception rate, diameter and age of the follicle from which ovulations occurred, duration of proestrus, and number of cows included in the first, second or third experiments from Mussard et al. (2003, 2007) investigating maturation of the follicle at ovulation and fertility

Conception rate (%)[a]	Follicle diameter at ovulation (mm)[b]	Age of follicle (d)[c]	Duration of proestrus (d)[d]	n	Experiment
4	11.1 ± 0.2	5.4	1.0 ± 0.1	45	Second
8	11.1 ± 0.2	5.1	1.0 ± 0.1	12	Third
57	13.6 ± 0.2	6.6	2.2 ± 0.1	54	Second
67	13.7 ± 0.2	6.1	2.0 ± 0.1	12	Third
76	10.7 ± 0.1	5.1	3.3 ± 0.1	29	First
100	12.0 ± 0.3	6.0	4.7 ± 0.2	24	First

[a]Percentage of animals determined to be pregnant by US at 30 d following insemination.
[b]Diameter of the largest ovulatory follicle from which ovulation occurred as determined by US conducted either at GnRH administration or estrus.
[c]Approximate developmental stage of the follicle from which ovulation occurred.
[c]Interval from PGF2a until GnRH administration.
First exp.: Cows were either induced with GnRH to have ovulations from a small (11mm) follicle or allowed to spontaneously exhibit estrus. Cows were inseminated 12 h following estrus or GnRH.
Second exp.: Cows were induced to ovulate from either a small (11mm) or large (13mm) ovarian follicle with GnRH. Animals were inseminated 12 h following GnRH administration.
Third exp.: Cows were induced to ovulate from either a small (11mm) or large (13mm) ovarian follicles with GnRH. Embryos from non-treated cows were then transferred 7 days after GnRH.
Reprinted with permission from Dr. M. L. Day. (Source: Bridgest et al. 2009. Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim. Reprod. Sci. doi:10.1016/j.anireprosci.2009.05.002. Table 1. Page 7)

CHAPTER 3
USE OF THE 5 D CO-SYNCH + CIDR TIMED ARTIFICIAL INSEMINATION PROTOCOL IN DAIRY HEIFERS AS A PLATFORM TO DETERMINE IF FLUNIXIN MEGLUMINE IMPROVES EMBRYO SURVIVAL AND PREGNANCY PER TIMED ARTIFICIAL INSEMINATION

Introduction

Artificial insemination allows for genetic improvement of replacement dairy heifers and enhances the value of pregnant heifers. Development of TAI programs has been based upon a thorough understanding of the factors controlling ovarian follicular growth (Thatcher et al., 2000). One program that has been successful for insemination of cows at a fixed time without the need for detection of estrus is the Ovsynch program, in which injections of GnRH are given 7 d before and 48 h after an injection of PGF2a, and cows are inseminated 16 to 20 h after the second injection of GnRH (Burke et al., 1996; Pursley et al., 1997). If TAI in the Ovsynch protocol is performed at the same time as the second GnRH injection, the protocol is referred to as Co-Synch (Geary and Whittier, 1998). An additional program is the Presynch/Ovsynch, in which presynchronization is achieved by PGF2a given twice at a 14-d interval and the Ovsynch program initiated 12 d after the second injection of PGF2a. In dairy cows, this program increased P/TAI by 17.3 percentage units at 72 d compared with cows receiving Ovsynch without presynchronization (Moreira et al., 2001). The Presynch/Ovsynch with the second GnRH injection at 56 h and TAI at 72 h had greater P/TAI (38.6%) compared with the Presynch/Co-Synch at 48 h (29.2%) or 72 h (25.4%) (Brusveen et al., 2008). Because of differences in number of follicular waves and follicle growth, heifers could have greater incidence of expression of estrus between the first and second administration of GnRH of an Ovsynch program (Pursley et al. 1995; Schmitt et al. 1996) compared to lactating cows. Inclusion of a CIDR insert (Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) insert during this period avoids premature ovulation, asynchrony of insemination and suppresses estrus without affecting

fertility, and the inclusion of a CIDR insert is recommended when detection of estrus is a limiting factor for AI programs in dairy heifers (Rivera et al., 2005).

The Co-Synch + CIDR protocol was modified by reducing the interval from the first GnRH and insertion of the CIDR insert to PGF2a administration and CIDR withdrawal to 5 d, and lengthening the interval from PGF2a to the second injection of GnRH and concurrent TAI to 3 d (5 d Co-Synch + CIDR). Because it is not known whether regression of accessory CL formed after the injection of GnRH would occur with a single injection of PGF2a, a second injection of PGF2a was administered approximately 12 h after the first PGF2a injection or with CIDR insert withdrawal (Bridges et al., 2008). The application of this protocol in beef cows resulted in an average P/TAI of 70.4%, which was 10.5% greater than achieved with a standard 7-d CO-Synch + CIDR program (Bridges et al., 2008). Based on the findings in beef cattle, the application of the 5 d Co-Synch + CIDR protocol may result in an effective reproductive management scheme when applied to dairy heifers.

Nearly 40% of total embryonic losses occur between d 8 to the time of CL maintenance which occurs around d 17 of pregnancy. The process of CL maintenance to sustain pregnancy involves extensive elongataion of the conceptus and secretion of IFN-τ. Embryo losses may occur because insufficient IFN-τ may not inhibit secretion of endometrial PGF2a (Thatcher et al., 2001). Flunixin meglumine (FM) is a potent nonsteroidal, antiinflammatory agent that inhibits cyclooxygenase and prevents conversion of arachidonic acid to PGF2a (Odensvik, 1995). Treatment with FM by intramuscular injections twice daily during the first 6 d postpartum partially inhibited the secretion of PGF2a, as indicated by a decrease in the concentration of 13, 14-dihydro-15-keto-PGF2a (PGFM) in the peripheral blood. (Guilbault et al., 1987).

It was hypothesized that the 5 d Co-Synch + CIDR protocol could be used as an efficient TAI protocol in dairy heifers and that treatment with FM during the period of CL maintenance would increase the late survival of embryos and P/TAI. Therefore, objectives of the two experiments were to determine: 1) P/TAI with the 5 d Co-Synch + CIDR protocol compared to a $PGF_{2\alpha}$/GnRH protocol and 2) to determine if FM administered on d 15.5 and d 16 after first TAI would increase P/TAI, using the 5 d Co-Synch + CIDR protocol with a new or used CIDR insert.

Materials and Methods

Heifers, Diets, Housing

The experiments followed the guidelines of the University of Florida Animal Use and Care Committee. From February to July, 2007 a total of 572 nulliparous Holstein heifers between 13 to 14 months of age from a commercial dairy farm located in south Florida were used. Heifers were housed in pasture with access to portable shades and trees and fed daily a TMR that met or exceeded the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg per d (NRC, 2001). The diet was based on the following ingredients: grass silage, lactation cow ration weighback, grass hay, distiller's grain, citrus pulp and a mineral and vitamin supplement. For implementation of blood collection, synchronization protocol, insemination, FM administration and pregnancy examination, heifers were handled in barns that contained self locking stanchions.

Experiment 1: 5 d Co-Synch + CIDR Protocol versus a PGF2a /GnRH Protocol

Two hundred forty-seven heifers were assigned randomly to either the PGF2a/GnRH protocol (n = 120) or the 5 d Co-Synch + CIDR protocol (n = 127). Heifers in the PGF2a/GnRH protocol received 2 injections of PGF2a (Lutalyse®, 25 mg i.m., Pfizer Animal Health Inc., New York, NY) given 14 d apart (d 0 and 14); a GnRH injection (Cystorelin®, 100 μg, i.m., Merial Inc., Ltd., Iselin, NJ) at 60 h (d 16.5) after the second PGF2a injection, and TAI at 60 h. Heifers

assigned to the 5 d Co-Synch + CIDR protocol received an intravaginal CIDR insert containing 1.38 g of progesterone and an injection of GnRH on d 0; 5 d later the CIDR insert was removed and PGF2a administered followed by a second injection of PGF2a 12 h later; on d 8, 72 h after CIDR insert removal, GnRH was administered and heifers were TAI (Figure 3-1).

Experiment 2: Administration of Flunixin Meglumine on d 15.5 and 16 post TAI in Heifers Synchronized with the 5 d Co-Synch + CIDR Protocol

Two sequential groups of heifers were used for this experiment. For the first group (n = 176) of heifers received CIDR insert and a GnRH injection at d 0. On d 5, the CIDR insert was removed, and heifers received two injections of cloprostrenol (Estrumate® 500 μg i.m., Schering-Plough, Animal Health Corp, Summit, NJ), the first at CIDR removal and the second 12 hrs later. On d 8, a second GnRH injection was administered concurrent with TAI. At initiation of the 5 d Co-Synch + CIDR protocol, heifers were assigned randomly to a FM group (Banamine®, Schering-Plough Corp, Summit, NJ) or remained as untreated controls. Heifers in the FM group (n = 87) received an i.m. injection of 400 mg of FM at d 23.5 and d 24; i. e., 15.5 and 16 d after TAI. Heifers in the control group (n = 89) were not treated with FM.

The same scheme was repeated sequentially with the second group of heifers (n = 147). The only difference for this group was that heifers received a 5 d used CIDR insert instead of a new CIDR insert in the 5 d Co-Synch protocol. As for the first group, heifers were assigned randomly to a FM group (n = 71) receiving an i.m. injection of 400 mg of FM at d 23.5 and d 24; i. e., 15.5 and 16 d after TAI, or to the control group (n = 76). The 5 d used CIDR inserts were disinfected with an iodine based solution, air dried, placed in autoclave bags (Tower DualPeel®, Allegiance Healthcare Corporation, McGaw Park, IL), and autoclaved for 15 min at 121 ◦C before re-use. Considering both the first and second group together a total of 158 heifers were assigned to the FM group, and 165 heifers to the control group.

Determination of Cyclic Status

In experiment 1 and in the first group of experiment 2, two blood samples were collected to measure plasma progesterone concentration. The first blood sample was taken 7 d before the initiation of the synchronization protocols (d -7). The second blood sample was taken on d 0, at the time of the first PGF2a administration to heifers receiving the PGF2a/GnRH protocol, or immediately before CIDR insertion in heifers receiving the 5 d Co-Synch + CIDR protocol. Progesterone concentrations in these samples were used to determine cyclic status of heifers at the time of initiation of treatments. Blood samples (~ 10 mL) were collected by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K_2 EDTA for plasma separation. Samples were placed immediately in ice and kept refrigerated until transported to the laboratory. Blood tubes were centrifuged at 3,000 × *g* for 15 min, and plasma frozen at −25°C until analyses. Analysis of progesterone in plasma was determined using a Coat-A-Count Kit (DPC® Diagnostic Products Incorporation, CA, USA) radioimmunoassay designed for the quantitative measurement of progesterone in plasma. Plasma concentrations of progesterone of known values were used in duplicates in every assay to calculate inter- and intra assay coefficients of variation. The known samples were plasma from an ovariectomized cow (0.4 ng/mL), a low progesterone sample (1.2 ng/mL) and high progesterone sample (4.2 ng/mL*).* The inter- and intra assay CV were: 5.1% and 12.5% for the sample with 0.4 ng/mL, 4.3% and 11.8% for the 1.2 ng/mL sample and 4.7% and 5.8% for the 4.2 ng/mL sample.

