ALTERING THE TIMING OF FIXED-TIME ARTIFICIAL INSEMINATION AND THE INTERVAL FROM PROGESTIN WITHDRAWAL TO PROSTAGLANDIN $F_{2\alpha}$ ADMINISTRATION IN ESTRUS SYNCHRONIZATION PROTOCOLS FOR BEEF HEIFERS

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The undersigned, appointed by the dean of the Graduate school, have examined the thesis entitled

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Dedication

This thesis is dedicated to Merrill (Rip) and Judith Redemer, my childhood neighbors and adopted family. Thank you for your unconditional love and kindness towards my family, and for always believing in me.

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List of Abbreviations

AI	Artificial insemination
Ca^{2+}	Calcium
CIDR	Controlled internal drug release
CL	Corpus luteum
CYP11A1	Cholesterol P450 side-chain cleavage enzyme
CYP17A1	17α-hydroxylase/17,20 lyase
CYP19A1	Aromatase
d	Day(s)
ET-1	Endothelin-1
FSH	Follicle-stimulating hormone
FTAI	Fixed-time artificial insemination
GnRH	Gonadotropin-releasing hormone
h	Hour(s)
HSD3ß2	3ß-hydroxysteroid dehydrogenase
HSD17ß	17ß-hydroxysteroid dehydrogenase

LEC	Luteal endothelial cell(s)
LH	Luteinizing hormone
LH-I	Liver homolog receptor
LHr	Luteinizing hormone receptor(s)
LLC	Large luteal cell(s)
MGA	Melengestrol acetate
mL	Milliliter(s)
mm	Millimeter(s)
ng	Nanogram(s)
PAPP-A	Pregnancy-associated plasma
P_4	Progesterone
pg	Picogram(s)
P/AI	Pregnancy rate to AI
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2

$PGF_{2\alpha}$, PG	Prostaglandin $F_{2\alpha}$
PKC	Protein kinase C
PKA	Protein kinase A
RIA	Radioimmunoassay
SAS	Statistical Analysis System
SD	Standard deviation
SE	Standard error
SF-I	Steroidogenic factor I
SLC	Small luteal cell(s)
StAR	Steroidogenic acute regulatory protein

Abstract

An experiment (Chapter 2) was designed to evaluate later timepoints for fixed-time artificial insemination (FTAI) of beef heifers, with the hypothesis that use of a later timepoint would allow a greater proportion of heifers to express estrus prior to FTAI and result in greater conception rates among estrous heifers inseminated with sex-sorted semen. Estrus was synchronized for 1640 heifers in 7 locations using the 14-d CIDR-PG protocol: insertion of an intravaginal progesterone-releasing insert (CIDR; 1.38 g progesterone) on Day -33 and removal on Day -19, and administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol sodium) on Day -3. Within location, heifers were blocked based on reproductive tract score and body weight and were randomly assigned to one of three FTAI timepoints: 66 h, 70 h, or 74 h after PG administration. Estrus detection aids (Estrotect) were applied at PG administration, with activation recorded at FTAI. Heifers that expressed estrus prior to FTAI were inseminated with sex-sorted semen (SexedULTRA 4M™). Heifers that failed to express estrus were inseminated with conventional semen. Gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered coincident with FTAI for heifers that failed to express estrus. The proportion of heifers that expressed estrus prior to FTAI (66 h: 62%; 70 h: 67%; 74 h: 71%) was greater when FTAI was performed at 74 h versus 66 h ($P < 0.01$). Treatments did not differ ($P > 0.10$) with respect to the conception rates of heifers that expressed estrus and were serviced with sex-sorted semen (66 h: 56%; 70 h: 53%; 74 h: 53%). Among heifers that failed to express estrus and were serviced with conventional semen, conception rates were greater ($P = 0.02$) when FTAI was performed at 66 h versus 70 h (66 h: 37%; 70 h: 25%; 74 h: 31%). Results indicate that performing FTAI later

following the 14-d CIDR-PG protocol increases the proportion of heifers that express estrus and are serviced with sex-sorted semen, but later timing of FTAI does not improve conception rates.

A series of experiments (Chapter 3) was designed to evaluate treatment schedules for control of the estrous cycle in which luteolysis is induced prior to atresia of the first follicular wave following a long-term progestin presynchronization treatment in beef heifers. The overarching hypothesis was that the proportion of heifers undergoing luteolysis and expressing estrus would be affected by the duration of treatment with an intravaginal progesterone-releasing insert (CIDR®; 1.38 g progesterone) and/or by the interval from CIDR removal to prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol) administration. In Experiment 1, heifers (n = 91) were blocked by reproductive tract score (RTS) and body weight (BW) and were assigned randomly within block to one of four treatments in a 2 x 2 factorial design: presynchronization via CIDR treatment for either 14 d (Treatments 14-8 and 14-9) or 18 d (Treatments 18-8 and 18-9) with administration of PG either 8 d (Treatment 14-8 and 18-8) or 9 d (Treatment 14-9 and 18-9) following CIDR removal. In Experiment 2, heifers (n = 63) were blocked by RTS and BW and randomly assigned to receive administration of PG either 9 d or 10 d following a 14 d CIDR presynchronization. In Experiment 3, heifers (n = 83) were blocked by RTS and BW and randomly assigned to receive administration of PG either 9 d or 16 d following a 14 d CIDR presynchronization. In all three experiments, estrus detection aids (Estrotect) were applied at the time of PG administration. In Experiments 1 and 2, heifers were observed for expression of estrus three times daily for 4 d following PG administration and received artificial insemination (AI) based on estrous response. For

heifers that failed to express estrus by 96 h after PG administration, timed AI was performed and gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered. In Experiment 3, Estrotect patch activation was recorded at 66 h, and heifers that expressed estrus by 66 h were inseminated at that timepoint. Heifers that failed to express estrus by 66 h were inseminated at 90 h. For heifers that failed to express estrus by 90 h, GnRH was administered at timed AI. In all three experiments, blood samples were collected at the time of PG administration and, for heifers that failed to express estrus, at timed AI to determine serum progesterone concentrations via radioimmunoassay. In Experiment 1, serum estradiol concentrations were also determined via radioimmunoassay. Transrectal ovarian ultrasonography was performed to determine CL status and to measure largest follicle diameter (LFD) at PG administration and, for heifers that failed to express estrus, at timed AI. Transrectal ultrasonography was performed 60-80 d after AI to determine pregnancy status. In Experiment 1, there was a tendency for a greater proportion of heifers to express estrus by 96 h after PG administration when PG administration occurred 9 d after CIDR removal versus 8 d after CIDR removal ($P < 0.07$; 8 d: 57% [26/46]; 9 d: 76% [34/45]). Additionally, serum E2 levels were greater at the time of PG administration when PG was administered 9 d after CIDR removal versus 8 d after CIDR removal following an 18-d CIDR treatment ($P < 0.006$; 18-8: 5.7 ± 1.0 pg/mL; 18-9: 9.6 ± 0.9 pg/mL). In Experiment 2, pregnancy rate to AI (P/AI) was greater among heifers receiving PG administration 9 d after CIDR removal versus 10 d after CIDR removal ($P < 0.03$; 14-9: 58% [18/31]; 14-10: 28% [9/32]). There was a tendency for LFD to be greater among non-estrous heifers in treatment 14-10 versus non-estrous heifers in treatment 14-9 when

measured at timed AI ($P < 0.08$; 14-9: 11.9 ± 1.6; 14-10: 13.6 ± 1.3). In Experiment 3, there was a tendency for serum progesterone concentrations at the time of PG administration to be greater among heifers in treatment 14-16 versus heifers in treatment 14-9 ($P = 0.07$; 14-9: 1.2 ± 0.2 pg/mL; 14-16: 1.8 ± 0.2 pg/mL). These results provide a preliminary evaluation of the feasibility of inducing luteolysis prior to atresia of the first follicular wave following long-term progestin presynchronization.

Chapter 1

Review of Literature

Bovine Estrous Cycle

Introduction

The estrous cycle is the duration between recurring periods of estrus and follicular ovulation. This cycle is characterized by physiological changes that are induced by reproductive hormones controlled by the hypothalamic-pituitary-ovarian axis. These changes are regulated by neuroendocrine and endocrine factors that involve a network of regulatory signals to control ovarian follicular development, atresia, ovulation, luteinization, luteolysis, and estrus. Cattle are polyestrous animals, meaning they have regularly occurring estrous cycles and can conceive during any time of the year, regardless of season. Once a heifer has reached puberty, she will cycle regularly. The average estrous cycle length in the cow is 21 days but varies between individual animals from 17 to 24 days (Hammond, 1927; Nellor & Cole, 1956; Hansel & McEntee, 1970; Wishart, 1972). Estrous cycle length is dependent on the timing of luteolysis and the number of follicular waves an animal has within each cycle. Phases of the estrous cycle are characterized by ovarian structure development and hormone production. One ovarian structure is the corpus luteum (CL), which is a transient endocrine gland that forms from an ovulated follicle. This structure produces progesterone (P_4) and is essential to the maintenance of pregnancy. Typically, a single CL will form following ovulation, and be maintained throughout the luteal phase of the estrous cycle before undergoing luteolysis and regression. Follicular development also occurs throughout the estrous cycle and is described in further detail in the "Folliculogenesis" section of this review.

The bovine estrous cycle consists of a follicular and a luteal phase. The follicular phase is the period from CL regression to ovulation and includes a proestrus and estrus stage. The dominant ovarian structure during this time is a large preovulatory follicle that is producing estradiol. The luteal phase is the period from ovulation to CL regression and includes metestrus and diestrus. During this phase, the dominant ovarian structure is a CL that is producing P_4, although follicular growth still occurs during this time.

Proestrus

Proestrus takes place during the follicular phase and is the period of CL regression and final maturation of the dominant preovulatory follicle. The declining action of P_4 on the uterus towards the end of diestrus triggers a cascade of events that lead to luteolysis and CL regression. Luteolysis occurs 16 to 19 days after estrus and is required for the initiation of a new estrous cycle (Ginther et al., 1989a). The uterus secretes the luteolysin that causes regression of the CL (Loeb, 1923; Wiltbank & Casida, 1956; Armstrong & Hansel, 1959; Anderson & Neal, 1961; Malven & Hansel, 1964; Anderson et al., 1965). Research involving the removal of the uterine horn ipsilateral to the CL has demonstrated that the luteolysin acts by a local effect (Ginther et al., 1967; Ginther, 1974; Hixon & Hansel, 1974; Ginther, 1981). Progesterone levels decline further as the CL regresses, allowing for increased estradiol action.

John Babcock suggested that prostaglandins may be the luteolytic factor controlling CL regression at "The Second Brook Lodge Workshop on Problems of Reproductive Biology" in May 1965 (Duncan et al., 1966). At the time, researchers understood that prostaglandins were a class of compounds released from the uterus that

had vasoconstrictive properties (Lauderdale, 2010). The identification of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as the mammalian luteolysin in the rat (Pharriss & Wyngarden, 1969) and ewe (McCracken et al., 1970, McCracken et al., 1972) led to $PGF_{2\alpha}$ being identified as the primary luteolytic compound in cattle (Hixon & Hansel, 1974; Lauderdale, 1974; Hansel et al., 1975). A countercurrent process between the uterine vein and ovarian artery transfers $PGF_{2\alpha}$ from the uterus to the ovary where $PGF_{2\alpha}$ acts on the CL to cause regression (McCracken et al., 1972; Hixon & Hansel, 1974; Walpole, 1975; Land et al., 1976). In cattle, $PGF_{2\alpha}$ can also act by a systemic effect. About 65% of $PGF_{2\alpha}$ is metabolized in one passage through the lungs, meaning the other 35% remains in circulation and can induce luteolysis (Davis et al., 1985). The development of an ovarian autotransplant model and method for autotransplant of the uterus and ovary together, in the ewe, was pivotal to the understanding of the local luteolytic interaction between the uterus and ovary (Goding & McCracken, 1966; Goding et al., 1967; McCracken et al., 1969; McCracken et al., 1970; McCracken et al., 1971).

Oxytocin is also involved in luteolysis and affects estrous cycle length (Armstrong & Hansel, 1959). As the luteal phase progresses, progesterone receptor levels in the endometrium decline due to a negative feedback effect of high levels of circulating progesterone (Schrader & O'malley, 1978; Zelinski et al., 1982; Vesanen et al., 1988). Estradiol levels increase with progressive follicular development and stimulate the formation of endometrial estradiol and oxytocin receptors (Roberts et al., 1976; Roberts et al., 1976; Koligian & Stormshak, 1977a; Koligian & Stormshak, 1977b; Clark et al., 1977; McCracken et al., 1981; McCracken et al., 1984; Hixon & Flint, 1987). Increased estradiol synthesis also increases the hypothalamic oxytocin pulse generator frequency,

increasing oxytocin secretion. Oxytocin is released from the posterior pituitary and interacts with oxytocin receptors in the endometrium. The interaction of neurohypophysial oxytocin with endometrial oxytocin receptors causes the secretion of endometrial luteolytic $PGF_{2\alpha}$ pulses (McCracken et al., 1984; Silvia et al., 1991). Uterine $PGF_{2\alpha}$ release will stimulate luteal oxytocin release, creating a positive feedback loop. Oxytocin is secreted by large luteal cells (LLCs) and binds to receptors on small luteal cells (SLCs). This activates the protein kinase C (PKC) second messenger pathway, which inhibits P_4 production and induces an intracellular calcium (Ca^{2+}) influx that causes apoptosis. The pulse magnitude of $PGF_{2\alpha}$ secretion increases as the luteolytic process proceeds, and the CL undergoes functional and structural luteolysis.