Pregnancy Diagnosis

Pregnancy diagnosis to first TAI was performed with a 5 MHz ultrasound scanner (Easi-Scan®, BCF Systems, Livingston, Scotland) at 32 d after the first TAI. The presence of an embryo with a heartbeat was the criterion used to determine pregnancy (Fricke et al., 1998).

Reconfirmation of pregnancy was performed at 45 d by transrectal palpation of the uterus and its contents. Pregnancy per TAI was defined as the percentage of all animals treated and TAI that became pregnant.

Statistical Analysis

The effect of treatments on P/TAI as a binary variable was analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Version 9.1 for Windows, SAS Institute, Cary, NC, USA). Terms with a significance value of $P > 0.20$ were removed from the complete model based on the Wald's statistics criterion, using the automatic stepwise backward elimination to derive the final reduced model for each variable.

. The initial model for P/TAI for experiment 1 included treatment (synchronization protocol), technician, sire and interactions between these variables. The model for experiment 2 included treatment (FM or control), technician, sire, group (including a new or used CIDR) and the appropriate interactions. A P-value of ≤ 0.05 was considered significant.

As there were no differences detected for technicians or sire, or interactions with these variables in either experiment, these terms were removed from the final analyses. Cyclic status was not included in the model because of the low proportion of prepuberal heifers (5.4%; 23/424) and that cyclic status was not monitored for the second group of heifers in experiment 2.

Results

Experiment 1: 5 d Co-Synch + CIDR Protocol versus a PGF2a /GnRH Protocol

For heifers receiving the PGF2a/GnRH protocol, P/TAI was 45.8% (55/120) at 32 d and 43.3% (52/120) at 45 d with a pregnancy loss of 5.5% (3/55). Heifers synchronized with the 5 d Co-Synch + CIDR protocol had a P/ TAI of 53.1% (68/128) at 32 d and 50.8% (65/128) at 45 d, with a pregnancy loss of 4.4% (3/68). There was no difference ($P = 0.26$) in P/TAI between treatments. Based on progesterone concentrations, 4.8% (12/248) of heifers were prepuberal at

the initiation of treatments. Four of the nine non-cyclic heifers in the 5 d Co-Synch + CIDR protocol became pregnant; in contrast, none of the non-cycling heifers (0/3) became pregnant in the PGF2a/GnRH group.

Experiment 2: Administration of Flunixin Meglumine on d 15.5 and 16 post TAI in Heifers Synchronized with the 5 d Co-Synch + CIDR Protocol

Overall P/ TAI in the control group was 60.0% (99/165) at 32 d and 59.4% (98/165) at 45 d with a pregnancy loss of 1.0% (1/99). In the FM group, P/ TAI was 60.8% (96/158) at 32 d and 59.5% (94/158) at 45 d with a pregnancy loss of 2.1% (2/96). There was no difference (P = 0.89) in P/TAI between heifers treated or not treated with FM. In the first group of experiment 2, 6% (11/176) of heifers were non-cyclic. Of the non-cyclic heifers, 36.3% (4/11) became pregnant.

In the first group of heifers in which a new CIDR was inserted, P/TAI was 59.7% (105/176) at 32 d and 59.1% (104/176) at 45 d, with a pregnancy loss of 0.9% (1/105). In the second group of heifers in which a 5 d used CIDR was inserted, P/TAI was 61.2% (90/147) at 32 d and 59.9% (88/147) at 45 d, with a pregnancy loss of 2.2% (2/90). There was no differences (P = 0.77) in P/TAI between groups, involving a new or used CIDR insert, and there was no group by treatment (i.e., FM) interaction (P = 0.91).

Discussion

The 5 d Co-Synch + CIDR protocol is a modified ovulation synchronization protocol that was developed based on a fundamental understanding of the bovine estrous cycle. The protocol increased P/TAI in beef cows compared with a 7 d Co-Synch + CIDR protocol (Bridges et al., 2008). In the present study, the 5 d Co-Synch + CIDR protocol was evaluated for the reproductive management of dairy heifers.

In experiment 1, P/TAI did not differ statistically between the 5 d Co-Synch + CIDR protocol (53.1%) with the lower cost PGF2a/GnRH protocol (45.8%). Nevertheless, the increase

of 8% in P/TAI observed in heifers bred to the 5 d Co-Synch + CIDR protocol, indicates that this protocol can be used in dairy heifers as initially hypothesized, and acceptable P/TAI of approximately 60% were achieved with the the 5 d Co-Synch + CIDR protocol in the two groups of experiment 2.

The application of the PGF2a/GnRH protocol is based on the luteolytic effect of PGF2a in cattle and can be used to synchronize estrus if given 11 to 14 d apart (Lauderdale et al., 1981). This interval allows sufficient time for those heifers with a CL that responded to the first PGF2a administration to have a new CL mature enough to respond to the second PGF2a administration. Furthermore, those heifers that did not have a CL at the time of the first PGF2a administration should have a mature CL capable of undergoing regression at the second PGF2a injection 11 or 14 d later. GnRH can be applied 48 h after the administration of PGF2a to induce ovulation in a two-dose PGF2a synchronization protocol (Slenning, 1992). Although, PGF2a is effective in inducing CL regression from d 6 to 16 of the estrous cycle, there is considerable variability in the intervals from treatment to estrus and ovulation (Odde, 1990). The administration of PGF2a at 5 or 8 d after ovulation resulted in ovulation of the dominant follicle of the first follicular wave in 2 or 3 d, whereas PGF2a given d 12 after ovulation resulted in ovulation of the dominant follicle of the second follicular wave in 5 d (Kastelic et al., 1990). If a new follicular wave emerges, LH receptors are not expressed in the granulosa cells of growing follicles during the first few d of the follicular wave (Xu et al., 1995). This is prior to the time of deviation in growth rates between the largest follicle and second largest follicle that occurs at d 2.8 after wave emergence, when the diameter of the dominant follicle is 8.5 mm and the largest subordinate follicle is 7.2 mm in diameter (Ginther et al., 1996). Consequently, if the second PGF2a injection is given in coincidence with the emergence of a new follicular wave, the ovulatory follicle from this

follicular wave would not be responsive to the GnRH when administered 60 h after the second PGF2a injection. This would result in a failure of synchronization. Therefore, the PGF2a/GnRH protocol would be more effective if the follicular wave is synchronized (Martinez et al., 2004).

The Co-Synch protocol uses GnRH and PGF2a to sequentially control ovarian follicular dynamics, luteolysis, the length of proestrus (interval from initiation of luteolysis to the GnRH-induced LH surge), and ovulation. The intent of the initial GnRH administration is to induce ovulation and reset follicular growth, leading to the synchronized development of estrogenic preovulatory follicles that are induced to ovulate by the second GnRH treatment given after PGF2a injection (Thatcher et al., 1989). The modification of the Co-Synch program by Bridges et al. (2008) was based on the hypothesis that reducing the interval from the initial GnRH treatment to PGF2a and CIDR insert withdrawal, and lengthening the interval until the second GnRH injection would increase estradiol secretion by the preovulatory follicle. A greater P/TAI was observed in cows with increased estradiol concentrations at induced ovulation (Perry et al., 2005). Younger dominant follicles, 4 d after emergence, have increased intra-follicular estradiol concentrations and a greater capacity to produce estradiol than dominant follicles evaluated later in the follicular wave (Valdez et al., 2005). Therefore, increased estradiol production by a younger follicle can be achieved if luteal regression and withdrawal of the CIDR insert occur earlier relative to follicular wave emergence, and the interval between PGF2a and the second GnRH administration is extended. Furthermore, if follicles fail to respond to the initial GnRH then the reduced period between GnRH and PGF2a and CIDR insert exposure reduces the probability of these follicles from becoming aged or to have a prolonged dominance.

The 5 d Co-Synch + CIDR protocol resulted in a numerical advantage in P/TAI than the PGF2a/GnRH protocol. Also, the pregnancy results from pre-pubertal heifers suggest that with

the use of this protocol some noncyclic heifers could respond and become pregnant; in contrast, the presence of a CL is required for the application of the PGF2a/GnRH protocol.

In the second experiment, the 5 d Co-Synch + CIDR protocol was used as a platform to evaluate the effect of a double injection of FM around the period that the CL is sustained to maintain pregnancy. Administration of FM did not improve P/TAI. The rationale for administering FM was to inhibit the activity of Prostaglandin G/H synthase-2 to reduce the synthesis of PGF2a by the uterus which would contribute to the antiluteolytic process during early pregnancy. Under this assumption, early embryonic losses would be reduced. The application of FM would inhibit the oxytocin-induced PGF2a secretion by the endometrium and decrease the number of PGF2a pulses (Odensvik et al., 1998). A previous study by Guzeloglu et al. (2007) reported that FM as a double injection 12 h apart on d 15.5 and 16 after TAI increased P/TAI by 54% in dairy heifers in Turkey (i.e., from 50% to 77% at d 29 after TAI). It is conceivable that the population of dairy heifers managed under the production systems in Turkey were under a greater stress such that FM treatment improved embryo survival. The present study, under the conditions of a commercial dairy heifer management system in the United States, with a large number of experimental animals, failed to detect an effect of FM on P/TAI or on embyo loss. The implication of management stress should not be overlooked. Merrill et al. (2007) reported that treatment of beef cows with FM increased P/AI irrespective of whether they experienced transportation stress. They speculated that even the stress associated with handling of cows for sample collection could produce a response and FM treatment would diminish the effect. However, it seems probably that heifers were not under stressful conditions in the present study, since the use of FM did not have an effect on P/TAI.

Other factors in addition to stressful conditions can affect fertility in dairy heifers, and nonpregnancy status could be either due lack of conception or embryonic loss. Donovan et al. (2003) examined animal and management factors associated with pregnancy to first AI at detected estrus in Holstein heifers in Florida. A considerable variation in pregnancy per AI was detected for technicians and bulls used. However, a higher risk for pregnancy was detected for heifers inseminated during the winter season, breeding to a spontaneous estrus and a bigger pelvic size during the warm season. In the present study, method of estrus detection was not a factor because all heifers were time inseminated. In contrast to the finding by Donovan et al. (2003), technicians and bulls did not have an effect on P/TAI in the present study. Prior to enrollment heifers for experiments 1 and 2 were evaluated to be of the appropriate age and weight for breeding at 13-14 months and 360 kg, respectively. Although cyclic status in heifers of group 2 in experiment 2 was not evaluated, progesterone results for the remaining experimental heifers indicated that the proportion of prepubertal animals was low and that CIDR insertion resulted in pregnancies in a certain proportion of prepubertal heifers that went through the 5 d Co-synch + CIDR protocol.

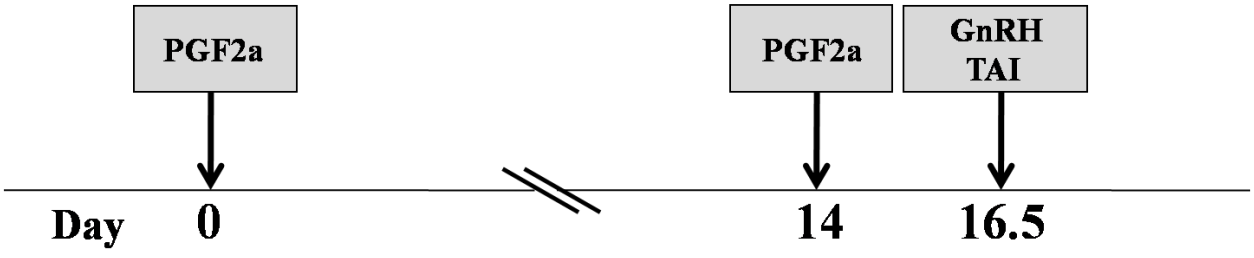
Both groups of experiment 2 had equal pregnancy/ TAI indicative that a 5 d autoclaved used CIDR insert did not alter fertility or modify the lack of a FM response. In a study by Zuluaga and Williams (2008) it was shown that a 7 d used autoclaved CIDR or a new CIDR maintain similar concentrations of progesterone during the 7 d of treatment period in ovarectomized beef cows, and the progesterone concentrations were greater for the new and used autoclaved CIDR than for a used disinfected CIDR. Cerri et al. (2009) evaluate progesterone concentrations after cows had initiated estrous cycles following calving and induction of estrous cycles in postpartum anovular dairy cows receiving a new CIDR or a 7 d used autoclaved CIDR.

In cows that had initiated estrous cycles, progesterone concentrations in plasma were sufficient to inhibit ovulation and maintain growth of the dominant follicle until insert removal if cows received either a new CIDR or a 7 d used autoclaved CIDR. Furthermore, resumption of estrous cycles was greater for cows treated with CIDR compared to control cows no receiving CIDR, and response did not differ between the new CIDR or a 7 d used autoclaved CIDR.

Across experiments P/TAI with the 5 d Co-Synch protocol was 58.3% at d 32 and 57.6% at d 46, which is acceptable compared to previous studies conducted with dairy heifers that reported a P/TAI of 35.1%, 42.5% and 45.5% with the Ovsynch protocol (Pursley et al., 1997; Stevenson et al., 2000; Schmitt et al., 1996) and from 29.5% to 45.1% with a 6 d Co-Synch 48 h protocol (Rivera et al., 2005; 2006).

Conclusion

The application of FM failed to improve P/TAI in dairy heifers. However, the 5 d Co-Synch + CIDR protocol with two doses of PGF2a approximately 12 h apart, results in an acceptable P/TAI in dairy heifers, either with a new or 5 d used CIDR insert.



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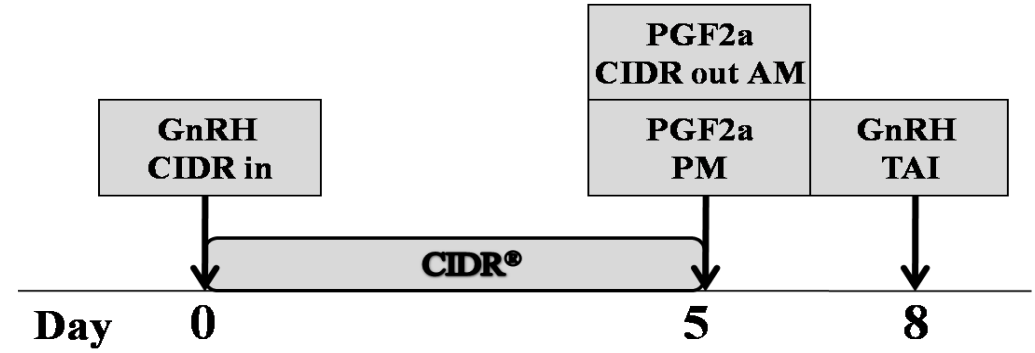

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Figure 3-1. Diagram of the PGF2α/GnRH protocol used in experiment 1 and the 5 d Co-Synch + CIDR used in experiment 1 and 2. A) PGF2a/GnRH: Heifers received 2 injections of PGF2a given 14 d apart (d 0 and 14); 60 h (d 16.5) after the second PGF2a injection, heifers received a GnRH injection and were TAI.

CHAPTER 4
APPLICATION OF ONE INJECTION OF PGF2A IN THE 5 D CO-SYNCH + CIDR PROTOCOL FOR ESTROUS SYNCHRONIZATION AND RESYNCHRONIZATION OF DAIRY HEIFERS

Introduction

A major limitation for use of AI in dairy replacement heifers is time and effort related with daily estrous detection (Erven and Arbaugh, 1987; Caraviello et al., 2006). Ovulation-synchronization protocols, such as Ovsynch, permit a fixed TAI without the need for estrous detection. This protocol consists of administering GnRH, followed 7 d later with an injection of PGF2a, and 48 h later a second administration of GnRH and TAI 0 to 24 h later (Pursley et al., 1995; Burke et al., 1996). When the Ovsynch protocol is modified with TAI performed at the same time as the second GnRH injection, it is referred to as a Co-Synch (Geary and Whittier, 1998). Such programs have been used successfully in lactating dairy cows but poorer results were obtained in dairy heifers (Pursley et al., 1995; 1997). Because of differences in follicular dynamics between cows and heifers (Pursley et al., 1997; Savio et al., 1988), dairy heifers could express estrus close to the PGF2a injection, thereby causing asynchrony at TAI (Rivera et al., 2004, 2005).

Inclusion of a CIDR insert (Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) containing progesterone in the Ovsynch or Co-Synch protocols suppresses ovulation during the days of CIDR insertion thereby allowing 100% submission rate of heifers for TAI (Rivera et al., 2005).

Bridges et al.(2008) modified the Co-Synch + CIDR protocol to a 5 d the interval from the first GnRH to PGF2a injection, with a final injection of GnRH and TAI 72 hrs from the PGF2a injection. This approach resulted in higher P/TAI compared with a 7 d Co-Synch + CIDR with the second GnRH and TAI occurring concurrently at 60 h (80.0% versus 66.7%, respectively).

Because of the shortened interval from the initial GnRH to PGF2a in the 5 d Co-Synch + CIDR protocol it is not known whether regression of an induced accessory CL occurs with one injection of PGF2a. Therefore, a second PGF2a injection was applied approximately 12 h after the first PGF2a injection, which results in additional animal handling and cost. However, two injections of PGF2a appear to be necessary when this protocol is applied to beef or lactating dairy cows.

The hypothesis of the present studies was that in dairy heifers, one injection of PGF2a at the time of CIDR insert withdrawal in the 5 d Co-Synch + CIDR protocol would regress CL present in the ovary and result in an acceptable P/TAI. The 5 d Co-Synch + CIDR protocol with one injection of PGF2a would simplify and reduce cost of dairy heifer reproductive management to optimize P/TAI under commercial conditions.

Objectives were to determine: 1) P/TAI to first TAI and CL regression in heifers receiving one or two injections of PGF2a in the 5 d Co-Synch + CIDR protocol, 2) P/TAI to the second service in heifers resynchronized with the 5-d CoSynch + CIDR protocol with one PGF2a injection versus insemination at detected estrus, 3) whether or not inclusion of a CIDR insert between the first GnRH and the single PGF2a injection is required for an effective PR to a resynchronized second TAI, and 4) field verification of the 5 d Co-Synch + CIDR program with one injection of PGF2a for reproductive management of first and second services in dairy heifers.

Materials and Methods

Heifers, Diets, Housing

A total of 1,013 nulliparous Holstein heifers between 13 to 14 months of age from two commercial dairy farms located in south Florida (SF) and north central Florida (NCF) were used in these studies. Heifers in both locations were managed on pasture, with access to portable

shades and trees, and fed once a d a TMR that met or exceeded the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg per d (NRC, 2001). The diet for each farm was based on the following ingredients: 1) NCF location; lactation cow ration weighback, grass hay, brewer's grain and a mineral and vitamin supplement, 2) SF location; grass silage, lactation cow ration weighback, grass hay distillers grain, citrus pulp and a mineral and vitamin supplement). For implementation of synchronization protocol, insemination, blood collection and pregnancy examination, heifers were handled in a barn in SF or in an open sided shaded barn in NCF that contained self locking stanchions.

Experiment 1: Pregnancy per First TAI and CL Regression in Dairy Heifers Receiving One or Two Injections of PGF2a

From August 2007 to September 2008, heifers located in the SF location (first replicate: n = 176; second replicate: n = 219) or the NCF location (n = 199) were assigned randomly to receive one (n = 295) or two (n = 298) injections of PGF2a in the 5 d Co-Synch + CIDR protocol. The protocol consisted of a first injection of GnRH (100 µg, Cystorelin®, Merial, Ltd., Iselin, NJ) and a CIDR insert containing 1.38 g of progesterone inserted at d 0; 5 d later (d 5) the CIDR was removed and one or two injections (12 h apart) of PGF2a (25 mg im, Lutalyse®, Pfizer Animal Health, New York, NY) were administered; 3 d later, on d 8 a second injection of GnRH was administered concurrently with TAI. Heifers were inseminated with a single sire in the NCF location. In the SF location, a single sire and four sires were used for inseminations of heifers in the first and second replicates, respectively. Inseminations were performed by four technicians at each dairy location.

The first replicate at SF (n = 176) was used only to compare P/TAI between one (n = 88) or two (n = 88) PGF2a injections. The second replicate of heifers from the SF location (n = 218) was also used to evaluate CL regression and P/TAI after one or two injections of PGF2a in the 5

d Co-Synch + CIDR protocol. Heifers were assigned randomly to receive one (n = 109) or two (n = 109) injections of PGF2a in the 5 d Co-Synch + CIDR protocol as previously described. In all heifers of the second replicate at SF, blood samples were collected 2 h after withdrawal of the CIDR insert and before the first injection of PGF2a. Ovaries also were scanned by ultrasonography to determine number of CL. On d 6 (i.e., 24 h after the first PGF2a injection), a second blood sample was collected.

Blood samples were taken to characterize plasma progesterone concentrations before and after PGF2a injections. Blood samples (~ 10 mL) were collected by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K_2 EDTA for plasma separation. Samples were placed immediately in ice and kept refrigerated until transported to the laboratory. Blood tubes were centrifuged at 3,000 × *g* for 15 min, and plasma frozen at −25°C until analyses. Analysis of progesterone in plasma was determined using a Coat-A-Count Kit (DPC® Diagnostic Products Incorporation,CA) radioimmunoassay designed for the quantitative measurement of progesterone in plasma. Samples with known low (0.42 ng/ml), medium (1.2 ng/ml) and high (4.3 ng/ml) concentrations of progesterone were run in duplicates before the experimental samples. Intrassay CV for each concentration was 3.9%, 9.1% and 3.5% respectively. Every sixth experimental sample was also duplicated. Duplicate plasma concentrations of progesterone were categorized into samples with progesterone ≥3.0 ng/mL and samples with progesterone ≥1.0 but <3.0 ng/mL. Duplicate samples with progesterone > 3 ng/mL had intra-assay CV of 8.4% and samples with progesterone between 1.0 and 3.0 ng/mL had a CV of 12.3%. Regression of CL was defined when progesterone concentration was > 1 ng/mL in the first sample, immediately before the first PGF2α and < 1 ng/mL 24 h later.