When $PGF_{2\alpha}$ is secreted by the endometrium, it binds to receptors on luteal endothelial cells (LECs) and large luteal cells (LLCs) (Milvae, 2000). The majority of $PGF_{2\alpha}$ receptors are found on LLCs (Fitz et al., 1982), and binding of $PGF_{2\alpha}$ to granulosa-derived LLCs activates the PKC second messenger pathway (Wiltbank et al., 1991; Niswender, 2002). Activation of PKC inhibits progesterone production and triggers a cascade of events that leads to an intracellular influx of Ca^{2+}, inducing apoptosis and cellular degeneration. Steroidogenic luteal cells contain receptors for many regulatory hormones and factors. Binding of $PGF_{2\alpha}$ to LECs activates the endothelin-1 (ET-1) gene in LECs, increasing the biosynthesis and secretion of ET-1. Endothelin-1 is a potent vasoconstrictor, and binding of ET-1 to receptors located on SLCs and LLCs leads to a decrease in basal and LH-stimulated progesterone production, a decrease in production of luteotropic prostaglandin I_2 (PGI_2), and an increase in luteolytic prostaglandin (Milvae, 2000). A reduction in blood supply to and within the CL results in complete functional

and structural luteolysis. Inhibition of progesterone secretion from LLCs by $PGF_{2\alpha}$ causes a cytotoxic effect that disrupts SLCs.

As the CL regresses, plasma progesterone levels decline. The removal of the negative feedback effect of progesterone on the hypothalamus leads to increased GnRH secretion, and a subsequent increase in LH and FSH secretion from the anterior pituitary. During this period, the increased concentration of plasma LH levels is characterized by pulses of high frequency and low amplitude (Rahe et al., 1980). A rise in LH secretion paired with an increase in LH receptor expression on the granulosa and theca cells of the dominant follicle induces an increase in estradiol synthesis and secretion by the follicle (Bao & Garverick, 1998; Ginther et al., 2001). Estradiol increases the sensitivity of the pituitary to GnRH and, in the absence of high levels of progesterone, induces the preovulatory LH surge (Allrich, 1994).

Estrus

Estrus is the period of sexual receptivity just prior to ovulation. The best indicator of estrus expression or sexual receptivity is "standing estrus", which is when the female stands to be mounted. The period of estrus lasts 12 to 16 h on average with a wide range from 3 to 28 h (Hurnik & King, 1987; Allrich, 1993; Allrich et al., 1994). Other behaviors are secondary indicators of this phase as heifers and cows in estrus will exhibit increased activity, increased vocalization, and attempt to mount other herd mates.

Estrus is induced by an elevation in plasma estradiol-17ß levels caused by increased follicular steroidogenesis when final maturation of the preovulatory follicle occurs (Hansel & Convey, 1983). The "two-cell two-gonadotropin model" illustrates that

synthesis and secretion of estradiol by pre-ovulatory follicles occurs through the combined action of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) with granulosa and theca interna cells located in the follicle wall (Armstrong & Papkoff, 1976; Fortune & Quirk, 1988). Both theca and granulosa cells are required for estradiol synthesis and secretion (Falck, 1959). The G-protein-coupled receptor for LH is present on theca cells. Binding of LH stimulates a cascade of events within theca cells triggering the activation of steroidogenic factor I (SF-I) which initiates transcription of a series of steroidogenic genes (Marsh, 1976; Fortune, 1986; Liu & Simpson, 1997). This activates enzymes for the transport of cholesterol into the mitochondrial membrane by steroidogenic acute regulatory protein (StAR) (Lin et al., 1995; Juengel et al., 1995) where cholesterol is converted to pregnenolone by cholesterol P450 side-chain cleavage enzyme (CYP11A1). Pregnenolone produced in the mitochondria enters the cytosol where it is converted to progesterone by 3ß-hydroxysteroid dehydrogenase (HSD3ß2), and progesterone is converted to androstenedione by 17α-hydroxylase/17,20 lyase (CYP17A1). Enzyme activation for these pathways is induced by SF-I activation. By a paracrine interaction, androstenedione diffuses across the basement membrane from theca cells to granulosa cells. In granulosa cells, aromatization of androgens occurs by stimulation of FSH (Dorrington et al., 1975). Binding of FSH to the G-protein coupled receptor for FSH, located on granulosa cells, triggers a cascade of events leading to activation of SF-I and liver homolog receptor I (LH-I) (Marsh, 1976; Fortune, 1986). This mediates enzyme expression of aromatase (CYP19A1) for catalysis of the conversion of androstenedione to estrone, and 17ß-hydroxysteroid dehydrogenase (HSD17ß) for catalysis of the conversion of estrone to estradiol. Androstenedione can

also be converted to testosterone by HSD17ß, and testosterone converted to estradiol by aromatase. Synthesized estradiol diffuses across the basement membrane to enter the bloodstream or enters follicular fluid.

When a given threshold of estradiol is met concomitantly with low progesterone levels, estradiol acts on the hypothalamus to induce estrous behavior (Allrich, 1994). An increase in circulating estradiol concentrations exerts a positive feedback effect on the pulsatile release of GnRH from the hypothalamus and LH secretion from the anterior pituitary gland (Goodman et al., 1994). Gonadotropin-releasing hormone stimulates the release of LH from the anterior pituitary gland (Schally et al., 1971) and estradiol stimulates synthesis of GnRH receptors, increasing the ability of the pituitary to respond to GnRH (Reeves et al., 1971; Kaynard et al., 1988). As progesterone levels decline and estradiol levels increase, GnRH pulse frequency increases and GnRH pulse amplitude decreases (Clarke et al., 1987). This creates a positive feedback loop as GnRH and LH pulses will stimulate a further increase in estradiol secretion by the follicle. High levels of estradiol will stimulate release of GnRH from the surge center of the hypothalamus at a high frequency and high amplitude, which induces the preovulatory surge of LH (Allrich, 1994; Karsch et al., 1997). The preovulatory LH surge occurs near the time of estrus onset, which precedes ovulation by 24 to 33 h (Hansel & Echternkamp, 1972; Walker et al., 1996). Gonadotropin-releasing hormone also stimulates the release of FSH from the anterior pituitary gland (Schally et al., 1971); however, estradiol and inhibin have a negative feedback effect on FSH release (Clarke et al., 1986). Removal of the negative feedback effect of inhibin and estradiol induces a transient rise in circulating FSH levels 4 to 12 h after the LH surge. This will induce recruitment of a new follicular wave.

Metestrus

The metestrus stage includes ovulation and early luteal development. Throughout the estrous cycle, LH is released in a pulsatile manner that fluctuates in frequency and amplitude. During the preovulatory surge, pulsatile LH is released at a high frequency and high amplitude and causes physiological and morphological changes that lead to ovulation (Walters & Schallenberger, 1984). The LH surge activates a cascade of proteolytic enzymes that cause the degradation and weakening of the cell wall of the preovulatory follicle (Reich et al., 1991). An increase in blood flow to the ovary and dominant follicle, and inflammatory, enzymatic, and morphological changes lead to increased pressure within the follicle (Espey, 1994). Intrafollicular forces cause the weakened cell wall of the follicle to rupture and release a haploid oocyte, capable of being fertilized.

After ovulation, a corpus luteum develops from the ruptured follicle and undergoes angiogenesis. Preovulatory gonadotropin surges of LH and FSH initiate the follicular cell changes associated with luteinization (Lipner et al., 1988; Niswender & Nett, 1988). Follicular cells differentiate to form luteal cells that synthesize and secrete progesterone (Hansel & Convey, 1983). The structural and functional changes of the granulosa and theca cells during luteinization shift these cells from estradiol- to progesterone-producing cells. Large luteal cells are derived from granulosa cells, and small luteal cells are derived from theca interna cells (Alila & Hansel, 1984; McCracken et al., 1999; Niswender et al., 2000). Small and large luteal cells differ in receptor content, second messenger function, and steroidogenic characteristics (Ursely & Leymarie, 1979; Koos & Hansel, 1981)

As development continues, the CL undergoes rapid growth, increasing from 640 mg on Day 3 of the cycle to 5.07 g on Day 14 of the cycle (Fields & Fields, 1996). This rapid increase in CL weight is largely due to hypertrophy of the LLCs, which comprise only 3% of the total number of luteal cells but make up 40% of the volume of the CL (O'Shea et al., 1989). Some SLCs will differentiate into LLCs as the CL ages (Alila & Hansel, 1984).

The capacity for P_4 synthesis and secretion is greater in LLCs than SLCs; however, SLCs are more sensitive to changes in LH because they contain more LH receptors (LHr; Fitz et al., 1982). Binding of LH to LHr on SLCs stimulates the production of P_4 by activating the protein kinase A (PKA) second messenger pathway (Hoyer & Niswender, 1985; Niswender, 2002). On LLCs, LH receptors are present but do not respond to receptor binding with an increase in progesterone production. The majority of $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2) receptors, which have luteolytic and luteotropic functions, are found on LLCs (Fitz et al., 1982). Other luteal cell types that are non-steroidogenic but important for CL function are fibroblasts, macrophages, and endothelial cells.

Diestrus

Diestrus is the longest of the four stages and occurs during the luteal phase of the estrous cycle. This period includes the majority of CL development and maintenance, and it ends with eventual luteolysis. As the CL progresses through development, P_4 synthesis and secretion greatly increases (Garverick et al., 1971). This increase in plasma progesterone concentration is highly correlated with luteal weight and volume (Wiltbank

et al., 1995). Vascularization and blood flow to and from the luteal ovary also increase at this time. This rapid vascularization process is required for the normal development of the CL (Smith et al., 1994; Reynolds et al., 2000). As the CL develops and is maintained throughout the luteal phase, blood flow to the luteal ovary increases from less than 1 ml/min to 3-7 ml/min (Niswender et al., 1976). Efficient blood flow increases the speed at which progesterone enters the bloodstream. During the mid-luteal phase, 65 to 95% of ovarian blood flows to the CL (Niswender et al., 1976). This is important for the exchange of proteins and hormones between the luteal cells and bloodstream through permeable luteal capillaries (Ellinwood, 1978).

Pulses of LH are necessary for normal CL development and function, including progesterone secretion (Summons & Hansel, 1964; Karsch et al., 1971; Baird, 1992; Peters et al., 1994). Early growth and development of the CL is dependent on LH action, but secretion of LH is not required for CL maintenance past Day 12 of the estrous cycle (Peters et al., 1994). Small luteal cells are sensitive to LH and respond with increased progesterone secretion (Ursely & Leymarie, 1979; Fitz et al., 1982; Hoyer & Niswender, 1985). Large luteal cells secrete progesterone independently of LH action, however, and because large luteal cells produce the majority of mid-cycle progesterone, CL function is largely not dependent on LH secretion during this time (Wiltbank, 1994).

During the mid-luteal phase, progesterone output by both cell types is dependent on lipoproteins. Normal progesterone production by the CL requires cholesterol for steroidogenesis, and serum lipoproteins are a major source of cholesterol. Cholesterol sources include low- and high-density lipoproteins, as well as stored cholesterol esters that are hydrolyzed by cholesterol esterase (Pate & Condon, 1982; O'Shaughnessy &

Wathes, 1985; Carroll et al., 1992). Efficient blood flow during the luteal phase delivers the lipoproteins for steroidogenesis, and sterol carrier proteins and cytoskeletal elements transport free cholesterol to the mitochondria. Steroidogenic acute regulatory protein (StAR), expressed in both LLCs and SLCs, transports cholesterol from the cytosol to the inner portion of the mitochondrial membrane (Lin et al., 1995; Juengel et al., 1995). In the mitochondria, cholesterol is converted to pregnenolone by cytochrome P450 cholesterol side chain cleavage enzyme complex (P-450scc). Pregnenolone is converted to progesterone by 3ß-hydroxysteroid dehydrogenase/ Δ^5, Δ^4 isomerase in the smooth endoplasmic reticulum (Niswender, 2002). Progesterone diffuses from the luteal cell to enter the bloodstream.

Folliculogenesis

Introduction

Folliculogenesis consists of a series of events that leads to the maturation of ovarian follicles. The progression from small primordial follicles to a large preovulatory follicle occurs in a wave-like process involving follicular recruitment, selection, and dominance (Fortune, 1994; Ginther et al., 1996b; Webb et al., 1999; Ginther et al., 2001; Jaiswal et al., 2009). Waves of ovarian follicular growth consist of the synchronous development of a cohort of follicles. Transrectal ultrasonography has demonstrated that most bovine estrous cycles consist of a two- or three-wave pattern (Sirois & Fortune, 1988; Savio et al., 1988; Ginther et al., 1989c). Each wave consists of one follicle that becomes dominant and a variable number of subordinate follicles that develop and undergo atresia.