Experiment 2: Pregnancy Per TAI or AI to Second Service in Heifers Resynchronized with the 5 d CO-Synch + CIDR Protocol with One PGF2a Injection and TAI or Inseminated at Detected Estrus

Heifers from the NCF location (n = 199) were used for this experiment. Heifers were synchronized with the 5 d Co-Synch + CIDR protocol for the first TAI. After the first TAI, heifers were allocated randomly into two groups, resynchronized TAI after diagnosed as nonpregnant (TAI group, n = 101), and daily observation of estrus and insemination at detected estrus (IDE group, n = 98) plus resynchronization and TAI if diagnosed nonpregnant and not detected previously in estrus. Therefore, after the first TAI all heifers in both groups underwent daily estrous detection with the use of tail chalk. Heifers with rubbed off chalk or that stood to be mounted were classified as being in estrus. Only those heifers seen in estrus in the IDE group (n = 40) were inseminated. At d 32 after the first TAI, pregnancy status was determined by ultrasonography in the TAI group and in those heifers not detected in estrus and not re-inseminated in the IDE group. Nonpregnant heifers were resynchronized with the 5 d Co-Synch + CIDR protocol with one injection of PGF2a for the second TAI (n = 46) at the time of pregnancy diagnosis. These heifers were 43 from the TAI group plus 3 heifers nonpregnant and not seen in estrus from the IDE group. One sire was used and heifers were inseminated by four technicians.

Experiment 3: Inclusion of the CIDR Insert in the 5 d Co-Synch Protocol with a Single Injection of PGF2a for Resynchronization of the Second TAI

Pregnancy status was determined at d 28 after the first TAI by ultrasonography and nonpregnant heifers from the SF location (n = 111) were assigned randomly to receive a CIDR insert (n = 55) between the first GnRH and PGF2a injection or not (n = 56) in the 5 d Co-Synch protocol for resynchronization of the second TAI. The protocols were initiated on the same d. Three sires were used for inseminations performed by four technicians.

Experiment 4: Field Verification of the 5 d Co-Synch + CIDR Protocol with One Injection of PGF2a

Two replicates of heifers from NCF location were synchronized for a first TAI with the 5 d Co-Synch + CIDR protocol with one injection of PGF2a (n = 203 and n = 214 for replicates 1 and 2, respectively). Nonpregnant heifers at 30 d after first TAI were resynchronized for a second TAI following the same protocol initiated on the same d when the diagnosis of nonpregnancy was performed. A single sire was used for the first TAI in both replicates and the second TAI of the first replicate, whereas three additional sires were used for the second TAI of the second replicate. Three and two technicians performed the AI for the first or second services, respectively.

Ultrasonography

In experiment 1 replicate 2 at SF, ovaries were scanned by ultrasound to determine CL number in heifers receiving one versus two injections of PGF2a. Ultrasonography was also used for pregnancy diagnoses to first TAI in all experiments using a 5 MHz ultrasound unit (Easi-Scan®, BCF Systems, Livingston, Scotland). Pregnancy diagnosis was determined between 28 to 32 d after first or second TAI and was based on the presence of an embryo with a heartbeat (Fricke et al., 1998). Reconfirmation of pregnancy was performed at 45 (SF location) or 60 (NCF location) d by transrectal palpation of the uterus and its contents.

Statistical Analysis

For the first experiment, binary variables such as P/TAI and CL regression were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS/STAT ver 9.1 for Windows, SAS Institute, Cary, NC, USA). Terms with $P > 0.20$ were removed from the complete model based in the Wald's statistics criterion in a stepwise manner to derive the final reduced model for each variable.

The initial model for P/ TAI as the dependent variable included treatment group (one or two PGF2a injections) technician, sire and location, and interactions between these variables. Treatment group and location were retained in the final model.

The area under the receiver operating characteristic (ROC) curve, also known as c-statistic, was used to estimate the best cut-off plasma progesterone concentration in the second sample as predictor of pregnancy at first TAI. Once the best cut off was obtained, cows were dichotomized as having progesterone concentration above or below that value as a response variable to model P/TAI for heifers in SF location. The independent variables in the model included treatment group (one or two PGF2a injections) technician, sire, and progesterone concentration 24 h after the PGF2a injection as above or below the best cut off and interactions between these variables. Progesterone concentration was retained in the final model.

The model for proportion of heifers that underwent CL regression included treatment, number of CL present and interaction between these variables. There was no significant variable to be retained in a final model.

Progesterone concentration 24 h after PGF2a was evaluated by ANOVA with the MIXED procedure of SAS. The model included the effects of treatment group (one or two PGF2a injections), number of CL present in each ovary and the interaction between both terms, progesterone concentration in the sample obtained before PGF2a as covariate, and the interaction treatment and CL number.

For the other experiments, P/ TAI was analyzed with the LOGISTIC procedure of SAS as described for the first experiment. Variables include in the initial model of the second and third experiments included treatment group, technician, sire and interactions between these variables. Only treatment group was retained in the final model. In experiment 4, the model for the first

TAI included replicate number, technician and the interaction. No significant effects were detected to include in a final model. For the second TAI, sire was added to the model. This term was retained in the final model. A P-value ≤ 0.05 was considered significant.

Results

Experiment 1: Comparison of One versus Two Injection of PGF2a in the 5 d Co-Synch + CIDR Protocol

Pregnancy to first TAI

Overall P/TAI for heifers receiving one or two PGF2a at 32 d were, respectively, 48.8% (144/295) and 50.7% (151/298), and overall pregnancy loss between 32 and 45 (SF) or 60 (NCF) d of pregnancy were 5.5% (8/144) and 4.0% (6/151). Pregnancy to first TAI in the SF location for the first replicate was 42.6% (75/176) at 30 d and 38.1% (67/176) at 45 d. In the NCF location, P/TAI to first TAI was 56.8% (113/199) at 32 d and 54.8% (109/199) at 60 d. Difference in P/TAI was significant (P = 0.001; odds ratio (OR): 0.51; 95% confidence interval (CI): 0.34 to 0.77) between locations. In the SF location, P/TAI to first service with one injection of PGF2a was 43.2% (38/88) at 30 d and 37.5% (33/88) at 45 d; and with two injections of PGF2a 42.0% (37/88) at 30 d and 38.6% (34/88) at 45 d. In the NCF location, P/TAI with one injection of PGF2a was 56.1% (55/98) at 32 d and 54.0% (53/98) at 60 d; and with two injections of PGF2a PR was 57.4% (58/101) at 32 d and 55.4% (56/101) at 60 d. There were no differences (P = 0.97) in P/TAI between one versus two PGF2a treatments and no interaction between treatment and location was detected (P =0.81)

Determination of CL regression

Proportions of heifers presenting 0, 1 or 2 CL in the ovary at the ultrasound scanning before PGF2a injections were 3.7% (8/218), 73.4% (160/218) and 22.9% (50/218) respectively. Considering the quantitative analysis of progesterone concentration in plasma, heifers with a

progesterone concentration in the first sample greater than 1 ng/mL were considered to have an active CL (168/218). Using this criteria, the percentage of heifers that regressed their CL (i.e., less than 1 ng/mL in the second sample) after 1 or 2 PGF2a injections were 86.9% and 92.8%, respectively (P =0.21). There were no effects of treatment (P = 0.22), number of CL (P = 0.96), or interaction between treatment and number of CL (P = 0.99) on the percentages of heifers undergoing luteolysis. For heifers with 1 and 2 CL, luteolysis were 88.8% and 91.5%, respectively (P = 0.69).

Progesterone concentrations according to treatment and CL number are presented in Table 4-1. There were no differences in progesterone concentrations in the second sample between PGF2a groups (P = 0.27), number of CL (P = 0.96) or an interaction between PGF2a and number of CL (P = 0.63).

Pregnancy per AI to first TAI with one injection of PGF2a was 46.8% (51/109) at 30 d and 45.9% (50/109) at 45 d; and with two injections of PGF2a 51.4% (56/109) at 30 d and 50.5% (55/109) at 45 d. There was no difference (P = 0.49) in P/TAI between one versus two PGF2a treatments. According to the c-statistics results, progesterone concentration in plasma 24 h after PGF2a that best predicted the probability of pregnancy and nonpregnancy was ≤ 0.2 ng/mL. At 0.2 ng/mL, the sensitivity was 37.4% and specificity 78.4%. This means that 37.4% of the pregnant heifers had a progesterone concentration on the second sample ≤ to 0.2 ng/mL, but 78.4% of the nonpregnant heifers had a progesterone concentration on the second sample > than 0.2 ng/mL. Using this cut off to model P/TAI, it was observed that the odds of pregnancy decreased (P = 0.01) for heifers with progesterone concentration > 0.2 ng/mL (OR = 0.46, 95% CI: 0.25 to 0.83).

Experiment 2: Pregnancy to Second Service in Heifers Resynchronized with the 5 d CO-Synch + CIDR Protocol with One PGF2a Injection and TAI or Inseminated at Detected Estrus

The distribution of detected estruses is shown in Figure 4-1. A total of 69 of 86 nonpregnant heifers from both the TAI and IDE groups were detected in estrus after the first TAI (69/86; 80.2%). Of these heifers, 79.7% (55/69) were detected between d 18 and d 24 after the first TAI. Only 3 heifers in the IDE group that were not detected in estrus after the first TAI were diagnosed as nonpregnant at 32 d by ultrasound. These heifers were resynchronized and TAI with the TAI group, and one of them became pregnant after the second TAI. In the TAI group, P/TAI was 53.5% (23/43) at 32 d after the second TAI and did not differ (P = 0.79) from the P/AI for heifers in the IDE group following insemination at detected estrus (55%; 22/40). Overall P/AI to the second TAI was 52.1% (24/46). At 60 d after insemination, P/AI to the second service was 52.1% (24/46) for heifers receiving TAI and 52.1% (21/40) for heifers in the IDE group (P =0.97)

Experiment 3: Inclusion of the CIDR Insert in the 5 d Co-Synch Protocol for Resynchronization of the Second TAI

Pregnancy per TAI at 32 d was 39.3% in the group without the CIDR insert (22/56) and 51.8% (28/54) in the group with the CIDR insert inserted between the first GnRH and the PGF2a injection (P = 0.11). There were no pregnancy losses between 32 and 45 d of gestation in both groups

Experiment 4: Field Verification of the 5 d Co-Synch + CIDR Protocol with One Injection of PGF2a

Pregnancy to the first TAI was 60.3% at 32 d (251/416) and 58.2% (242/416) at 60 d. Pregnancy loss between d 32 and 60 of gestation was 3.6% (9/251). For the second service, P/TAI was 52.5% (86/164) at 32 d and 47.5% (78/164) at 60 d. Pregnancy loss was 4.9% (8/164). Pregnancy to the second TAI at 60 d was 58.4% (45/77) for the sire used in the first

replicate. In the second replicate, sire affected (P = 0.01) P/TAI to the second TAI, and they were 11.1% (1/9), 50.0% (14/28), and 36% (18/50) at 60 d after AI for sires 1, 2, and 3, respectively.

Discussion

A single injection of 25 mg of PGF2a was as effective as two injections when utilizing the 5 d Co-Synch + CIDR protocol in dairy heifers. Heifers receiving one injection of PGF2a had a P/TAI of 48.8%, whereas heifers receiving two injections achieved 50.7%. Lack of differences in first service P/TAI indicated equal effectiveness of one or two injections of PGF2a to induce CL regression at the time of CIDR insert withdrawal. Of concern was whether one injection of PGF2a would regress a newly formed CL in response to the injection of GnRH given at the time of CIDR insertion. Compared to lactating cows, heifers have a faster rate of follicular growth (Pursley et al., 1997) and a higher frequency of thee wave follicular cycles (Savio et al., 1988). When the first GnRH of the protocol is given at the beginning of a follicular wave, a dominant follicle is not present so ovulation frequency to the GnRH injection is expected to be low (Moreira et al., 2000). If heifers began the 5 d Co-Synch + CIDR protocol at random stages of the cycle and there were predominately three wave follicular cycles, then approximately 43% of the estrous cycle would have a dominant follicle capable of undergoing an ovulation to form a new CL either in the presence (luteal phase) or absence of an original CL (proestrus/estrus phases). Of the 298 heifers injected with GnRH at the time of CIDR insertion, 22.9% (50/218) had two CL. Indeed the occurrence of CL regression and basal concentrations of progesterone 24 h after the first injection of PGF2a did not differ between the one or two injections of PGF2a. Even when heifers were presynchronized with GnRH 6 d before initiating the TAI protocol, ovulation to the first GnRH was still low. Rivera et al. (2006) observed that only 39.3% (63/160) of the heifers ovulated to the first GnRH of the TAI protocol regardless of presynchronization. Stevenson et al. (2008b) observed that administration of GnRH at random stages of the estrous

cycle only induced ovulation in 14.3 to 30.7% of the heifers. Collectively, the equivalent P/TAI and similar efficacy of CL regression indicate that one injection of PGF2a is adequate when applying the 5 d Co-Synch + CIDR protocol to dairy heifers. The single injection of PGF2a is sufficient to regress multiple CL and effectively synchronize ovulation in dairy heifers as part of a 5 d Co-Synch + CIDR protocol for TAI.

In contrast to the results of the present study, in lactating dairy cows a difference in CL regression was found when one versus two injections of PGF2a was used in a 5 d Co-Synch 72 h protocol (58.7% versus 95.8% for one versus two injections, respectively) (Chebel et al., 2008). If lactating cows have higher frequency of ovulation in response to the first GnRH injection, they will have a higher frequency of accessory CLs. Therefore, if the period between the GnRH and PGF2a is shortened, the accessory CL will not be responsive to the PGF2a injection therefore, an extra injection of PGF2a 12 hrs after the first PGF2a injection to successfully induce luteolysis of the initially unresponsive CL would be required. Luteal regression could be induced by an exogenous injection of PGF2a given after d 7 of the normal estrous cycle but the same luteolytic dose of PGF2a did not induce luteolysis before d 5 (Schallenberger et al., 1984). When exogenous PGF2a is administered, there is an immediate increase in luteal oxytocin secretion as a result of degranulation of the large luteal cells of the CL (Fields et al. 1992). Maximal level of luteal oxytocin is reached within 5 to 10 min and its secretion drops to basal values within 12 h (Wathes et al. 1984). Oxytocin then causes the pulsatile release of uterine PGF2a, which travels to the CL via the counter-current mechanism to elicit further lysis of the CL. In lactating dairy cows it has been shown that two PGF2a injections at an 8 h interval were more effective in inducing luteolysis than a single injection (Archbald et al., 1993; Repasi et al., 2005). This is probably related to the subsequent stimulation of oxytocin release and endogenous PGF2a.

Although, these studies were done with mature CL during mid diestrus, similar mechanisms can induce luteolysis of an early CL, even 5 d after formation, when 2 injections of PGF2a are administered 12 hr apart. In beef cattle, it has been reported that either in the 5 or 7 d Select Synch + CIDR protocol with a single PGF2a treatment, reproductive performance was similar between treatments for yearling beef heifers (Helser et al., 2006). For beef cows, the authors concluded that similar mechanism observed in lactating cows may determine the use of a double injection of PGF2a in the 5 d Co-Synch + CIDR protocol (Kasimanickan et al., 2009).

The use of a single injection of PGF2a in the protocol represents a distinct advantage for the implementation of TAI in dairy heifers from an economic and practical point of view. Differences in P/TAI to the first TAI were found between farm locations. In the farm in SF with the lower P/TAI, TAI was performed during the summer season; therefore, heat stress could have affected fertility. A study from tropical Australia showed that fertility decreases significantly when temperature increases above 27.5 ºC (Orr et al., 1993). In another study conducted in Florida, heifers AI during the summer season were 76% less likely to become pregnant to the first AI than those inseminated during winter (Donovan et al., 2003). Despite a lower P/TAI in the SF location, no interaction was observed between location and number of PGF2a treatments on P/TAI, thereby indicating that a single PGF2a was as effective as 2 PGF2a injections in both locations.

In experiment 2 involving resynchronization, no difference in P/AI was detected between TAI and AI at detected estrus. Estrous detection requires more labor per animal and therefore can increase costs compared with a synchronization program (Olynk and Wolff, 2008). In an overall economical analysis of reproductive management strategies on United States commercial dairy

farms, synchronization programs emerged as having greater expected net present values (NPV) than visual estrous detection programs (Olynk and Wolff, 2008).

Most heifers came into estrus between d 18 to 24 after the first TAI, so the inter-ovulatory interval was in agreement with the commonly accepted 21 d average. Consequently, nonpregnant heifers would be between d 4 to 14 of their estrous cycle when the second resynchronization protocol was initiated between 28 to 32 d after the first TAI. Most of these heifers could be in a stage of the cycle in which a potential ovulatory follicle would grow under high concentrations of progesterone (Moreira et al., 2000). However, those nonpregnant heifers that failed to be synchronized after the first TAI program would be in a random stage of the estrous cycle when the second resynchronization program is initiated and therefore, the inclusion of a CIDR insert in the resynchronization program would be justified.

In the third experiment, resynchronization of nonpregnant heifers with the use of a CIDR insert between the first GnRH and PGF2a injections tended to increase P/TAI. Including the CIDR insert during the TAI protocol is expected to improve synchronization of ovulation by avoiding heifers from coming into estrus and ovulating before the day of TAI. Another possibility is that a certain number of non-pregnant heifers experienced either early or late embryonic mortality (Van Cleeff et al., 1996) and may benefit from a 5 d period of progesterone exposure to reset the hypothalamic-pituitary-reproductive tract axis in a manner that would improve P/TAI to the subsequent synchronized service. The inclusion of CIDR insert during in the resynchronization program should be used to maximize P/TAI after the second TAI.

The field verification studies indicated that the 5 d Co-Synch + CIDR protocol can be successfully used with a single injection of PGF2a either for the synchronization of first TAI or resynchronization for second TAI. The P/TAI obtained for the first TAI was 60.3% at 32 d, with

a low (3.6%) embryonic loss between d 32 and 60 of pregnancy. This result is comparable to the overall P/TAI obtained in the experiments described in Chapter 3, in which two injections of PGF2a were used in the 5 d Co-Synch + CIDR program (58.3% at d 32 and 57.6% at d 46). However, the P/TAI to the second TAI was lower (52.5%) compared with the results of the first TAI. Fertility in the second TAI was affected by sire. Herd-level or animal-level factors affect fertility in dairy heifers (Donovan et al., 2003), and most herd level variation in P/TAI in heifers is caused by variation among inseminators and service sires (Ron et al., 1984).

The significant effects of service sires on P/TAI in the field studies may be attributed to differences in post-thaw sperm viability, progression of spermatozoa in the female internal genital tract affecting the resultant sperm reservoir, capacitation, acrosome reaction and fertilizing capacity (Januskauskas et al., 1999; Hunter, 2003). Seminal differences that decrease P/AI can be categorized as compensable or uncompensable. Males with compensable deficiencies require higher number of sperm to increase pregnancy, since these defects affect sperm transport and function in the female reproductive tract, including initiation of the fertilization process and the block to polyspermy. When differences in fertility among males or inseminates are independent of sperm dosage the seminal deficiencies are known as uncompensable. Such deficiencies are important to the maintenance of the fertilization event and subsequent embryogenesis (Saacke et al., 2000; Saacke, 2008a,b). Because of compensable deficiencies, there are differences among bulls in response to time of insemination (Dalton et al., 2001). If the bull has little to no compensable deficiencies it would easily meet the threshold numbers of sperm to the cow by AI, perform as well at low sperm dosages as at normal, and be less vulnerable than other bulls to inseminator error in semen placement and handling. Therefore, it would perform well over a broad time span relative to ovulation time. Instead, if seminal

compensable deficiencies are high, bulls would be more vulnerable to dilution rates, inseminator competence, and timing of insemination, requiring later breeding to optimize their efficiency in sperm access to the ovum (Saacke, 2008b). Our results, along with these studies, highlight the importance of semen quality in a TAI protocol to optimize P/TAI.

Conclusion

In dairy heifers, the modified 5 d Co-Synch + CIDR protocol with one injection of $PGF_{2\alpha}$ at the time of the CIDR insert withdrawal and GnRH/TAI 72 h later was an efficient reproductive management program to achieve acceptable P/TAI in dairy heifers. This is an alternative program for managing reproduction in dairy heifers in herds that are not capable to perform daily observation of estrus efficiently.

Table 4-1. Proportion of corpus luteum (CL) regression and least squares means for plasma progesterone concentrations (ng/mL) in heifers that received one or two injections of PGF2a in the 5 d Co-Synch + CIDR protocol (second replicate from SF location; first experiment)

	PGF2a injection	
	One (n =109)	Two (n = 109)
CL regression (%)		
One CL	85.9	93.2
Two CL	88.2	93.3
Progesterone (ng/mL) 24 h after PGF2a		
One CL	0.52 ± 0.03	0.40 ± 0.04
Two CL	0.54 ± 0.07	0.38 ± 0.05
Decrease in progesterone after PGF2a (ng/mL)		
One CL	3.01 ± 0.24	3.37 ± 0.27
Two CL	3.03 ± 0.50	3.64 ± 0.40

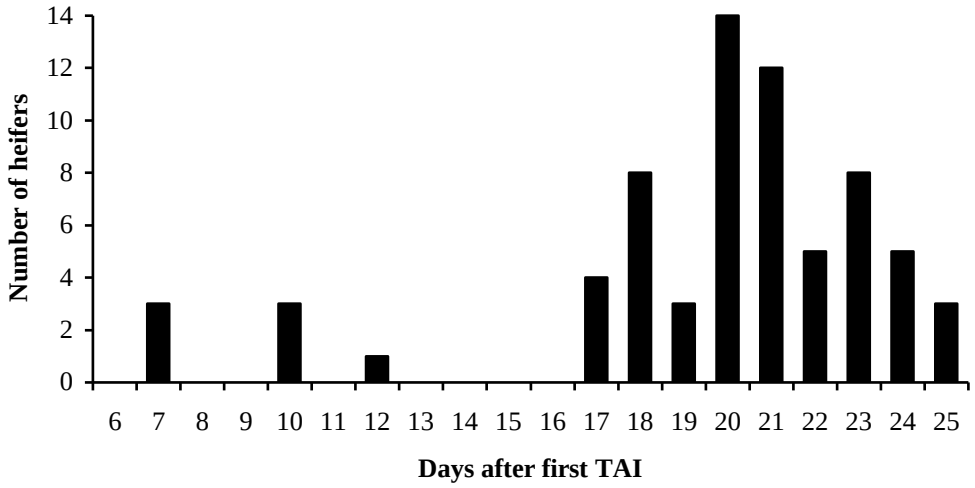

Figure 4-1. Distribution of return to estrus after the first 5 d Co-Synch + CIDR protocol (experiment 2). All heifers underwent daily estrous detection with the use of tail chalk and those heifers with rubbed off chalk or that stood to be mounted were classified as being in estrus.