The dominant follicle will either undergo atresia or become the ovulatory follicle depending on the stage of the estrous cycle. If cattle are in the luteal phase of the estrous cycle, the dominant follicle will be non-ovulatory and eventually undergo atresia (Matton et al., 1981; Ireland & Roche, 1983). The ovulatory dominant follicle originates from the final follicular wave in the estrous cycle and will ovulate if luteal regression occurs during the growing phase of the dominant follicle (Kastelic et al., 1990; Sirois & Fortune, 1990; Lucy et al., 1992). Follicular waves also occur during the prepubertal period in heifers (Evans et al., 1994), during gestation (Ginther et al., 1989b; Ginther et al., 1996a; Adams, 1999), and during postpartum anestrus (Ginther et al., 1996a).

Early Follicular Development

There are approximately 150,000 primordial follicles present in the bovine ovary at birth and a few hundred growing follicles at any one time (Erickson, 1966). During fetal development, primordial germ cells from the epithelium of the fetal yolk sac migrate to the gonadal ridge (Smitz & Cortvrindt, 2002). During migration and upon reaching the gonadal ridge, the primordial germ cells undergo a limited number of mitotic divisions (Smitz & Cortvrindt, 2002). Primordial germ cells cease mitotic division when they are internalized into the gonadal ridge through the surface epithelium. Once primordial germs cells are enclosed in germ cell cords made up of epithelial cells, they are referred to as oogonia (Russe, 1983). As fetal development progresses, these oogonia undergo developmental changes to form primary oocytes. Primordial follicles are characterized by a single layer of flattened cuboidal cells surrounding a primary oocyte arrested in prophase I of meiosis (Fortune, 1994). Primordial follicles are stored in a “resting pool”

and continuously recruited into the growing pool of follicles; however, the fate of more than 99% of all follicles is atresia (Ireland, 1987).

Follicular growth begins with the transition of primordial follicles to primary follicles. Primordial follicles consist of one layer of squamous granulosa cells surrounding a single oocyte. The transition starts with the phenotypic shift of granulosa cells on the primordial follicle from a flattened shape to a cuboidal proliferative phenotype (Pedersen & Peters, 1968; Braw-Tal & Yossefi, 1997). Gonadotrophs do not initiate this process (Dufour et al., 1979), and the bovine follicle can grow to 4mm without FSH (Garverick et al., 2002). Development of FSH receptors occurs in primary and secondary bovine follicles, and follicular growth is dependent on FSH after development to the primary follicle stage (Bao & Garverick, 1998). Follicle-stimulating hormone promotes continued follicular growth and contributes to the increase in estradiol synthesis by follicles. Follicular growth continues with granulosa cell proliferation and an increase in oocyte size.

Recruitment

Recruitment is a gonadotropin-dependent event that occurs when a cohort of follicles acquire the ability to respond to gonadotropins and begin to mature in response (Hodgen, 1982; Goodman & Hodgen, 1983). A small rise in circulating FSH initiates the recruitment of each wave (Adams et al., 1992a; Ginther et al., 1996a). Gonadotropin-releasing hormone stimulates the release of FSH from the anterior pituitary gland (Schally et al., 1971); however, estradiol and inhibin have a negative feedback effect on FSH release (Clarke et al., 1986). Regression of the dominant follicle at the end of a

follicular wave or ovulation at the end of the estrous cycle causes a transient elevation in circulating FSH levels. Secretion of FSH stimulates locally produced growth factors, and expression of mRNA for steroidogenic enzymes cytochrome P450 side chain cleavage (P450scc) and cytochrome 450 aromatase (P450arom) in granulosa cells (Bao & Garverick, 1998). These enzymes are necessary for steroidogenesis and will promote continued follicular growth and development (Xu et al., 1995). With each follicular wave, a new cohort of follicles is recruited and starts to grow. A cohort typically consists of 8 to 41 small follicles that develop at a similar rate for 2 to 3 days, until selection occurs (Savio et al., 1988; Sirois & Fortune, 1988; Ginther et al., 1989a; Ginther et al., 1996b). Inhibin and estradiol, produced by the growing follicles, have a negative feedback effect on FSH secretion (Clarke et al., 1986; Adams et al., 1992b; Adams et al., 1993; Gibbons et al., 1997). Emergence of the first follicular wave within the estrous cycle takes place on the day of ovulation, or Day 0 (Adams, 1999; Aerts & Bols, 2010). The day of subsequent follicular wave induction is dependent on the number of follicular waves within the cycle. Two-wave cattle will have two separate surges of FSH to stimulate follicular recruitment, and these typically occur on Day 0 and on Day 9 or 10 of the estrous cycle. Three-wave cattle will have three FSH surges to initiate follicular recruitment and these typically occur on Day 0, Day 8 or 9, and Day 15 or 16 of the estrous cycle (Adams, 1999; Aerts & Bols, 2010).

Selection

Follicle selection is when one follicle from a cohort of growing medium-sized follicles is selected to continue to develop toward ovulation. Follicles undergo a period known as the common-growth phase for 2 to 3 days after their initial emergence at 4.0 mm (Ginther, 2000). After the common-growth phase, deviation begins. Deviation is a central event in follicle selection and is described as the initial deviation in growth rates between dominant and subordinate follicles. There is an increase in growth of the largest follicle, which becomes the dominant follicle, and a reduction in growth of smaller follicles, which become subordinate follicles (Ginther et al., 1997). Events contributing to selection include decreased FSH secretion by the pituitary, increased expression of LH receptors within the granulosa cells of the selected follicle, and increased estradiol production by the dominant follicle (Ireland & Roche, 1983; Ireland, 1987; Gong et al., 1996; Sartori et al., 2001; Beg et al., 2001; Ginther et al., 2001). As the wave progresses, follicles produce increasing levels of estradiol and inhibin, selectively inhibiting the release of FSH from the anterior pituitary and reducing FSH to basal levels through negative feedback (Adams et al., 1992b; Adams et al., 1993; Gibbons et al., 1997). The largest follicle expresses receptors for both LH and FSH. Selective inhibition of FSH will limit the growth of subordinate follicles as only the selected follicle has receptors for LH. Without FSH, subordinate follicles cease growth and eventually become atretic.

Dominance

There are two models that have been developed to explain how follicular dominance is acquired; the Missouri model and the Cornell model. The Missouri model was developed at the University of Missouri-Columbia and describes a transition from

FSH dependence to LH dependence by the dominant follicle (Xu et al., 1995). During recruitment, follicles are FSH dependent, and the ability of granulosa cells to bind FSH does not vary significantly with follicle size (Ireland & Roche, 1983). The dominant follicle that arises from the recruited pool acquires LH receptors in granulosa and theca interna cells. This enables a dominant follicle to "starve" subordinate follicles by selectively inhibiting FSH levels through estradiol and inhibin negative feedback mechanisms (Xu et al., 1995). Secretion of LH is not inhibited by these mechanisms and thus can support the continued growth of the dominant follicle.

The Cornell model is based on the premise that one follicle within a particular wave has a developmental advantage over the other follicles in that wave (Fortune et al., 2004; Lucy, 2007). With this model, it is theorized that mRNA expression for LH receptors and expression of LH receptors in granulosa cells only increases after selection of the dominant follicle has occurred (Evans & Fortune, 1997). The initiating event is the increase in pregnancy-associated plasma protein-A (PAPP-A). The FSH that initiates the recruitment of a follicular wave induces synthesis of PAPP-A, also known as insulin-like growth factor binding protein (IGFBP) protease. This protease acts in follicular fluid to degrade IGFBP-4 and IGFBP-5. These binding proteins bind insulin-like growth factor-1 (IGF-1), so a decrease in these binding proteins leads to an increase in free IGF-1 within the follicular fluid. Estradiol synthesis by the follicle is stimulated by the coaction of IGF-1 and FSH. Estradiol exerts a negative feedback effect on FSH production by the anterior pituitary, allowing the dominant follicle to continue to grow in response to FSH while "starving" subordinate follicles into atresia.

It is likely that the mechanisms hypothesized in both the Missouri and Cornell models are involved in selection and are ultimately required by the dominant follicle. The dominant follicle possesses receptors for both LH and FSH at this time, and both are involved in steroidogenesis. Around the time of selection, LH receptor expression in granulosa cells, concentrations of free IGF-1, and estradiol production by the follicle increase significantly in the selected follicle (Beg & Ginther, 2006). The selected dominant follicle escapes initial atresia, continues to grow, and suppresses further development of subordinate follicles (Goodman & Hodgen, 1983). Dominance is characterized by decreasing FSH levels and increasing LH levels. Dominant follicles from both anovulatory and ovulatory waves secrete estradiol and inhibin, which selectively inhibit secretion of FSH from the pituitary gland (Clarke et al., 1986; Sunderland et al., 1994; Arai et al., 1996). Beg and Ginther (2006) have proposed that the increase in LH receptor expression and free IGF-1 occur at approximately the same time and that both theories are likely involved in acquisition of dominance by a follicle.

Estradiol is a major contributor to the initiation of the preovulatory surge of LH, while progestins have a negative feedback effect on LH release (Hobson & Hansel, 1972; Chenault et al., 1975). Estradiol synthesized and secreted by the dominant follicle has a priming effect on the pituitary (Kesner et al., 1981, 1982). Estradiol can induce the preovulatory surge of LH by increasing GnRH pituitary sensitivity and GnRH release from the surge center of the hypothalamus. Luteal regression must occur during the growing phase of the dominant follicle in order for ovulation to occur (Kastelic et al., 1990; Sirois & Fortune, 1990; Lucy et al., 1992). When luteolysis occurs and plasma progesterone levels decline, LH pulsatility will increase (Rahe et al., 1980). Acquisition

of enough LH receptors is required for ovulation of the dominant follicle. The dominant follicle acquires LH receptors in granulosa and theca interna cells. The increase in pulsatile LH will increase LH receptor expression in granulosa cells, increasing the ability of the dominant follicle to respond to the LH surge and ovulate (Bao & Garverick, 1998).

The dominant follicle reaches an advanced stage of development even during the luteal phase of the estrous cycle. Large luteal phase follicles are capable of ovulating if stimulated. This was demonstrated by an experiment in which it was observed that heifers treated with 1500 IU of hCG could ovulate a large, luteal-phase follicle (Price & Webb, 1989). A subsequent experiment demonstrated that 75-80% of heifers treated with 500 μg synthetic GnRH on Day 6 of the estrous cycle ovulated (Webb et al., 1992). However, without exogenous stimulation, if luteolysis does not occur during the growing phase, the dominant follicle will undergo atresia due to insufficient LH pulsatility (Sirois & Fortune, 1990; Lucy et al., 1992).

The Development of Estrus Synchronization Protocols

Introduction

Exceptional growth in the use of artificial insemination (AI) in the cattle industry occurred in the 1940s and 50s as technological advancements in the production of semen extenders (Lardy & Phillips, 1940; Salisbury et al., 1941; Almquist et al., 1949; Foote & Bratton, 1950) and eventually, cryopreservation practices were made (Smith & Polge, 1950; Polge & Rowson, 1952; Polge, 1953; Foote, 2002). A major limitation to the wider use of this technology, especially within the beef industry, was the requirement of daily

estrus detection. The development of methods to control the bovine estrous cycle enabled wider commercialization of AI. Progestogens, as well as luteolytic and luteotropic compounds, were used in this pursuit. Initially, fertility following estrus synchronization was very poor, even when cattle were inseminated based on detection of estrus. As the understanding of the physiology and endocrinology of the bovine estrous cycle improved, more effective estrus synchronization protocols were developed. Today, cattle can be inseminated at a fixed timepoint following estrus synchronization with similar fertility to that expected following estrus detection. The development of more effective ways to control luteal and follicular development has led to this capability. Research to minimize the number of cattle handlings, the cost associated with estrus synchronization, and the difficulty of application continue.

Progestogens

The understanding that corpus luteum (CL) lifespan is a critical factor in determining estrous cycle length led to the administration of exogenous progestogens to control the estrous cycle. Progestogens can be used to mimic the effect of progesterone endogenously produced by the CL and, when administered at sufficient concentrations, can inhibit estrus and ovulation. Progestogens do not alter the secretory capacity of the CL, so progestogen treatments were initially designed to be as long as the luteal phase (Thimonier et al., 1975). Later, transrectal ultrasonography demonstrated that the bovine estrous cycle consists of wave-like patterns of follicular development (Sirois & Fortune, 1988; Savio et al., 1988; Ginther et al., 1989c), suggesting a need for the development of

estrus synchronization protocols that could control not only CL lifespan but follicular development as well.

Exogenous progesterone can be used to establish an artificial luteal phase and control the length of the estrous cycle, as administration of exogenous progesterone inhibits follicular growth, estrus, and ovulation (Christian & Casida, 1948; Ulberg et al., 1951; Nellor & Cole, 1956; Hansel et al., 1961; Lamond, 1964a). Early research in the use of progestogens to control the estrous cycle involved administering exogenous progesterone and progestins at varying doses. This included daily injections of progesterone (Christian & Casida, 1948; Ulberg et al., 1951; Trimberger & Hansel, 1955), microcrystalline suspensions (Nellor & Cole, 1956), and the development of oral progestins that could be fed to groups of animals (Hansel et al., 1966; Zimbelman & Smith, 1966). Inhibition of follicular growth and the interval from the end of treatment to estrus and ovulation varied by progesterone dose and among animals.