CHAPTER 5
TIMED ARTIFICIAL INSEMINATION WITH SEXED SEMEN IN DAIRY HEIFERS SYNCHRONIZED WITH THE 5 D CO-SYNCH + CIDR PROTOCOL

Introduction

The goal of sexed semen use in AI is to obtain offspring of a preferred gender. In sexed semen, the fractions of X bearing and Y bearing sperm have been modified from the natural mix through a sorting and selection process using a fluorescence-activated cell-sorting approach described by Johnson et al. (1987a,b, 1999). In 2003 commercial application of sexed semen in the United States began in earnest with the granting of a sorting license to Sexing Technologies Inc. (Navasota, TX). Based on available developmental research, it was anticipated that P/AI would be 70 to 75% of conventional semen (De Jarnette et al., 2009).

Because sexed semen usually results in less P/AI than conventional semen, and to increase the probability of acceptable pregnancy results, recommendations for use of sexed semen have been restricted to first or second AI at detected estrus in virgin heifers. Use of sexed semen in lactating cows or in heifers with fixed TAI is discouraged (De Jarnette et al., 2009).

Ovulation synchronization protocols that allow for TAI without the need for estrous detection consist of the application of reproductive hormones to control ovarian follicular dynamics, luteolysis and ovulation. In lactating dairy cows the most commonly used TAI protocol is Ovsynch, in which injections of GnRH are given 7 d before and 48 h after an injection of PGF2a, and cows are inseminated 16 to 20 h after the second injection of GnRH (Pursley et al., 1995; Burke et al., 1996). In the Co-Synch protocol, TAI is performed at the same time that the second GnRH injection is given (Geary and Whittier, 1998). Another widely used program in lactating cows is the Presynch/Ovsynch, in which presynchronization is achieved by PGF2a given twice at a 14-d interval and the Ovsynch program initiated 12 d after the second injection of PGF2a. In cyclic dairy cows, this program increased P/TAI by 17.3 percentage units

at 72 d compared with cows receiving Ovsynch without presynchronization (Moreira et al., 2001). A CIDR insert (Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) insert containing progesterone is often used during the interval from the initial GnRH to PGF2a injection in the Ovsynch or Co-Synch protocol to prevent estrus after the first GnRH treatment. Inclusion of a CIDR insert is recommended in protocols for TAI of dairy heifers to improve synchronization of ovulation (Rivera et al., 2005). A modified Co-Synch protocol, known as the 5 d Co-Synch + CIDR protocol, increased P/TAI in beef cows compared with the standard 7 d Co-Synch + CIDR protocol (Bridges et al., 2008). Either in beef cows (Kasimanickam et al., 2009) or lactating dairy cows (Chebel et al., 2008), two injections of PGF2a are necessary at CIDR insert withdrawal to improve P/TAI with the 5 d Co-Synch + CIDR protocol. In contrast, similar P/TAI were obtained with dairy heifers with one or two injections of PGF2a within the 5 d Co-Synch + CIDR protocol (Chapter 4). One injection of PGF2a at CIDR withdrawal in the 5 d Co-Synch + CIDR protocol is an efficient reproductive management program in dairy heifers that can be used to achieve acceptable P/TAI.

Considering the challenges faced by dairy producers to raise dairy heifers, an efficient reproductive management program should improve pregnancy results and increase the number of females born. The utility of TAI combined with the commercial availability of sexed semen, could prove to be an effective reproductive management program of dairy heifers if acceptable P/TAI are obtained, especially in herds with inefficient estrous detection (De Vries et al., 2008). However, there is a lack of research on the application of TAI protocols using sexed semen in dairy heifers.

It was hypothesized that the 5 d Co-Synch + CIDR protocol with one injection of PGF2a would be an acceptable reproductive management platform for TAI of dairy heifers with sexed

semen. Objectives were to compare P/TAI using conventional or sexed semen for the first TAI and conventional semen for second TAI in heifers synchronized with the 5 d Co-Synch + CIDR protocol (Experiment 1) and to evaluate P/TAI using a TAI reproductive management program with sexed semen for the first TAI and sexed or conventional semen for second TAI in heifers synchronized with the 5 d Co-Synch protocols (Experiment 2).

Materials and Methods

Heifers, Diets, Housing

Holstein heifers between 13 to 14 mo of age from two commercial dairy farms located in SF and NCF were used in a series of experiments. Heifers in both locations were housed in pasture with access to portable shades and trees and fed once daily a TMR that met or exceeded nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg (NRC, 2001). The diet for each farm was based on the following ingredients: 1) NCF location; lactation cow ration weighback, grass hay, brewer's grains and a mineral and vitamin supplement, 2) SF location; grass silage, lactation cow ration weighback, grass hay, distiller's grains, citrus pulp and a mineral and vitamin supplement. For implementation of synchronization protocol, insemination, and pregnancy examination, heifers were handled in a barn in SF or in an open sided shaded barn in NCF that contained self locking stanchions.

Experiment 1: Pregnancy per TAI using Conventional or Sexed Semen for the first TAI and Conventional Semen for the Second TAI

From May to June 2008, 198 heifers from the SF location were assigned randomly to be TAI with conventional (n = 98) or sexed (n = 100) semen using the 5 d Co-Synch + CIDR protocol with one injection of PGF2a. The protocol consisted of an injection of GnRH (100 µg i.m., Cystorelin®, Merial, Ltd., Iselin, NJ) and a CIDR insert containing 1.38 g of progesterone inserted at d 0; 5 d later (d 5) the CIDR insert was removed and one injection of PGF2a (25 mg,

i.m., Lutalyse®, Pfizer Animal Health, New York, NY) administered; 3 d later (d 8) a second injection of GnRH was administered concurrent with TAI. Commercial straws with sexed or conventional semen were obtained from each of the two sires for TAI and heifers were inseminated by four technicians.

Nonpregnant heifers (n = 103), at 32 d after first TAI diagnosed by ultrasound (**US**), were resynchronized for a second TAI with conventional semen following the same protocol initiated on the same d when the US diagnosis was performed. Semen from two sires were used (one of them was also used for the first TAI), and TAI was performed by four technicians (two were also inseminators for the first TAI).

Experiment 2: Pregnancy per TAI Using a TAI Reproductive Management Program with Sexed Semen for the First TAI and Sexed or Conventional Semen for the Second TAI

From February to March and from November to December 2008, four groups with a total of 802 heifers were used. In the SF location, only one group was used (group 1, n = 192); in the NCF location, three groups were used (group 2, n = 272; group 3, n = 172; and group 4, n = 166). Heifers in all groups at both locations were synchronized for a first TAI with the 5 d Co-Synch + CIDR protocol with one dose of PGF2a. All heifers were inseminated with sexed semen from a total of ten sires (four at the SF location and six at the NCF location) by thirteen technicians (four from the SF location and nine from the NCF location).

For the second TAI, nonpregnant heifers diagnosed by US on d at 32 after first TAI were resynchronized with the same protocol. These heifers were inseminated with sexed semen from two sires (SF location, group 1, n = 114) or conventional semen from five sires (NCF location, group 2, n = 182; group 3, n = 103; and group 4, n = 88). In SF location, inseminations for the second TAI were performed by four technicians, two of them also inseminated heifers in the first TAI. For the NCF location, the second TAI was performed by the same nine technicians that

inseminated heifers in the first TAI. In groups 3 and 4, heifers were assigned randomly to receive either new (n = 95) or 5 d used CIDR (n = 96) inserts for resynchronization in the second TAI. Heifers in groups 1 and 2 received all new CIDR inserts

Pregnancy Diagnosis

Pregnancy diagnoses either for the first or second TAI in both experiments were performed with a 5 MHz US (Easi-Scan®, BCF Systems, Livingston, Scotland) at 32 d after the first TAI. The presence of an embryo with a heartbeat was the criterion used to determine pregnancy (Fricke et al., 1998). Reconfirmation of pregnancy was performed at 45 d (SF) or at 60 d (NCF) by transrectal palpation of the uterus and its contents to determine pregnancy loss. Pregnancy per TAI was defined as the percentage of all heifers that received TAI that became pregnant.

Statistical Analysis

The binary variable, P/TAI to first and second TAI, was analyzed by logistic regression using the GLIMMIX procedure of SAS (SAS Version 9.1 for Windows, SAS Institute, Cary, NC, USA), with heifer treated as a random effect. For model reduction, variables with a P-value > 0.20 were manually removed from the initial model, retaining variables of interest and significant variables in the final model.

In the first experiment, the initial statistical model for P/TAI as the dependent variable for first TAI included semen (conventional or sexed semen), technician, sire and interactions between these variables. Variables retained in the final model were type of semen, sire, technician and the interaction between type of semen and technician. For the second TAI, the model included technician, sire and the interaction of technician and sire.

In the second experiment, the statistical model for P/TAI, using sexed semen for the first TAI, included group and technician by sire nested within group. Orthogonal contrasts were as follows: group 1 versus groups 2, 3 and 4 (i.e., examine SF and NSF location effect); group 2

versus 3 and 4 and group 3 versus 4. Sire by technician was nested in groups because not all sires and technicians were used in each of the groups.

For the second TAI of the second experiment, sexed semen was used at the SF location (group 1) and conventional semen at the NCF location (groups 2, 3 and 4). The statistical model for P/TAI as the dependent variable included group and technician by sire nested within group. Orthogonal contrasts were as follows: group 1 versus groups 2, 3 and 4 (i.e., examine SF and NSF location or sexed versus conventional semen); group 2 versus 3 and 4 (new versus new or used CIDR insert) and group 3 versus 4. Sire by technician was nested in groups because not all sires and technicians were used in each of the groups. An additional analysis was conducted exclusively with groups 3 and 4 to examine the main effects of new and used CIDR inserts, mathematical model for P/TAI included group, CIDR insert, group by CIDR insert, sire by technician nested in group by CIDR insert.

A P-value of ≤ 0.05 was considered significant, and a P-value of ≤ 0.10 and > 0.05 was considered a tendency.

Results

Experiment 1: Pregnancy per TAI using Conventional or Sexed Semen for the first TAI and Conventional Semen for the Second TAI

For the first TAI, P/TAI with conventional semen was 53.1% (52/98) at 32 d and 51.0% (50/98) at 45 d after insemination. Pregnancy loss was 3.8% (2/52). Pregnancy/TAI with sexed semen was 43.0% (43/100) at 32 d and 42.0% (42/100) at 45 d, and pregnancy loss was 2.3% (1/43). There was a tendency (P = 0.10) for a lower P/TAI with the use of sexed semen. However, the main effect of semen was not significant because of the marked difference between the two sires (P = 0.01, OR: 2.23; 95% CI: 1.17 to 4.29) and the interaction (P = 0.03) of technician by type of semen on P/TAI (Table 5-1). Technician D had a higher P/TAI for sexed

semen than conventional semen. For the second TAI only with conventional semen, P/TAI was 55.3% (57/103) at 32 d and 52.4% (54/103) at 45 d. Pregnancy loss was 5.2% (3/57).