Progestogen Injections

Progestogen injections were used to synchronize estrus in groups of cattle; however, at the administered dose, pregnancy rates to AI (P/AI) at the time of synchronized estrus were generally poor (Trimberger & Hansel, 1955; Nellor & Cole, 1956). In some cases, ovulation was observed in response to the removal of progestogen treatment, but accompanying expression of estrus occurred less frequently (Lamond, 1964b). One method of exogenous progestogen treatment was the administration of daily subcutaneous injections of progesterone in a corn oil carrier (Christian & Casida, 1948; Trimberger & Hansel, 1955). In a trial conducted by Trimberger & Hansel (1955) in

dairy cows, progesterone injections of 50, 75, or 100 mg were administered subcutaneously for 3, 7, 9, or 13 days. The average interval from final progesterone injection to estrus was 4.6 days and the pregnancy rate of synchronized estrus was 12.5% (Trimberger & Hansel, 1955). Normal estrus expression and pregnancy rates occurred the following cycle, suggesting there was no long-term effect of progesterone treatment on reproduction.

A progesterone crystalline suspension was also developed. In a study conducted by Nellor & Cole (1956), beef heifers were injected subcutaneously with a starch emulsion of 540-560 mg or 700-1120 mg crystalline progesterone. Estrus was prevented for 15 to 23 days following the injection. Of the heifers treated, 89% ovulated 15 to 19 days after the 560 mg injection and 95% ovulated 15 to 23 days after the 700 mg injection (Nellor & Cole, 1956). This method was also used in conjunction with a 750 IU or 2140 IU equine gonadotropin (eGonado) injection 15 days after the crystalline progesterone injection. Of the heifers treated, 90% expressed estrus within 1 to 4 days after the gonadotropin injection. Conception rates at synchronized estrus were very poor, however, being around 17%. Nellor & Cole (1956) suggested that progesterone has a role in initiating estrus in some non-cycling heifers. This was based on results indicating that previously non-cycling heifers expressed estrus in response to the crystalline progesterone treatment.

Oral Progestins

Oral progestins can be used to inhibit estrus during the feeding period. The first progestational compound was 6-methyl-17-acetoxyprogesterone (MAP) (Hansel et al.,

1961; Zimbelman, 1963; Brunner et al., 1964; Hulet, 1966). Treatment with MAP inhibits ovulation from occurring in treated animals, but allows for follicular development to continue (Zimbelman, 1963). Hansel et al. (1961) fed MAP in soybean meal to 32 cows for 20 days. Estrus was inhibited during the feeding period. Half (16) of the cows treated with MAP expressed estrus 3 to 4 days after the removal of MAP from the feed source. Of those females not expressing estrus, 13 had "silent" ovulations. All 32 cows were inseminated, but only 25% conceived to first service AI. Half of the cows were injected with estradiol at the time of insemination, but no improvement in P/AI was noted as a result of this treatment (Hansel et al., 1961). In another study, conducted by Zimbelman (1963), 86% of beef cows and heifers fed 120-180 mg of MAP daily for 18 days expressed estrus within 1 to 6 days after the last day of feeding. As a result of this trial, the effective oral dose of MAP for cattle was determined to be 180 mg fed daily for 18 days (Zimbelman, 1963).

Chlormadinone acetate (CAP) was also investigated as an oral progestin to synchronize estrus in beef cattle. On an 18-day feeding schedule, 87% of beef cows fed CAP by liquid feed or in pellets expressed estrus within 1 to 9 days following removal of the progestin from the feed source (Hansel et al., 1966). When compared to MAP, similar estrus expression rates were detected; however, pregnancy rate to AI was only 31% at CAP-synchronized estrus and 49% at MAP-synchronized estrus (Hansel et al., 1966). The development of Repromix® followed this work, with the Upjohn Company commercializing the development and sale of MAP as a feed source to synchronize estrus (Lauderdale, 2010). Repromix® was the first estrus synchronization product created for commercial use in the cattle industry. Dosage, developed by Zimbelman's group, was

180 mg daily for 18 days. The sale of Repromix® took place from 1965 to 1967. Cost limited the use of this product in the commercial industry, and it was later taken off the market.

Another oral progestin that was developed during this time is melengestrol acetate (MGA). A trial, conducted by Zimbelman & Smith (1966), determined that 0.4 mg of MGA daily would inhibit ovulation and CL development, but allow for continued follicular growth. Heifers treated with MGA had improved feed efficiency and weight gain (Bloss et al., 1966; Zimbelman & Smith, 1966). This was believed to be related to estrogen output. Initially, this product was approved by the FDA for use in feedlot heifers to suppress estrus, improve feed efficiency, and increase weight gain. In 1997, MGA was approved for use in synchronizing estrus in breeding heifers. The recommended dosage for this product was and still is to feed heifers 0.5 mg/ day for no more than 24 days. Research indicates that feeding progestins for an extended period of time does not have a lasting negative effect on reproductive performance (O'Brien & Zimbelman, 1970). Heifers in a trial conducted by O'Brien & Zimbelman (1970) were fed 0.35-0.5 mg of MGA daily for up to 63 days without long-term effects on cyclicity. Like other long-term progestin protocols, fertility of the first synchronized estrus was low. Conception rates of MGA-fed heifers at the time of the second estrus after MGA removal are much greater than those obtained at the initial synchronized estrus.

Feeding MGA to feedlot heifers still occurs today and is effective at inhibiting estrus, improving feed efficiency, increasing average daily gain, improving carcass quality grade, and decreasing disease mortality rates (Perrett et al., 2008). Feedlot rations incorporate MGA at a rate of 0.25-0.5 mg/ head/ day. Estrus synchronization of breeding

heifers is still conducted through the use of MGA but often involves additional exogenous hormones to synchronize the second, rather than first, estrus following the end of MGA treatment. This increases the length of the protocol but also increases the conception rate of first service AI. There are two melengestrol acetate products currently available that can be incorporated into feed rations to improve feedlot performance or synchronize estrus in breeding heifers: HeifermaX®, marketed by Elanco, and MGA®, marketed by Zoetis.

Progestogen-Estrogen Treatments

To reduce variation in estrus synchronization, progestogens are used in combination with the administration of other exogenous hormones, including gonadotropins and luteolytic compounds like estrogen and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Exogenous estrogen administration can induce the early regression of the CL, with most corpora lutea regressing within 2 to 7 days of estradiol valerate (EV) injection (Wiltbank et al., 1961; Kaltenbach et al., 1964). Initial trials that combined EV treatment with progestogen treatment showed that a shortened progestogen treatment could synchronize estrus with greater fertility at first induced estrus than longer progestogen treatments (Wiltbank & Kasson, 1968; Wiltbank et al., 1971). These shortened progestogen treatments included the use of progesterone injections, feeding the oral progestin DHPA, and the development of a synthetic progesterone implant containing norgestomet.

An oral progestin, 16-alpha-17-dihydroxyprogesterone acetophenonide (DHPA), was developed and used to synchronize estrus in cattle cooperatively with an EV injection. Wiltbank et al. (1967) synchronized estrus in cattle by feeding DHPA at a rate

of 500 mg/d for 20 days or 400 mg/d for 9 days along with the administration of 5 mg of EV on the second day of the feeding period (Wiltbank et al., 1967). Of the heifers treated in the 9-day treatment group, 84% expressed estrus within 96 hours of each other (Wiltbank et al., 1967). In a similar trial, 66 heifers were fed 400 mg of DHPA daily for 9 days and received a 5 mg injection of EV on the second day of the feeding period. Estrus was synchronized in 95% of the heifers, and 54% of those heifers conceived to synchronized estrus (Wiltbank & Kasson, 1968). Control heifers in this study, inseminated at spontaneous estrus, had a similar first-service conception rate.

An ear implant was developed as a convenient method to administer progestin and synchronize estrus (Burrell et al., 1972; Whitman et al., 1972; Miksch et al., 1978; Spitzer et al., 1978). The ear implant contained 6 mg 17α-acetoxy-11ß-methyl-19-nor-preg-4-ene-3, 20-dione (norgestomet) and was administered with an injection containing 5 mg of EV and 1 or 3 mg norgestomet. Estrous response was high in treated heifers. A large field trial including 744 beef heifers from 5 different locations had an overall average of 95% of heifers expressing estrus by 120 h after implant removal (Miksch et al., 1978; Spitzer et al., 1978). Conception rates to synchronized estrus were slightly lower than first service conception rates of control heifers in two of the five trials but did not differ in the other three trials (Spitzer et al., 1978).

Based on convenience and the low cost associated with the preceding estrus synchronization treatments, Syncro-Mate-B® (SMB) was developed. The SMB treatment included a 6 mg norgestomet poly-hydroxy polymer ear implant subcutaneously inserted for 9 days and a 2 mL i.m. injection containing 3 mg norgestomet and 5 mg EV administered at the time of implantation. In 1982, data led to the approval of SMB by the

Food and Drug Administration (FDA) for synchronization of estrus in beef cattle and non-lactating dairy heifers (Spitzer et al., 1976; Spitzer et al., 1978; Miksch et al., 1978; Spitzer et al., 1981; Lauderdale, 2010). A large field trial, including 958 beef heifers, was conducted to evaluate the breeding management implications of synchronization of estrus by SMB within a 45-day breeding season. First service pregnancy rate of synchronized SMB-treated heifers was 55%. Non-treated control heifers had a 67% first service conception rate; however, the proportion of heifers that conceived within the first 27 days of the breeding period was 6% higher in SMB-treated heifers (Spitzer et al., 1981). Pregnancy rate at the end of the 45-day breeding window did not differ between treated and control heifers (Spitzer et al., 1981).

Many prepubertal heifers and anestrous cows expressed estrus in response to SMB treatment but had reduced conception rates compared to cycling animals. Anestrous cattle that responded to SMB and failed to conceive would often fail to continue cycling thereafter (Spitzer, 1982). McGuire et al., (1990) conducted an experiment to evaluate the effect of SMB treatment on ovariectomized cows and heifers. Observation of estrus following treatment showed that 55.2% of ovariectomized cows and 56.7% of ovariectomized heifers exhibited estrus in response to SMB treatment (McGuire et al., 1990). This indicated that SMB could act at the level of the brain to induce estrous behavior in cows and heifers independently of ovarian status. Expression of estrus following SMB treatment was therefore not a reliable indicator of an animal's ability to ovulate and conceive. Ultimately, with the development of other methods to effectively synchronize estrus (Brown et al., 1988; King et al., 1988), Syncro-Mate-B® was taken off the market.

Intravaginal Progesterone-Releasing Inserts

Intravaginal inserts were developed to dispense progesterone internally over several days. Polyurethane sponge pessaries impregnated with 3 g progesterone or 0.15 g SC-9880 (cronolone) were developed as a method to control the estrous cycle by intravaginal progestogen treatment (Robinson, 1965; Wishart & Hoskin, 1968; Scanlon & Burgess, 1972; Sreenan, 1974; Sreenan & Mulvehill, 1975; Sreenan, 1975; Mulvehill & Sreenan, 1978). Long- and short-term treatments were applied with or without the use of other exogenous hormones. Investigators found that synchronization of estrus with pessaries was successful; however, retention of pessaries for the full period of treatment was variable. In a trial conducted by Sreenan (1975), 78.3% of heifers treated with progesterone pessaries and 84.8% of heifers treated with SC-9880 pessaries retained the pessary in position for the full 20-day treatment period. In an attempt to increase retention rates, coils containing progesterone were developed and used to synchronize estrus in beef and dairy cattle (Roche, 1976a). The coils were 10% progesterone and composed of stainless steel coated in silastic rubber. Retention rates were over 90% on average for treatments from 7 to 18 days long (Roche, 1976a).

Further research into the development of intravaginal inserts led to the production of the progesterone-releasing intravaginal device (PRID) (Webel, 1976). Originally, the device contained 2 g of P_4 in silastic rubber and was shown to release 1 g of P_4 over 14 days (Sprott et al., 1984). The PRID® DELTA contains 1.55 g of P_4, is composed of ethyl vinyl acetate and polyamide, and is now typically recommended for 7-day treatments (Werven et al., 2013). A similar device, controlled internal drug release (CIDR), was developed in New Zealand in the 1980s (Macmillan et al., 1991). The CIDR is t-shaped

with a nylon spine and a silicone cover that is impregnated with progesterone. The device is inserted vaginally with a CIDR applicator and releases progesterone, maintaining plasma progesterone concentrations of greater than 2 ng/ ml until removed (Macmillan et al., 1991; Rathbone & Burke, 2013). Progesterone content of this device varies globally; however, the FDA approved dosage for CIDR devices used in the U.S. is 1.38 g. Originally, the device contained 1.9 g of micronized progesterone, but a reduction to 1.38 g has not reduced the effectiveness of this product at preventing estrus and ovulation while in place (Rathbone et al., 2002). With a device treatment period of 4 to 15 days, the retention rate in heifers is 99% (Macmillan et al., 1988, Macmillan et al., 1991). A series of large field trials determined that treatment with a CIDR for 7 days in combination with an injection of $PGF_{2\alpha}$ one day before CIDR removal could effectively synchronize estrus in beef heifers, beef cows, and dairy heifers (Lucy et al., 2001). The Eazi-Breed™ CIDR® was FDA-approved for synchronization of estrus in beef cattle and dairy heifers in 2002. It is currently the only exogenous progestogen treatment available for synchronization of estrus in both beef cows and heifers in the U.S., as MGA products are not label-approved by FDA for use in mature cows. Further research has led to the development of estrus synchronization protocols that involve treatment with a CIDR for various lengths of time, from 5 to 18 days.

Induction of Cyclicity

Along with synchronization of estrus, progestogens can be used to induce cyclicity in peripubertal heifers and anestrous cows postpartum. During anestrus, LH pulse frequency is low and follicular dynamics mirror those present during the luteal

phase (Short et al., 1990). Follicles develop in a wave-like pattern but become atretic and ovulation does not occur. Prior to initiation of puberty in heifers and resumption of a normal estrous cycle following parturition, plasma progesterone concentrations rise (Prybil & Butler, 1978; Berardinelli et al., 1979; Rawlings et al., 1980). During anestrus, estradiol has a negative feedback effect on GnRH release from the hypothalamus. An increase in plasma progesterone concentrations reduces the concentration of estradiol receptors in the hypothalamus and therefore reduces the sensitivity of the hypothalamus to the negative feedback effect of estradiol. This reduction in hypothalamic estradiol receptors leads to increased GnRH and subsequent LH secretion (Day & Anderson, 1998). This system will effectively re-establish the positive feedback relationship between estradiol and LH within the hypothalamic-pituitary-gonadal axis, which is necessary for puberty attainment and the return to normal estrous cyclicity postpartum.

Treatment with exogenous progesterone can also induce this effect. Sub-luteal concentrations of progesterone increase LH pulse frequency to a level that is similar to what occurs during the follicular phase of the estrous cycle (Roberson et al., 1989). This cascade of events stimulates follicular growth, resulting in greater estrogen production by ovarian follicles (Henricks et al., 1973; Wetteman and Hafs, 1973; Sheffel et al., 1982; Garcia-Winder et al., 1986). Progestin treatment can also mimic endogenous progesterone by stimulating expression of LH receptors on the dominant follicle (Garcia-Winder et al., 1987; Rhodes et al., 2001). An increase in LH receptor expression on the granulosa and theca cells of the dominant follicle will increase estradiol synthesis and secretion by the follicle (Bao & Garverick, 1998; Ginther et al., 2001). Removal of a progestogen further accelerates LH pulse frequency (Hall et al., 1994; Anderson et al.,

1996; Imwalle et al., 1998), and can lead to pubertal ovulation and functional CL formation (Manns et al., 1983). In anestrous cows, exposure to short-term progestin treatments can mimic the short luteal phase that occurs after a cow's first postpartum ovulation (Perry et al., 1991). This effect can help reestablish normal estrous cyclicity in postpartum cows.

Persistent Follicles

In cycling animals, treatment with exogenous progestins will provide a sub-luteal dose of progesterone capable of maintaining an LH pulse frequency sufficient for dominant follicle maintenance for an extended period of time (Kojima et al., 1995). The high LH pulse frequency will prevent the dominant follicle from undergoing atresia (Lucy et al., 1990; Sirois & Fortune, 1990; Savio et al., 1993). Thus, a persistent follicle can develop as a result of progestin treatment. This is a follicle that remains in dominance for an extended period of time and generally reaches a larger size than is typical in a normal estrous cycle. Prolonging the lifespan of the dominant follicle may cause premature maturation of the oocyte by the continued progression of meiosis to metaphase II even before the LH surge (Mihm et al., 1994; Revah & Butler, 1996). This reduction in oocyte quality explains the poor pregnancy rates associated with the first synchronized estrus following long-term progestin treatments (Kinder et al., 1996).

Prostaglandins

Prostaglandin $F_{2\alpha}$ and synthetic analogs of $PGF_{2\alpha}$ can be used to induce luteolysis of the corpus luteum (CL) of cycling animals during certain periods of the estrous cycle (Lauderdale, 1972; Liehr et al., 1972; Louis et al., 1972; Rowson et al., 1972; Louis et al., 1974; Hafs et al., 1975; Jackson et al., 1979; Herschler, 1983; Maffeo et al., 1983). During early development of the CL, $PGF_{2\alpha}$ is not effective at inducing luteolysis (Lauderdale, 1972; Rowson et al., 1972; Jackson et al., 1979; Battista et al., 1984; Kiracofe et al., 1985); however, after Day 5 of the estrous cycle, the CL will obtain the capacity to respond to $PGF_{2\alpha}$ (Lauderdale, 1972). By induction of luteolysis, $PGF_{2\alpha}$ and analogs of $PGF_{2\alpha}$ can be used to synchronize estrus among cycling heifers and postpartum cows. Early trials using $PGF_{2\alpha}$ and synthetic analogs of $PGF_{2\alpha}$ included intrauterine infusions, intramuscular injections, and subcutaneous injections.

Non-surgical infusion of $PGF_{2\alpha}$ into the uterine horn ipsilateral to the CL can induce luteolysis if the treatment is administered after Day 4 of the estrous cycle (Liehr et al., 1972; Louis et al., 1972; Louis et al., 1974). A trial conducted by Louis et al. (1974), found that intrauterine infusion of $PGF_{2\alpha}$ on Days 7, 11, or 15 of the estrous cycle caused a decrease in CL diameter, decline in serum progesterone concentrations and an increase in serum estradiol concentrations that led to the initiation of an LH surge, subsequent estrus expression, and ovulation. This trial also determined that contralateral $PGF_{2\alpha}$ infusion can also induce luteolysis (Louis et al., 1974).

When administered between Days 5 and 16 of the estrous cycle, intramuscular injection of a $PGF_{2\alpha}$ analog is just as effective as non-surgical intrauterine infusion of $PGF_{2\alpha}$ at inducing luteolysis (Lauderdale, 1972; Tervit et al., 1973). The effective dose of

an i.m. injection of $PGF_{2\alpha}$ was determined to be 25 mg (Lauderdale et al., 1977). A method of estrus synchronization involving 2 injections of $PGF_{2\alpha}$ administered 10 to 12 days apart was developed. This method can effectively synchronize estrus among a group of cycling cattle who are at random stages of the estrous cycle at the start of treatment (Cooper, 1974). Approximately 70% of cycling cattle should respond to the first injection of $PGF_{2\alpha}$ within this treatment if cattle are evenly distributed across days of the estrous cycle. Those females responding to the first injection will be at a stage in their estrous cycle in which they will respond to a second injection 10 to 12 days later. Those females not responding to the initial injection due to the stage of their estrous cycle should be at a stage where they have the capacity to respond to $PGF_{2\alpha}$ at the time of the second injection. This method of estrus synchronization has been evaluated for effectiveness when detecting estrus and performing timed AI. In a study conducted by Manns et al. (1976), heifers were treated with two injections of $PGF_{2\alpha}$ (25 mg, Upjohn Company) administered 12 days apart and were inseminated 75 h, 80 h, or 85 h after the second injection. Animals in this study were at or near Days 9 to 13 of the estrous cycle at the time of the second injection, depending on stage of cycle at the time of the first injection. Heifers near Day 13 of the estrous cycle had greater P/AI (49.5%) than those heifers near Day 9 (39.8%) on the day of the second injection. The 85 h interval between the final $PGF_{2\alpha}$ injection and timed AI resulted in lower conception rates compared to the other two timepoints (75 h: 43.7%; 80 h: 46.8%; 85 h: 34.2%), which were similar to the conception rate of control heifers inseminated based on expression of estrus over a 30-day observation period (46.2%) (Manns et al., 1976).

Currently, there are several $PGF_{2\alpha}$ products approved for commercial use in estrus synchronization; however, only some products have label approval for use in conjunction with other estrus synchronization products. This includes Lutalyse and Lutalyse highcon (dinoprost tromethamine), Estrumate (cloprostenol sodium), and Estroplan (cloprostenol sodium). Prostamate (dinoprost tromethamine) is also FDA approved for commercial use in estrus synchronization, though it is technically not approved for this purpose when used in conjunction with other products.

Progestogen-Prostaglandin Treatments

Shortened progestogen treatments result in greater conception rates to the first synchronized estrus compared to long-term progestogen treatments but require a luteolytic compound to regress the CL (Wiltbank & Kasson, 1968; Wiltbank et al., 1971; Smith & Vincent, 1973). Administration of a norgestomet ear implant was first used in combination with an EV and norgestomet injection administered at the time of norgestomet implant (Burrell et al., 1972; Whitman et al., 1972). A similar protocol was developed using $PGF_{2\alpha}$ rather than EV to induce luteolysis. The norgestomet implant was administered subcutaneously in the ear and remained in place for 5 to 9 days, with $PGF_{2\alpha}$ administered by i.m. injection or intrauterine infusion before or at the time of implant removal (Heersche Jr et al., 1974; Wishart, 1974; Thimonier et al., 1975; Roche, 1976b; Heersche Jr et al., 1979). Conception rates to synchronized estrus with the EV-progestogen treatment and progestogen- $PGF_{2\alpha}$ treatment were similar to those resulting from insemination following spontaneous estrus (Mares et al., 1977; Smith et al., 1984). Similar estrous response and conception rates were obtained among cows and heifers

treated with the EV-progestogen and those treated with progestogen- $PGF_{2\alpha}$ (Beal et al., 1984; Brown et al., 1986; Whittier et al., 1986).

With the progestogen- $PGF_{2\alpha}$ treatment, a tighter degree of synchrony of estrus expression is obtained by injecting $PGF_{2\alpha}$ 2 days prior to removal of the norgestomet implant rather than on the day of implant removal (Odde et al., 1984). A similar study, using a PRID rather than norgestomet implant had similar results. Heifers treated with $PGF_{2\alpha}$ one day prior to PRID removal had a greater degree of synchrony of estrus compared to those heifers treated with $PGF_{2\alpha}$ on the day of PRID removal, when PRID treatment was for 7 days (Smith et al., 1984). When compared to two $PGF_{2\alpha}$ injections administered 11 days apart, heifers treated with a PRID for 7 days and a $PGF_{2\alpha}$ injection on Day 6 had a greater estrous response and conception rate to timed AI (Smith et al., 1984). This protocol was later evaluated using a CIDR. A CIDR was in place for 7 days with $PGF_{2\alpha}$ administered one day prior to CIDR removal. This method proved to be effective at synchronizing estrus among beef cows, and dairy and beef heifers. When compared to a single $PGF_{2\alpha}$ injection, this protocol had a greater degree of synchrony and similar conception rates to first service AI. This protocol also had similar conception rates as compared with those obtained among females inseminated following spontaneous estrus (Lucy et al., 2001).

Oral progestins can also be used in combination with $PGF_{2\alpha}$ to synchronize estrus. Beal & Good (1986) fed melengestrol acetate (MGA), an oral progestin, for 5, 7, or 9 days and administered $PGF_{2\alpha}$ on the last day of MGA feeding. They found that MGA needed to be fed for at least 7 days to be effective when used with $PGF_{2\alpha}$. Feeding of MGA for 9 days with $PGF_{2\alpha}$ administered on Day 9 was compared to the 9-d

norgestomet- $PGF_{2\alpha}$ protocol. Both treatments induced estrus among anestrous cows, however, the MGA protocol had a longer interval to estrus among these cows (Beal & Good, 1986). Cows bred after synchronized estrus with a 9-day MGA treatment and $PGF_{2\alpha}$ administration on Day 9 had lower conception rates than control cows bred after a spontaneous estrus (Beal et al., 1988). This was largely due to reduced conception rates among cows in late stages of the estrous cycle at the start of treatment (14 to 20 days), presumably as a result of persistent follicle formation.

Long-term Progestin- $PGF_{2\alpha}$ Treatments

Rather than breeding at the time of first synchronized estrus following progestogen treatment, a protocol was developed to synchronize the second estrus following removal of progestogen. This protocol involved feeding MGA for 14 days and administering $PGF_{2\alpha}$ 16 to 18 days after the last day of MGA feeding (Brown et al., 1988). The rationale for this approach was that cattle would be in the late luteal phase at the time of $PGF_{2\alpha}$ administration, which had proven to be more effective at inducing estrus than $PGF_{2\alpha}$ administered early in the luteal phase (King et al., 1982; Tanabe & Hann, 1984; Watts & Fuquay, 1985). This treatment was compared to the 7-day MGA treatment with $PGF_{2\alpha}$ administered on Day 7 and resulted in greater pregnancy rates among beef heifers (Mauck et al., 1988). When compared to SMB in heifers, the two treatments had a similar estrous response rate; however, the 14-day MGA- $PGF_{2\alpha}$ treatment resulted in greater P/AI (Brown et al., 1988). A similar treatment in cows, involving a 14-day norgestomet implant with alfaprostol administered 16 days after implant removal, was compared to

SMB. Estrous response was greater for cows treated with SMB, but pregnancy rate to synchronized estrus was similar between the two groups (King et al., 1988).