Experiment 2: Pregnancy per TAI Using a TAI Reproductive Management Program with Sexed Semen for the First TAI and Sexed or Conventional Semen for the Second TAI

For each group, P/TAI to first TAI with sexed semen and P/TAI to second TAI with sexed or conventional semen is shown in Table 5-2. For the first TAI with sexed semen, overall P/TAI for the four groups was 39.3% (315/802) at 32 d and 35.9% (288/802) at pregnancy reconfirmation. Pregnancy loss was 8.6% (27/315). Group 2 had a lower P/TAI than groups 3 and 4 ($P<0.01$; Table 5-2).

For the second TAI overall P/TAI was 56.9% (277/487) at 32 d with 3.6% (10/277) of pregnancy loss. There was a significant effect of location or sexed semen ($P = 0.01$, Table 5-2) on P/TAI, with a reduced P/TAI at the SF location with sexed semen (42.1%) compared to the NCF location with conventional semen (61.4%) at 32 d. In analyses restricted to groups 3 and 4, there was no difference ($P = 0.18$) in P/TAI between the use of a new versus a used CIDR insert. Overall P/TAI was 61.7% at 32 d with a pregnancy loss of 5.3% (7/118).

Discussion

It is evident that the use of sexed semen for TAI of dairy heifers decreases P/TAI compared with the use of conventional semen. In the present series of experiments, overall P/TAI for the use of sexed semen was 81% of that achieved with conventional semen. Despite of this reduction in P/TAI the use of sexed semen on dairy farms could be justified because it enhances producer's ability to efficiently obtain replacement heifers thus mitigating some of the effects of high culling rates and poor reproductive efficiency. Furthermore, AI virgin dairy heifers with X-bearing sperm would decrease the incidence of calving difficulty, because female calves are smaller than males (Weigel, 2004).

Because the sorting procedure separates X and Y sperm and the reduced sperm number and viability per dose, P/AI with sexed semen is less than with the use of conventional semen. In a retrospective study by DeJarnette et al. (2009), that analyzed farm records from AI with sexed semen, P/AI was 80% of that obtained with conventional semen. In another study, that involved six field experiments with sexed or conventional semen for AI at detected estrus in dairy heifers, P/AI at 60 d with sexed semen average 74.5% of that obtained with conventional semen (Seidel and Schenk, 2008).

In the first experiment of the present study, heifers were synchronized with the 5 d Co-Synch + CIDR protocol, and P/TAI after first TAI with sexed and conventional semen was evaluated under similar conditions, i. e., TAI was performed during the same time period by the same technicians using semen from the same sires. Consistent with previous reports (DeJarnette et al., 2009; Seidel and Schenk, 2008), P/TAI at 32 d with sexed semen in our study was 81.0% of conventional semen (43.0% and 53.1% for sexed and conventional semen respectively). As shown in Table 5-1, sire 1 had a greater P/TAI for either sexed or conventional semen compared with sire 2 for both types of semen. This finding supports previous reports and highlights the importance for careful selection of bulls used for AI based on accurate analysis of fertility under field conditions (Bodmer et al., 2005; Cerchiaro et al., 2007). Until now, monitoring fertility results of sexed semen and using bulls with the highest fertility is the best way to optimize the overall fertility of sexed semen (Frijters et al., 2009).

In Experiment 1, differences in P/TAI obtained by individual technicians varied depending upon type of semen. Technicians A and B obtained consistent P/TAI results with both conventional and sexed semen; whereas, technician C obtained a reduced P/TAI with sexed

semen and technician D obtained a greater P/TAI with sexed semen than with conventional semen.

The variability in P/AI from AI in heifers has been related to technician and service sire (Ron et al., 1984). Many factors related to the successful use of AI are adequate health and nutritional management of the herd combined with knowledge, technical expertise, semen handling, and correct semen placement in the uterus during the AI process (Barth, 1993). Some of these factors could have contributed to the low performance of technician C with sexed semen. Nevertheless, and in concordance with previous studies, P/TAI obtained with sexed semen was less than with conventional semen that was evident when evaluated at the first TAI (experiment 1) or the second TAI (experiment 2, group 1 versus groups 2, 3, and 4). The lower fertility obtained with sexed semen can be attributed to the lower insemination dose and by physical and chemical stresses that occur during the sorting process. These stresses include high dilution of gametes, staining with the DNA binding dye Hoechst 33342, mechanical forces during sorting, light from the UV laser beam, and projection into the collection tube under high pressure and centrifugation (Garner, 2006).

In the second experiment, the 5 d Co-Synch + CIDR protocol was used as a reproductive management program with sexed semen for the first TAI. A significant lower P/TAI for group 2 was found (Table 5-2). A possible explanation for this difference was the greater number of heifers managed for TAI in group 2, which was 61% higher than in the other two groups for the same farm. Consequently, there was pen overcrowding with more heifers not locking in stanchions which could have affected TAI protocol compliance and insemination pressure on the technicians. Recommendations were made for handling a lower number of animals in the subsequent groups. Pregnancy per TAI to the first TAI in the second experiment ranged from

33.1% to 47.0%, with an overall P/TAI of 39.3% and a pregnancy loss of 8.6%. Our results are comparable to results from several field trials from 1999 to 2004 conducted in virgin heifers (Weigel, 2004). Pregnancy per AI in these field trials typically ranged from 35 to 40% with sexed semen, compared with 55 to 60% with conventional semen. Seidel and Shenk (2008) performed a number of field trials in beef and Holstein heifers with sexed semen. Field trials with Holstein heifers were performed at two locations. In one of the locations, a multi-year study included 611 heifers with an overall P/AI of 38.5% at d 56-58 with a 13% pregnancy loss. No differences were detected between 1.0 x 10^6 or 3.0 x 10^6 sperm per insemination dose. Pregnancy per AI with sexed semen was 70% of controls. In the other location, data were collected from 719 inseminates in four herds. Pregnancy per AI ranged from 21% to 48% with a 38.2% average for 1.5 x 10^6 sperm per insemination dose. Pregnancy loss was not reported. In contrast to our study, heifers in these trials (Weigel, 2004; Seidel and Shenk, 2008) were AI at standing estrus, with or without PGF2a injections for estrous synchronization. The use of TAI instead of AI at detected estrus is an economical advantage for the dairy producer, because estrous detection requires more labor per animal and therefore more sensitive to increases in labor costs compared to TAI program. A TAI program has a greater expected net present value (NPV) than a visual estrous detection program (Olynk and Wolff, 2008).

Results from the second TAI with conventional semen from both experiments were similar to a previous study where the 5 d Co-Synch protocol was applied to both first and second TAI with conventional semen (Chapter 4). In a study by Osawa et al., (2009), a resynchronization protocol was initiated at 26 d after the first TAI using the Ovsynch protocol in Holstein cows. The authors found that during the resynchronization period at 26 d , the diameter of the dominant follicle and the plasma estradiol concentration were significantly greater and the plasma

progesterone concentration significantly lower at the time of the second GnRH injection compared with the time of the PGF2a injection. These differences were not detected during the first synchronization period when animals were at different stages of the estrous cycle, concluding that the stage of the estrous cycle may be more consistent among animals during the second synchronization period than during the first. The acceptable P/TAI obtained in the present study after the second TAI with conventional semen indicates that the 5 d Co-Synch protocol can be used successfully for resynchronization of nonpregnant heifers after the first TAI in a timely efficient manner.

For the second TAI in experiment 2, there was a lower P/TAI for group 1, this difference could be attributed to either sexed semen or the SF location (Table 5-2). The difference is due likely to the use of sexed semen because sexed semen had a lower P/TAI compared to conventional semen for the first service conducted at the same SF location (experiment 1). Furthermore use of conventional semen at second service in experiment 1 (SF location) resulted in a P/TAI of 55.3% comparable to the 61.4% at the NF location (groups 2 to 4, experiment 2).

Results from this and other studies clearly indicate that application of sexed semen results in a decreased P/TAI compared with conventional semen. The P/TAI with sexed semen in the present study was comparable to reported by others (Weigel, 2004; Seidel and Shenk, 2008) demonstrating that a TAI protocol like the 5 d Co-Synch + CIDR can be used by a dairy producer that prefers to inseminate with sexed semen. This could become especially important in those heifer management systems with estrous detection problems.

Although the intention of the present research was not to promote the implementation of AI with sexed semen in a dairy farm, the second experiment of this study could be used to estimate the potential advantages of using sexed semen. Sexed semen technology has the

potential to skew the sex ratio of offspring toward 90% of the desired sex, i.e., use of female biased sexed semen results in 90% female calves (Johnson, 2000). Considering the results in the second experiment, group 1 heifers were TAI with sexed semen for both the first and second TAI. Total pregnancy per heifers enrolled after the first and second TAI with sexed semen was 60.4% (116/192). Hypothetically, if 90% of the born calves are heifers, it would be expected that from the total of heifers receiving one or two inseminations, 54.7% (105/192) of the subsequent calves would be female. In the same experiments groups 2, 3 and 4 were TAI with sexed semen for the first TAI and conventional for second TAI. Total pregnancy per heifers enrolled was 72% (439/610). Female calves born would be 90% from the first TAI and theoretically 50% for the second TAI. Consequently, from the total of heifers receiving two inseminations, 50.1% of them would have female calves after an accumulated two services (306/610). In this experiment there was not a group using conventional semen for both inseminations. Nevertheless, if conventional semen was used for both first and second TAI and P/TAI is 60% for the first and second TAI, then 84% of the heifers enrolled would become pregnant after two TAI services. From the total of calves born, 50% would be heifers or 42% of heifers enrolled would be calving female calves after two services. The 12.7 percentage units of difference in heifers born after using exclusively sexed semen versus conventional semen for first and second TAI could be justified in large commercial herds focused on raising replacement heifers.

Conclusion

A reduced P/TAI is expected when AI was observed with sexed compared to conventional semen. Despite the decline in P/TAI, application of the 5 d Co-Synch + CIDR protocol with one dose of $PGF_{2\alpha}$, as a reproductive management platform for TAI of dairy heifers was able to

achieve an acceptable P/TAI with sexed semen. Sexed semen can be used with TAI to effectively manage reproduction in dairy heifers by removing the challenges of estrous detection and increase the number of females born.

Table 5-1. Pregnancy per timed AI (P/TAI) by sire and technician used in heifers synchronized with the 5 d Co-Synch + CIDR protocol, TAI with conventional or sexed semen from the same sires for first service.

	Conventional semen (n = 98)	Sexed semen (n = 100)
32 d P/TAI (%)		
Sire 1	63.3 (31/49)[a]	50.0 (25/50)[a]
Sire 2	42.9 (21/49)[b]	36.0 (18/50)[b]
Technician A	60.9 (14/23)[c]	56.0 (14/25)[c]
Technician B	61.5 (16/26)[c]	52.0 (13/25)[c]
Technician C	56.0 (14/25)[c]	12.5 (3/24)[d]
Technician D	33.3 (8/24)[d]	50.0 (13/26)[c]
45 d P/TAI[1] (%)		
Sire 1	61.2 (30/49)[a]	48.0 (24/50)[a]
Sire 2	40.8 (20/49)[b]	36.0 (18/50)[b]
Technician A	56.5 (13/23)[c]	56.0 (14/25)[c]
Technician B	61.5 (16/26)[c]	48.0 (12/25)[c]
Technician C	56.0 (14/25)[c]	12.5 (3/24)[d]
Technician D	29.2 (7/24)[e]	50.0 (13/25)[c]

[a - b] Means differ between sires (P = 0.01)
[c - d] Means differ semen by technician interaction (P = 0.04)
[1]Pregnancy was reconfirmed at 45 d

Table 5-2. Pregnancy per timed AI (P/TAI) for the first and second TAI by group in heifers synchronized with the 5 d Co-Synch + CIDR protocol and TAI with sexed semen (SS) for the first TAI and sexed (group 1) or conventional (CS) semen (groups 2, 3 and 4) for the second TAI.