In heifers, extending the interval between MGA removal to $PGF_{2\alpha}$ administration from 17 to 19 days resulted in a shorter interval to estrus following $PGF_{2\alpha}$ administration, a greater estrous response by 72 hours after $PGF_{2\alpha}$, a greater proportion of heifers in the late luteal phase at the time of $PGF_{2\alpha}$, and higher overall estrous response rate (Deutscher, 2000; Lamb et al., 2000). This higher degree of synchrony is likely related to the size and maturity of the largest ovarian follicle at the time of treatment (Scaramuzzi et al., 1980) and has implications for the effectiveness of timed AI following these treatments. The 14-day MGA treatment with 19 days from MGA removal to $PGF_{2\alpha}$ administration was compared to a similar treatment with a 14-day CIDR and 16 days between CIDR removal and $PGF_{2\alpha}$ administration. Interval to estrus was shorter for CIDR-treated animals, but pregnancy rates did not differ between the two treatments (Tauck et al., 2007). The addition of a GnRH injection administered 7 days prior to $PGF_{2\alpha}$ administration resulted in improved synchrony among MGA-treated heifers (Wood et al., 2001) but did not improve synchrony among CIDR-treated heifers (Mallory et al., 2011).

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) agonists were developed by altering the chemical structure of native GnRH molecules. This synthetic form of GnRH, when administered by intracarotid or intramuscular injection, stimulates the secretion of FSH and LH from the anterior pituitary gland (Kaltenbach et al., 1974). An increase in circulating LH and FSH occurs within 2 to 4 h following GnRH administration (Chenault

et al., 1990; Rettmer et al., 1992; Stevenson et al., 1993). This can indirectly induce luteinization or ovulation of the dominant follicle(s), if present, and thus allows for subsequent recruitment of a new follicular wave (Lofstedt et al., 1981; Thatcher et al., 1989; Macmillan & Thatcher, 1991; Rettmer et al., 1992; Stevenson et al., 1993). Follicle maturity and stage of follicular development will determine the capacity of a follicle to respond to GnRH injection (Prescott et al., 1992; Silcox et al., 1993). A physiologically mature follicle must obtain LH receptors on both granulosa and theca cells to respond to an LH surge (Ireland & Roche, 1983; Bao & Garverick, 1998). Typically this occurs after the follicle reaches 10 mm in diameter (Sartori et al., 2001). A new, synchronized follicular wave will emerge within 2 days of GnRH treatment (Twagiramungu et al., 1994).

Initially, GnRH agonists were used to treat ovarian cysts in dairy cattle (Food & Drug Administration, 1986). However, when used in combination with progestogens and/or $PGF_{2\alpha}$, these products can also be used to synchronize estrus. When used in combination with $PGF_{2\alpha}$ in a synchronization program, GnRH increases the precision of estrus synchronization (Thatcher et al., 1989; Twagiramungu et al., 1992), although precision of estrus is dependent on large follicle population status at the time of $PGF_{2\alpha}$ - induced luteolysis (Sirois & Fortune, 1988; Ginther et al., 1989a; Sirois & Fortune, 1990).

When administered to cycling postpartum cows at random stages of the estrous cycle, GnRH can be used to synchronize luteal function; however, GnRH administration is not as effective in virgin heifers (Pursley et al., 1997). Ovsynch was developed to synchronize estrus and ovulation in dairy cows by using GnRH and $PGF_{2\alpha}$ injections. This

treatment consists of administration of GnRH at a random stage of the estrous cycle, with $PGF_{2\alpha}$ administered 7 days later to induce luteolysis. A second injection of GnRH is administered 0, 24, or 48 h after $PGF_{2\alpha}$ injection, and AI is performed 24 h after the final GnRH injection. The development of this treatment schedule eliminated the need for estrus detection and, instead, relies on timed AI. In a trial conducted by Pursley et al. (1995), conception rates varied by time from $PGF_{2\alpha}$ to second GnRH injection (0 h: 11%; 24 h: 55%; 48 h: 46%), and the interval was later changed to 30 to 36 h between $PGF_{2\alpha}$ and final GnRH injection (Pursley et al., 1995, 1997). Conception rates to timed AI following Ovsynch (38.9%) were comparable to those obtained by estrus detection and AI based on the AM-PM rule in lactating dairy cows following a single or double $PGF_{2\alpha}$ injection administered 14 days apart (37.8%) (Pursley et al., 1997). The same trial conducted in virgin dairy heifers resulted in a greatly reduced conception rate among heifers treated with Ovsynch. Heifers treated with Ovsynch had reduced conception rates (35.1%) compared to heifers receiving two $PGF_{2\alpha}$ injections to synchronize estrus (74.4%). The measurement of serum progesterone concentrations at the time of each injection indicated that the first injection of GnRH failed to synchronize luteal function among heifers (Pursley et al., 1997).

A method of estrus synchronization, similar to Ovsynch and more popular in the beef industry is CO-Synch. With this protocol, the final GnRH injection is administered at the time of AI rather than in advance of AI. This reduces the number of cattle handlings required and does not require estrus detection; however, one experiment suggested this may also result in less than maximal P/AI among beef cows when compared to Ovsynch (Geary et al., 1998). Several similar estrus synchronization

protocols have been developed using this method. A common practice now is to utilize estrus detection aids and limit administration of the final GnRH injection to only those females that fail to express estrus by FTAI (Bishop et al., 2016).

Sex-sorted Semen

Introduction

Sex-sorted semen can be a powerful tool for genetic advancement within both beef and dairy herds. This technology enables producers to skew the calf sex ratio and/or selectively produce offspring of the desired sex from a particular mating. The original and most widely used method of sorting sperm cells is via differentiation of sperm cells based on DNA content as determined using flow cytometry (Johnson et al., 1989; Seidel, 2007). Flow cytometry was initially used to measure differences in DNA quantity and to determine the ratio of X- and Y-bearing sperm in a collection (Pinkel et al., 1982; Garner et al., 1983; Johnson & Pinkel, 1986; Johnson et al., 1987). In time, flow cytometry combined with cell sorting was used to produce populations of sorted sperm cells based on X or Y chromosome content (Johnson & Clarke, 1988). The first live births from sorted sperm cells were successfully accomplished in rabbits (Johnson et al., 1989).

Many improvements have been made since the initial application of this technology, with a current sorting capability of greater than 90% accuracy for either sex (Sharpe & Evans, 2009; Seidel, 2014). However, a major limitation to the wider use of sex-sorted semen within the beef industry is the reduction in fertility compared to conventional semen. Damage to the sperm cells during the sorting process and

subsequent cryopreservation impact fertility, and the severity of this effect can vary from bull to bull based on sperm quality.

Flow Cytometry

In cattle, the DNA content of X-chromosome-bearing sperm cells is about 3.8% greater than that of Y-chromosome-bearing sperm cells (Moruzzi, 1979), with some variability among breeds (Garner et al., 1983). Flow cytometry sex-sorting relies on the use of Hoechst 33342, a fluorescent dye that permeates the cell membranes and selectively binds DNA stoichiometrically (Penfold et al., 1998; Garner & Seidel, 2008; Garner, 2009). After staining, the sperm cells enter the flow cytometer and are separated into single droplets, which pass through the laser beam individually. When excited by the laser, DNA can be quantified by fluorescence detectors that measure the signal intensity of H33342 dye bound to DNA (Garner & Seidel, 2008; Garner, 2009). A charge is applied to sperm cells of the desired sex based on DNA content of the cells and a charged plate is used to sort the droplets. As the sperm cells flow through the sorter, charged sperm cells are attracted to the oppositely charged plate, and sperm cells are collected into one of two holding tubes: sperm cells of the desired sex (charged) or unsorted sperm cells (uncharged).

A sorting accuracy of 90% is regularly achieved, although sorting speed is a function of the accuracy setting on the equipment (Schenk et al., 1999; Seidel et al., 1999a; Seidel, 2003). The higher the accuracy that is set, the slower the sorting speed will be. Characteristics of the ejaculate also influence sorting speed: if more dead sperm cells are present and must be discarded, sorting speed will be slower. In recent years,

efficiency of the sorting process has improved with the advancement of digital processing, automation, and the addition of multiple heads (Evans, 2009; Evans, 2010; Vishwanath, 2014; Vishwanath et al., 2014).

Challenges

One limiting factor to the wider use of this technology within the beef industry is lower P/AI compared to non-sorted sperm (Schenk et al., 2009). This is believed to be a result of fewer sperm cells per insemination (Bodmer et al., 2005) as well as damage to the sperm cells as a result of the sorting process (Schenk & Seidel, 2007; Frijters et al., 2009). During the sorting process, sperm cells may be damaged by Hoechst 33342 DNA-binding dye, electromagnetic energy, mechanical damage, pressure, osmotic concentration, oxidative agents, and post-sorting centrifugation and cryopreservation (Seidel & Schenk, 2008; Carvalho et al., 2010). Induced changes in sperm cell membranes, premature sperm capacitation, and acrosomal alterations that occur as a result of the semen sorting process and subsequent cryopreservation may reduce the lifespan of sex-sorted cells in the female reproductive tract (Mocé et al., 2006; Schenk et al., 2009; Carvalho et al., 2010). The degree to which these challenges impact sperm cell viability appears to vary from bull to bull (Den Daas et al., 1998). Certain sires may be incapable of producing fertile sex-sorted semen even if an ejaculate from that same bull can be cryopreserved as conventional semen.

The insemination dose of sex-sorted semen is typically between 2 x 10^6 to 4 x 10^6 sperm cells per insemination, much lower than the 20 x 10^6 sperm cell dose used with conventional semen. The lower dose increases the number of straws per collection that

can be produced when sorting sperm cells, which is necessary for commercial application of sex-sorted semen for expense and efficiency reasons (Amann, 1999; Seidel et al., 1999b; Seidel, 2007). This may contribute to the reduced conception rates resulting from the use of sex-sorted semen (Bodmer et al., 2005). There have been mixed results in regard to the effect of insemination dose on conception rates (DeJarnette et al., 2010), but it is ultimately unlikely that a change in dosage can fully overcome the reduction in fertility caused by the sorting process (Frijters et al., 2009).

Advancements

The original flow cytometer sex-sorting technology used was XY Legacy technology (Johnson & Welch, 1999; Schenk et al., 1999; Seidel et al., 1999a). Recently, improvements to this technology have led to SexedULTRA sex-sorting. SexedULTRA sex-sorting technology is also based upon flow cytometry but the media used for initial holding and preparation of the sperm cells for staining, collection, and freezing has been redesigned. This media is more effective at managing buffer conditions, pH, and oxidative load throughout the sorting process (Gonzalez-Marin et al., 2016; Lenz et al., 2016), and has led to improved sperm motility and acrosome integrity compared to the XY Legacy technology (Gonzalez-Marin et al., 2016). In practice, this has led to improved conception rates to AI (Vishwanath, 2015) and, when used for in vitro fertilization, a greater number of freezable embryos (Gonzalez-Marin et al., 2016). This technology also improves sorting speed and reduces sperm cell loss, which can facilitate the use of higher sperm cell numbers per inseminate (Amann, 1999; Seidel et al., 1999a; DeJarnette et al., 2008).

Two large field trials have elucidated the fertility advantage of SexedULTRA sex-sorting technology over the previously used XY sorting technology. Semen from 8 Holstein bulls was used to inseminate 6,930 Holstein heifers across 41 commercial locations, based on expression of estrus, with sexed semen sorted by SexedULTRA or XY technology. Pregnancy rates were 4.5% greater among heifers inseminated with semen sorted by SexedULTRA technology (46.1% vs 41.6%). In another trial using greater sperm cell doses, semen from 5 bulls was used to inseminate 7,855 Holstein heifers with sex-sorted semen produced using XY technology at a dose of 2.1 x 10^6 sperm cells per straw or produced using SexedULTRA technology at a dose of 2.1 x 10^6, 3.0 x 10^6, or 4.0 x 10^6 sperm cells per straw. Another 62,398 heifers were inseminated with conventional semen at a dose of 15 x 10^6 sperm cells per straw, produced using contemporary ejaculates from the same bulls. Results indicated that SexedULTRA sex-sorted semen, when administered at a dose of 4.0 x 10^6 sperm cells per insemination achieved pregnancy rates (66.73%) comparable to those achieved with conventional semen at a dose of 15 x 10^6 sperm cells per insemination (65.66%). These data also indicated a dose-response with sex-sorted semen, with SexedULTRA semen at a dose of 4 x 10^6 sperm cells per straw resulting in a greater P/AI (66.73%) than SexedULTRA semen at a dose of 2.1 x 10^6 (59.95%) or 3.0 x 10^6 (60.02%) sperm cells per straw (Lenz et al., 2016).

Timing of Artificial Insemination

Introduction

Many factors influence P/AI, including the time at which AI is performed relative to the time at which ovulation occurs (or as a proxy, the time at which expression of estrus occurred). Early research in this area, conducted by Trimberger & Davis (1943) and Trimberger (1948), determined that P/AI in dairy cattle is maximized when AI is performed during midestrus or a few hours after the end of behavioral estrus. This work led to the development of the AM-PM rule, in which cattle are bred 12-18 h following observed estrus (Trimberger & Davis, 1943; Trimberger, 1948). More recently, research has indicated that timing of AI impacts both fertilization rate and embryo quality (Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001a; Dalton et al., 2001b; Saacke, 2008). This research indicates that insemination too early relative to the time of ovulation results in high embryo quality but may reduce fertilization rates due to lower numbers of viable sperm present at the time of ovulation. Conversely, insemination too late relative to the time of ovulation results in a high fertilization rate by ensuring sufficient numbers of available sperm cells but may lead to reduced embryo quality as the oocyte ages before fertilization. Dalton et al. (2001a) evaluated three timepoints for AI following estrus onset: 0 h, 12 h, and 24 h. Embryo quality was adversely affected when AI did not occur until 24 h following estrus onset; however, fertilization rate increased as the interval from estrus onset to AI increased. Dalton et al. (2001a) recommended using the 12 h timepoint, as it provides a compromise between lower fertilization rates at 0 h and lower embryo quality or an increase in degenerate embryos at 24 h (Dalton et al., 2001a).

Sperm cells acquire the ability to fertilize oocytes after capacitation, or about 8 h following insemination (Hunter & Wilmut, 1983). The interval between onset of estrus and time of ovulation is approximately 28 h (Walker et al., 1996). A trial measuring fertilization rate and embryo quality when AI is performed at different timepoints relative to ovulation found that insemination 12 to 24 h prior to ovulation, or 2 to 14 h after estrus onset, optimizes both fertilization rate and embryo quality (Roelofs et al., 2006). Fertilization rate declines when AI occurs less than 4 h prior to ovulation, and the proportion of good embryos declines when AI is performed less than 12 h prior to ovulation. This is consistent with other studies that have found the optimal timepoint for AI to be 6 to 17 h (Maatje et al., 1997), 4 to 16 h (Dransfield et al., 1998), and 5 to 17 h (Roelofs et al., 2005) following estrus onset. These data support previous observations that sperm cells can survive for a longer period of time following insemination than the oocyte can survive following ovulation (Hawk, 1987). Sperm survivability may vary from bull to bull, however, and this variation could presumably result in bull-specific differences in the sensitivity of fertility to timing of AI (MacMillan & Watson, 1975).

Fixed-time Artificial Insemination

By eliminating the need for estrus detection, fixed-time artificial insemination (FTAI) can be used to reduce the time and labor associated with AI. With FTAI, all females are serviced at a predetermined time, regardless of estrus expression. When using FTAI, it is important to select an estrus synchronization protocol that offers a high degree of control over the estrous cycle, as this aids in eliminating variance in timing of estrus expression (and ultimately ovulation) among treated females. Not every animal will

express estrus prior to FTAI; however, those that do express estrus within 24 hours of FTAI typically have a larger diameter dominant follicle than those that fail to express estrus (Perry et al., 2007). A larger diameter follicle correlates to greater serum estradiol concentrations. Estradiol produced by the dominant follicle induces estrus expression, prepares follicular cells for luteinization, aids in sperm transport within the female reproductive tract, and regulates expression of estradiol and progesterone receptors in the endometrium (Hawk, 1987; Pohler et al., 2012; Atkins et al., 2013; Jinks et al., 2013). These factors influence uterine environment and ultimately maintenance of pregnancy. For those females that fail to express estrus prior to timed AI, ovulation can be induced by exogenous GnRH administration (Sartori et al., 2001); however, fertility is reduced among this subset of animals (Perry et al., 2005; Richardson et al., 2016). It is therefore high-priority to maximize the proportion of females expressing estrus with a mature preovulatory follicle at the time of FTAI (Atkins et al., 2010).

Timing of Fixed-Time AI

An increase in the use of timed AI protocols presents another challenge for determining when to perform AI, as the optimal timepoint for AI must be determined for an entire group of cattle rather than one individual. The benefit of using fixed-time artificial insemination (FTAI) is a reduction in the time and labor required to facilitate AI of a group of females. However, there is a 27% reduction in AI conception rates among females that fail to express estrus prior to AI compared to those that express estrus (Richardson et al., 2016). This indicates the importance of a high estrous response rate when performing timed AI. To overcome the potential reduction in pregnancy rates

associated with performing FTAI rather than performing AI based on detected estrus, a FTAI timepoint must be identified that is late enough that a large proportion of females express estrus by the time of AI, yet early enough that insemination does not occur too late for those cattle that express estrus earliest in the synchronized group.

Recommendations related to timing of FTAI for a specific protocol have been based on experimental efforts to characterize timing of estrus expression as well as research in which successful pregnancy rates have been obtained as a result of FTAI at a given timepoint; however, direct comparisons between FTAI timepoints for specific protocols have been limited. Based on the distribution of estrus expression following a given estrus synchronization protocol, shifting the FTAI timepoint for an entire group of cattle can be detrimental to overall fertility (Busch et al., 2008). Following synchronization of estrus using the 14-day CIDR- $PGF_{2\alpha}$ protocol, the recommended time for FTAI of beef heifers is 66 h after $PGF_{2\alpha}$ administration (Leitman et al., 2009a; Mallory et al., 2010; Mallory et al., 2011). The timing of estrus expression relative to $PGF_{2\alpha}$ administration has been recorded using radiotelemetric devices (HeatWatch) among mixed groups of heifers receiving this treatment (Leitman et al., 2009a; Leitman et al., 2009b; Mallory et al., 2010; Mallory et al., 2011). Data from these trials were used to identify the optimal timepoint to perform FTAI following this protocol.

Timing of AI with Sex-sorted Semen

Generally, the use of sex-sorted semen is not recommended for use in FTAI protocols due to reduced pregnancy rates, especially among females that fail to express estrus (Hall et al., 2010; Rhinehart et al., 2011; Meyer et al., 2012; Sá Filho et al., 2012;

Cooke et al., 2014; Thomas et al., 2014a). Both the use of sex-sorted semen (Seidel, 2007; Schenk & Seidel, 2007; Schenk et al., 2009; DeJarnette et al., 2009; Seidel, 2014) and failure of an animal to express estrus reduce fertility (Richardson et al., 2016). A management method used to address these challenges while maintaining the use of FTAI is limiting the use of sex-sorted semen to only cattle that express estrus by timed AI. This requires a means of determining estrous response so that all females that fail to express estrus are inseminated with conventional semen. This remains one of the most effective ways of managing the reduced P/AI associated with the use of sex-sorted semen.

Another way to improve pregnancy rate to FTAI is to increase the proportion of females that express estrus prior to AI. Split-time artificial insemination (STAI) was developed to increase the proportion of cattle expressing estrus prior to insemination following an estrus synchronization protocol. Cattle that express estrus by the standard FTAI timepoint are serviced at that time, and insemination of non-estrous females is delayed by 20 to 24 hours. This method increases the total proportion of females expressing estrus by the time of insemination and can improve overall pregnancy rate to synchronized estrus when using sex-sorted (Thomas et al., 2014a; Thomas et al., 2017; Thomas et al., 2019) or conventional semen (Thomas et al., 2014b).

The optimal timing of insemination with sex-sorted semen may differ from conventional recommendations due to the reduced lifespan of sex-sorted sperm cells in the female reproductive tract (Maxwell et al., 2004), fewer sperm cells per insemination (DeJarnette et al., 2008), and increased incidence of precapacitation (Lu & Seidel, 2004). These factors may narrow the window of fertility with regard to timing of insemination relative to ovulation (Sales et al., 2011; Bombardelli et al., 2016). Research in this area

has explored this concept with regard to timing of ovulation and within FTAI protocols. The data obtained from these studies suggest that pregnancy rates to AI with sex-sorted semen are improved when animals are inseminated closer to the time of ovulation (Sales et al., 2011; Bombardelli et al., 2016). However, results have been mixed when delaying timing of FTAI with sex-sorted semen until later than typically recommended when using conventional semen. Some experiments have suggested modest improvements in P/AI with sex-sorted semen when timed AI is delayed (Sales et al., 2011; Oosthuizen et al., 2021) whereas others have observed no improvement (Hall et al., 2017; Drake et al., 2020; Ketchum et al., 2021; Oosthuizen et al., 2021).

Sales et al. (2011) found an improvement in P/AI among Jersey heifers inseminated with sex-sorted semen at a later timed AI timepoint following an 8-day CIDR, with EV administered at CIDR insertion and $PGF_{2\alpha}$ administered at CIDR removal. Another trial indicated an improvement in P/AI among suckled postpartum Nellore cows when insemination with sex-sorted semen occurred closer to the time of ovulation (Sales et al., 2011). However, when suckled postpartum Nellore cows were inseminated at a fixed timepoint following an estradiol-progestin estrus synchronization protocol, P/AI with sex-sorted semen was not improved with the application of a later timepoint (Sales et al., 2011). Bombardelli et al. (2016) synchronized estrus in lactating Jersey cows using 3 $PGF_{2\alpha}$ injections administered 14 days apart. Insemination with sex-sorted semen was performed at different timepoints relative to animals reaching activity threshold (AT) during estrus, based on activity monitoring with heat-rumination long-distance collars. This trial indicated that inseminating cows 23 to 41 h following the attainment of AT resulted in greater P/AI with sex-sorted semen than timepoints less than 23 h or greater

than 41 h. This range is later than what previous studies indicate is the optimal timing with conventional semen (Stevenson et al., 2014).

A trial conducted in beef heifers that administered PG 7 days prior to 7-d CO-Synch + CIDR had greater P/AI when insemination with sex-sorted semen occurred at 72 h vs 54 h (Oosthuizen et al., 2021). In the same study, heifers treated with only 7-d CO-Synch + CIDR had similar conception rates to sex-sorted semen when timed AI occurred at 54 h and 72 h. Inducing luteolysis by PG injection will result in approximately 70% of cycling heifers, at random stages of their estrous cycle, to respond and express estrus in 3 days (Lauderdale et al., 1974; Louis et al., 1974). In order to respond to the GnRH injection administered at CIDR insertion, these heifers need to have a physiologically mature follicle (Prescott et al., 1992; Silcox et al., 1993). If expression of estrus occurs only 3 to 4 days prior to GnRH administration, there is likely a large proportion of females that do not have a dominant, LH-responsive follicle when GnRH is administered. Given the lack of control over stage of follicular development, optimal timing of FTAI following PG presynchronization prior to 7-d CO-Synch + CIDR may be later than what is recommended following just the 7-d CO-Synch + CIDR protocol. The reduction in estrus expression by FTAI when PG presynchronization is used compared to heifers that only received the 7-d CO-Synch + CIDR treatment further illustrates this point (Oosthuizen et al., 2018, 2021). In several trials conducted using estrus synchronization protocols with a well-established optimal FTAI timepoint, later timing has not been shown to improve P/AI when using sex-sorted semen. This includes the 7-d CO-Synch + CIDR treatment group in the preceding trial where P/AI was not improved when insemination with sex-sorted semen was delayed from 54 h to 72 h (Oosthuizen et al.,

2021). Another trial, conducted in lactating beef cows, indicated no improvement in P/AI when timed insemination with sex-sorted semen took place at a later timepoint following the 5-d CO-Synch + CIDR protocol (Hall et al., 2017). Another trial was conducted to evaluate two timepoints for STAI when using sex-sorted semen in beef heifers following the 14-day CIDR- $PGF_{2\alpha}$ protocol (Ketchum et al., 2021). In this experiment, expression of estrus was recorded at 66 h or 72 h following $PGF_{2\alpha}$ administration. Heifers that expressed estrus by their assigned timepoint were inseminated at that time and the remaining heifers were inseminated 20 to 24 h later. A larger proportion of heifers expressed estrus prior to timepoint 1 when timed AI was performed at 72 h vs 66 h. However, there was no difference in P/AI with sex-sorted semen between the two timepoints.

Several studies have cited a marked improvement in the proportion of females that have expressed estrus prior to FTAI when using a later timepoint for FTAI (Hall et al., 2017; Ketchum et al., 2021; Oosthuizen et al., 2021), as one would anticipate since more of the distribution of estrus expression is observed and the cumulative proportion of estrous females should therefore be greater. Based on the mixed results across the published literature when using later timing of FTAI following varying estrus synchronization protocols, the degree to which delaying timing of FTAI may be advantageous or disadvantageous when using sex-sorted semen may be protocol dependent.

Conclusion

Effective estrus synchronization programs generate highly synchronous expression of estrus among a large proportion of the treated females. This decreases the

labor and expense associated with estrus detection and facilitates the use of assistive reproductive technologies such as FTAI or embryo transfer. When FTAI is performed, females that express estrus prior to timed AI have greater conception rates than animals that fail to express estrus. Thus, an ideal estrus synchronization protocol induces a large proportion of females to express estrus within a very narrow window of time. Determining a FTAI timepoint that optimizes the proportion of females that express estrus prior to FTAI—without being too late for those that express estrus earliest in the synchronized group—may help minimize the reduction in P/AI associated with the use of FTAI versus AI performed following observed estrus. Likewise, an estrus synchronization protocol that minimizes variance in timing of estrus expression among the synchronized group may facilitate improvements in pregnancy rates to FTAI.

Despite improvements in sex-sorting technologies, pregnancy rates to AI when using sex-sorted semen are still reduced compared with those expected when using conventional semen. Some data suggest that fertility with sex-sorted semen may be improved if insemination is performed closer to the time of ovulation than would be recommended when using conventional semen. This may have implications when considering the optimal timepoint for FTAI when using sex-sorted semen. With these questions in mind, the following experiments were designed to identify the optimal timepoint for FTAI following the 14 d CIDR-PG protocol when using sex-sorted semen and to determine the effect of a shortened interval from progestin removal to $PGF_{2\alpha}$ administration following progestin presynchronization.