	First TAI (SS) (n = 802)	Second TAI (n = 487)
32 days P/TAI (%)		
Group 1	40.6 (78/192)[a]	(SS) 42.1 (48/114)[c]
Group 2	33.1 (90/272)[b]	(CS) 62.6 (114/182)[d]
Group 3	40.1 (69/172)[a]	(CS) 62.1 (64/103)[d]
Group 4	47.0 (78/166)[a]	(CS) 61.4 (51/88)[d]
60 days P/TAI[1](%)		
Group 1	36.5 (70/192)[a]	(SS) 40.4 (46/114)[c]
Group 2	31.3 (85/272)[b]	(CS) 60.4 (110/182)[d]
Group 3	34.9 (60/172)[a]	(CS) 58.3 (60/103)[d]
Group 4	44.0 (73/166)[a]	(CS) 58.0 (51/88)[d]

[1]Pregnancy was reconfirmed at 45 d in group 1 and at 60 d in groups 2, 3, and 4.
[a - b] Means differ between groups for the first TAI (P=0.01)
[c- d] Means differ between groups for the second TAI (P=0.01)

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

The main conclusion from this series of studies is that the modified 5 d Co-Synch + CIDR protocol with one injection of PGF2a at the time of the CIDR insert withdrawal and GnRH/TAI 72 h later, is an efficient reproductive management program to achieve acceptable P/TAI in dairy heifers. Ovulation synchronization protocols that allow for TAI avoiding estrous detection have a major impact on the use of AI for breeding dairy heifers. In addition to the advantages obtained from the use of AI for genetic improvement, the implementation of an ovulation synchronization protocol for TAI represents an economic advantage. Detection of estrus requires more labor per animal and therefore increases costs compared to a synchronization program in a dairy farm (Olynk and Wolff, 2008). In an overall economical analysis of reproductive management strategies used in the United States commercial dairy farms, synchronization programs have greater expected net present value (NPV) than visual estrous detection programs (Olynk and Wolff, 2008). In a more specific economic analysis, Moreira (2009) analyzed the economics for breeding dairy heifers, comparing estrous detection with the 5 d Co-Synch + CIDR protocol. With a TAI management program, heifers would spend less time from puberty to conception, representing a lower negative cash flow due to a decrease in feeding cost, and higher positive cash flow for an increase in the lifetime profit of heifers. Also, a faster accumulation of pregnancies with a TAI program occurs compared to estrous detection within a breeding period. Therefore, a TAI program results in 12% reduction in breeding costs and an 28% improvement in NPV per heifer, including that feed and time savings, provide a positive return on the investment with the 5 d Co-Synch + CIDR program.

The development of the 5 d Co-Synch protocol by Bridges et al. (2008) was based on the hypothesis that an increase in P/TAI would be achieved if the Co-Synch + CIDR protocol were

modified by reducing the interval to 5 d from the first GnRH treatment to the PGF2a injection and CIDR insert withdrawal and lengthening the proestrus interval from PGF2a to the second injection of GnRH/TAI to 3 d. The reason for this modification was based on a series of experiments that demonstrated that a longer proestrus period is related with increased fertility after AI (Mussard et al., 2003, 2007). In addition, greater P/AI was observed in cows with increased estradiol at induced ovulation (Perry et al., 2005). Younger dominant follicles, 4 d after emergence, have increased intra-follicular estradiol concentrations and a greater capacity to produce estradiol than dominant follicles evaluated later in the follicular wave (Valdez et al., 2005). They deduced that increased estradiol production by a younger follicle could be achieved if luteal regression and withdrawal of the CIDR occur earlier relative to the follicular wave emergence, and the interval between PGF2a and the second GnRH administration is longer. Furthermore, if follicles fail to respond to the initial GnRH then the reduced period between GnRH and PGF2a and CIDR exposure reduces the probability of these follicles from becoming aged or to have a prolonged dominance.

A second PGF2a injection was applied approximately 12 h after the first PGF2a injection at the time of the CIDR withdrawal, to make sure that luteolysis also occur in accessory CLs. Bridges et al. (2008) successfully confirmed their hypothesis that tested the application of the 5 d Co-Synch + CIDR protocol in beef cows, because an increased in P/TAI were achieved. In addition, other study by Bridges et al., (2009) confirmed that cows with longer proestrus (2.25 d) had grater P/TAI, higher concentration of progesterone and less occurrence of short luteal phase in the subsequent luteal phase, and higher concentration of estradiol during the proestrus period, compared with cows with shorter proestrus (1.25 d).

Results from the studies performed in the current thesis showed that acceptable P/TAI can be obtained with the application of the 5 d Co-Synch + CIDR protocol in dairy heifers. An important finding from these studies was that a single injection of 25 mg of PGF2a at the time of CIDR withdrawal was as effective as two injections 12 h apart to induce CL regression. In contrast, in lactating dairy cows a difference in CL regression was found when one versus two injections of PGF2a was used in the 5 d Co-Synch 72 h protocol (58.7% versus 95.8% for one versus two injections, respectively; Chebel et al., 2008). The main reason for the difference obtained in CL regression can be related to differences in follicular dynamics between heifers and cows. Compared to lactating cows, heifers have a faster rate of follicular growth (Pursley et al., 1997) and a higher frequency of three wave follicular cycles (Savio et al., 1988). When the first GnRH of the protocol is injected at the beginning of a follicular wave, a dominant follicle is not present, therefore ovulation frequency to the GnRH injection is expected to be low (Moreira et al., 2000). Thus, heifers present a lower probability to ovulate a dominant follicle in response to the first GnRH in the Ovsynch protocol compared to cows (Pursley et al., 1995, 1997). If lactating cows have a higher frequency of ovulation in response to the first GnRH injection, they will have a higher frequency of accessory CLs. Therefore, if the period between the GnRH and PGF2a is shortened, the accessory CL will not respond to the PGF2a injection requiring an additional injection of PGF2a 12 h after the first PGF2a injection to successfully induce luteolysis of the initially unresponsive CL. The use of a single injection of PGF2a in the protocol represents a distinct advantage for the implementation of TAI in dairy heifers from an economic and practical point of view.

The 5 d Co-Synch + CIDR protocol with one PGF2a injection was evaluated as an ovulation synchronization protocol for TAI of dairy heifers with sexed semen. Pregnancy per

TAI with sexed semen was lower compared to P/TAI obtained with conventional semen. Nevertheless, P/TAI was similar to what is reported in other studies with sexed semen, in which heifers were inseminated at detected estrus. A decrease in P/TAI is expected because of a lower sperm dosage and the sorting procedure, which reduces fertility in sexed semen compared to conventional semen. The application of the 5 d Co-Synch + CIDR protocol with one dose of PGF2a for synchronization and TAI of dairy heifers with sexed semen results in an acceptable P/TAI for this type of semen. Sexed semen can be used with TAI to effectively manage reproduction in dairy heifers by removing the challenges of estrous detection and increasing the number of females born.

In a number of experiment of the present thesis, the 5 d Co-Synch + CIDR protocol with one PGF2a injection was also applied for resynchronization of nonpregnant heifers initiated at the day of the pregnancy diagnosis. Pregnancies per TAI obtained were similar to that from the first TAI, resulting also in an acceptable P/TAI after resynchronization. The main significance of this finding is that the majority (~ 75%) of heifers became pregnant after implementing the synchronization and resynchronization protocols, requiring in total 2 periods of 8 d with 3 d of animal handling each one (protocol application and pregnancy diagnosis). A reduction in animal handling within the breeding period after heifers reach puberty, would allow producer to plan ahead the d for protocol application, optimizing the use of labor with the advantage of AI.

In conclusion, the modified 5 d Co-Synch + CIDR protocol with one injection of PGF2a at the time of the CIDR withdrawal and GnRH/TAI 72 h later is an efficient reproductive management program that dairy producers could use for breeding dairy replacement heifers for first TAI and second TAI of nonpregnant heifers. This could have a positive impact in the organization of the dairy farm, especially in those herds with inefficient estrous detection.

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BIOGRAPHICAL SKETCH

Maria Belen Rabaglino was born in the nice city of Rio Cuarto, Cordoba, Argentina. After a wonderful childhood and a great high school experience at the Agronomy School of Rio Cuarto, in 1998 she enrolled in the National University of Rio Cuarto (UNRC) to obtain a degree as veterinarian. Early in her veterinary education she developed an interest in reproduction physiology and the use of the laboratory as a tool to investigate physiologic process or its application in the clinical setting. Consequently, she trained to become Laboratory Technician, to complement her education in veterinary medicine.

Maria Belen obtained her degree as Laboratory Technician in February, 2003; and as Veterinarian in August, 2003. After this, from July 2004, she attended graduate courses at the National University of Cordoba (UNC) and the Animal Reproduction Institution of Cordoba (IRAC) and in August 2006, received the degree of Specialist in bovine reproduction.

As a professional, Maria Belen has always been interested in academic and research activities. She obtained a scholarship for two years from the government at the UNRC as an undergraduate student to be a research assistant in the Animal Pathology Department. After graduation as Laboratory Technician, she obtained an employment contract in the department of Clinical Analyses at the UNRC. After obtaining her veterinary degree, Maria Belen acquired a position in May 2004 as head teaching assistant in the Animal Reproduction Department at the UNRC and a contract to teach Physiopathology of Reproduction at a private University in Mendoza, Argentina.

During her tenure at UNRC and the private University, Maria Belen participated in research and service activities related to canine, equine and ruminant theriogenology, as well as organizing and teaching of graduate courses.

All these activities provided her with an excellent opportunity to develop her personal skills conducive for a teamwork approach for research and the stimulus to continue improving her knowledge in a foreign country. It is known that USA is at the cutting edge in research and technology so, in order to fulfill her goals, Maria Belen applied and obtained in August 2006, a special Fulbright scholarship (Faculty Development program). This scholarship gave her the opportunity to select any University in the United States to pursue a Master program beginning in August 2007, which opened a new chapter in her life.

Maria Belen chose University of Florida due to its prestige and, as she has been always interested in the events related with gestation, parturition and the postpartum period, to conduct research in these areas with Dr. Carlos Risco as advisor. Due to circumstances related to funding, she conducted her MS thesis research in the area of reproductive management of dairy heifers. However, research in these areas has been very satisfactory for her and has added more expertise to her formation as a scientist. Maria Belen received his Master degree from University of Florida in the summer 2009. The Master program has stimulated her to continue with graduate studies in a PhD program at the University of Florida from August 2009. Maria Belen hopes to fully utilize in her doctoral studies, the invaluable skills acquired as a Master student in the Department of Large Animal Clinical Sciences.