Chapter 2

Evaluation of later timepoints for fixed-time artificial insemination of beef heifers when using sex-sorted semen following the 14-d CIDR-PG protocol

Abstract

An experiment was designed to evaluate later timepoints for fixed-time artificial insemination (FTAI) of beef heifers, with the hypothesis that use of a later timepoint would allow a greater proportion of heifers to express estrus prior to FTAI and result in greater conception rates among estrous heifers inseminated with sex-sorted semen. Estrus was synchronized for 1640 heifers in 7 locations using the 14-d CIDR-PG protocol: insertion of an intravaginal progesterone-releasing insert (CIDR; 1.38 g progesterone) on Day -33 and removal on Day -19, and administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol sodium) on Day -3. Within location, heifers were blocked based on reproductive tract score and body weight and were randomly assigned to one of three FTAI timepoints: 66 h, 70 h, or 74 h after PG administration. Estrus detection aids (Estrotect) were applied at PG administration, with activation recorded at FTAI. Heifers that expressed estrus prior to FTAI were inseminated with sex-sorted semen (SexedULTRA 4M™). Heifers that failed to express estrus were inseminated with conventional semen. Gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered coincident with FTAI for heifers that failed to express estrus. The proportion of heifers that expressed estrus prior to FTAI (66 h: 62%; 70 h: 67%; 74 h: 71%) was greater when FTAI was performed at 74 h versus 66 h ($P < 0.01$). Treatments did not differ ($P = 0.67$) with respect to the conception rates of heifers that expressed estrus and were serviced with sex-sorted semen (66 h: 56%; 70 h: 53%; 74 h: 53%).

Among heifers that failed to express estrus and were serviced with conventional semen, conception rates were greater ($P = 0.02$) when FTAI was performed at 66 h versus 70 h (66 h: 37%; 70 h: 25%; 74 h: 31%). Results indicate that performing FTAI later following the 14-d CIDR-PG protocol increases the proportion of heifers that express estrus and are serviced with sex-sorted semen, but later timing of FTAI does not improve conception rates.

Introduction

Sex-sorted semen can be used in artificial insemination (AI) programs to skew the calf sex ratio and/or selectively produce offspring of the desired sex from a particular mating, with greater than 90% accuracy for either sex (Sharpe & Evans, 2009; Seidel, 2014). Sex-sorted semen is produced using a flow cytometry-based method to sort X- and Y- chromosome-bearing sperm cells based on a DNA content difference of approximately 3.8% (Moruzzi, 1979; Pinkel et al., 1982; Garner et al., 1983; Johnson & Pinkel, 1986; Johnson et al., 1987). A major limitation to the wider use of this technology in the beef industry is a reduction in fertility compared to conventional semen (Schenk et al., 2009). This is believed to be a result of fewer sperm cells per insemination (Bodmer et al., 2005) as well as damage to the sperm cells as a result of the sorting process (Schenk & Seidel, 2007; Frijters et al., 2009). Induced changes in sperm cell membranes, premature capacitation, and acrosomal alterations that occur as a result of the semen sorting process and subsequent cryopreservation may reduce the lifespan of sex-sorted cells in the female reproductive tract (Mocé et al., 2006; Schenk et al., 2009; Carvalho et al., 2010). It has been suggested that these characteristics of sex-sorted semen impact the

optimal timing of AI relative to the onset of estrus and ovulation. Some research indicates that conception rates with sexed semen may be improved if insemination is performed at a timepoint closer to ovulation, later than the timepoint that would be typically employed when using conventional semen (Sales et al., 2011; Bombardelli et al., 2016).

The 14-d CIDR-PG protocol is an effective and widely used protocol for control of the estrous cycle among both pubertal and peri-pubertal heifers. This protocol results in a large proportion of heifers expressing estrus with a high degree of synchrony, enabling the use of FTAI rather than requiring detection of estrus (Busch et al., 2007; Leitman et al., 2009a, 2009b; Mallory et al., 2010, 2011). Previous efforts to characterize timing of estrus expression relative to PG administration using HeatWatch among mixed groups of pubertal and peri-pubertal heifers receiving this treatment resulted in the recommendation that FTAI be performed at 66 h after PG administration for optimal pregnancy rates using conventional semen (Leitman et al., 2009a; Leitman et al., 2009b; Mallory et al., 2010; Mallory et al., 2011).

Previous efforts to evaluate optimal timing for FTAI when using sex-sorted semen following the 14-d CIDR-PG protocol have been limited, although alternative timed AI approaches such as split-time AI (STAI) have been proposed as strategies to use sex-sorted semen more effectively (Thomas et al., 2014). Recently, alternative sets of timepoints for STAI were evaluated when using sex-sorted semen in beef heifers following the 14-d CIDR-PG protocol (Ketchum et al., 2021). In that experiment, expression of estrus was recorded at 66 h or 72 h following PG administration. Heifers that expressed estrus by their assigned timepoint were inseminated at that time, and the remaining heifers were inseminated 24 h later. A larger proportion of heifers expressed

estrus prior to timepoint 1 when timed AI was performed at 72 h vs 66 h. There was no difference in the conception rate of estrous heifers receiving sex-sorted semen at 72 vs 66 h at that power of test, but the estrous response and pregnancy rates observed highlighted the need for further investigation of the optimal timing of FTAI when using sex-sorted semen. The following experiment was designed to test the hypothesis that use of a later timepoint for FTAI of beef heifers following the 14-d CIDR-PG protocol would allow a greater proportion of heifers to express estrus prior to FTAI and result in greater conception rates among estrous heifers inseminated with sex-sorted semen.

Materials & Methods

Animals and Estrus Synchronization

Estrus was synchronized using the 14-d CIDR-PG protocol (Figure 2.1) for 1640 beef heifers in 7 Missouri locations during the 2021 spring and fall breeding seasons. Heifers were treated with a 1.38 g progesterone-releasing intravaginal insert (CIDR) on Day -33 of the protocol, followed by removal of the device on Day -19, and by administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol sodium) on Day -3. Individual animal body weights (BW) and reproductive tract scores (RTS; (Andersen et al., 1991; Rosenkrans & Hardin, 2003; Holm et al., 2009) were recorded at CIDR insertion. Using a randomized complete block design, heifers within each location were blocked based on RTS and BW and were randomly assigned to one of three FTAI timepoints: 66 h, 70 h, or 74 h after PG administration.

Estrus Detection

Estrus detection aids (Estrotect™, Rockway Inc, Spring Valley, WI) were applied at the time of PG administration on Day -3, and activation was recorded at the time of FTAI on Day 0. Estrus detection aid activation was scored on a scale of 0-4 (0 = missing patch; 1 = 0-25% activated; 2 = 25-50% activated; 3 = 50-75% activated; 4 = 75-100% activated; Pohler et al., 2016b). Heifers were considered to have expressed estrus when 50% or greater of the estrus detection aid was activated (patch score 3 or 4; n = 1071) or when the estrus detection aid was missing at FTAI (patch score = 0; n = 18). Heifers were considered to have failed to express estrus if less than 50% of their patch was activated (patch score = 1 or 2; n = 551).

Artificial Insemination

Heifers that expressed estrus prior to FTAI were serviced with sex-sorted semen (SexedULTRA 4M). Heifers that failed to express estrus were serviced with conventional semen and administered gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) at the time of FTAI. Time of PG administration on Day -3 and time of FTAI on Day 0 was recorded for each heifer and used to calculate individual time intervals between PG administration and FTAI. Two technicians serviced heifers at each location. To limit potential confounding effects of technician, heifers were blocked based on treatment, RTS, and BW and randomly preassigned within block to technician.

Semen

Semen from four bulls was collected at commercial facilities (STgenetics, Navasota, TX) and processed to produce units of sex-sorted and conventional semen. Both sex-sorted and conventional semen from each bull were assessed and met the standard quality control criteria set for the respective semen types. SexedULTRA™ Genesis III sorting technology (Sexing Technologies, Navasota, TX) was used to sort sex-sorted units to contain X or Y chromosome-bearing sperm cells at >90% accuracy for the desired sex. Sex-sorted units were packaged to contain 4.0 x 10^6 live sperm cells per 0.25 mL straw prior to freezing, and conventional units were packaged to contain 20.0 x 10^6 live sperm cells per 0.25 mL straw prior to freezing.

Sire varied between locations; however, within location, the same bull(s) were used across all three treatments. Heifers from Location 1 and Locations 3-7 were inseminated with semen from a single bull. At Location 2, heifers were inseminated with semen from one of two bulls, with heifers blocked based on treatment, RTS, and BW and randomly preassigned within block to bull to ensure an even distribution of each sire across the three treatments in that Location. Heifers failing to express estrus by the time of FTAI were inseminated with conventional semen from the same preassigned sire. Heifers were exposed to natural service bulls beginning 14 days after AI.

Pregnancy Diagnosis

Pregnancy rate to AI (P/AI) was determined by transrectal ultrasonography 75-90 days after timed AI. Pregnancies resulting from AI were distinguished from those resulting from natural service based on fetal size, as heifers were not exposed to natural

service bulls until 14 days after AI. In Locations 2, 3, and 5, fetal sex was determined for pregnancies resulting from AI.

Statistical analysis

A mixed model (Proc GLIMMIX of SAS) using the binomial distribution link logit function was used to evaluate the proportion of heifers expressing estrus prior to FTAI and the pregnancy rate to FTAI for heifers inseminated with sex-sorted and conventional semen. Fixed effects tested for inclusion in the mixed model were treatment, RTS, BW, RTS × treatment interaction, and BW × treatment interaction. Location was included as a random effect. In the design of this experiment, expression of estrus determined if heifers were inseminated with sex-sorted or conventional semen, so conception rate could of course not be compared based on semen type.

The proportion of heifers expressing estrus prior to FTAI and the conception rate to AI for heifers inseminated with sex-sorted semen were also evaluated as a function of the interval from PG administration to AI, analyzed as a continuous variable. Regression equation coefficient estimates were used to model the effect of PG to AI interval on expression of estrus and AI conception rates among heifers inseminated with sex-sorted semen, using an average RTS of 4.0 and average body weight of 320.0 kg. Statistical inferences were made based on the LS means derived from the mixed models. Figure 2 illustrates the modeled relationship between PG to AI interval and estrus expression and conception rate to AI when using sex-sorted semen.

Results

Location Summary

Data for average RTS, BW, and PG to AI interval by treatment are shown in Table 2.1. There were no differences in RTS and BW among treatments, as this information was used for blocking. The actual intervals (mean ± SD) from PG administration to AI by treatment were 66.1 ± 1.1 h, 69.9 ± 1.1 h, and 73.6 ± 0.9 h respectively.

Estrus Expression

Data for the proportion of heifers expressing estrus is summarized in Table 2.2. The proportion of heifers that expressed estrus prior to FTAI (66 h: 62% [340/551]; 70 h: 67% [361/542]; 74 h: 71% [388/547]) was greater at 74 h versus 66 h ($P < 0.01$). The proportion of heifers expressing estrus was also affected by the time interval from PG administration to AI ($P = 0.01$) when analyzed as a continuous variable. Figure 2.2 illustrates the anticipated expression of estrus prior to FTAI based on average RTS (4.0) and BW (320.0 kg) across heifers in the experiment: as PG to AI interval increases, the cumulative proportion of heifers expected to have expressed estrus by that timepoint also increases. The proportion of heifers expressing estrus was also affected by RTS ($P < 0.0001$) and BW ($P < 0.0001$); however, there was no RTS × treatment interaction ($P = 0.22$) or BW × treatment interaction ($P = 0.98$).

Pregnancy Rate

Data for P/AI are summarized in Table 2.3. Conception rate did not differ (P = 0.67) among treatments for heifers that expressed estrus and were serviced with sex-sorted semen (66 h: 56% [189/340]; 70 h: 53% [192/361]; 74 h: 53% [206/388]). Among heifers that failed to express estrus and were serviced with conventional semen, conception rates were greater (P = 0.02) when FTAI was performed at 66 h versus 70 h (66 h: 37% [79/211]; 70 h: 25% [46/181]; 74 h: 31% [49/159]).

Discussion

Many factors influence pregnancy rate to AI, including the time at which AI is performed relative to the time at which ovulation occurs (or as proxy, the time at which expression of estrus occurred). Early research in this area, conducted by Trimberger & Davis (1943) and Trimberger (1948), determined that pregnancy rates to AI in dairy cattle are maximized when AI is performed during midestrus or a few hours after the end of behavioral estrus. This work led to the development of the AM-PM rule, in which cattle are bred 12-18 hours following observed estrus (Trimberger & Davis, 1943; Trimberger, 1948). More recently, research has indicated that timing of AI impacts both fertilization rate and embryo quality (Dransfield et al., 1998; Saacke et al., 2000; Dalton, Nadir, Bame, Noftsinger, & Saacke, 2001; Saacke, 2008). This research indicates that insemination too early relative to the time of ovulation results in high embryo quality but may reduce fertilization rates due to lower numbers of viable sperm present at the time of ovulation. Conversely, insemination too late relative to the time of ovulation results in a high fertilization rate by ensuring sufficient numbers of available sperm cells but may

lead to reduced embryo quality as the oocyte ages before fertilization. With this understanding, the optimal time for insemination may involve a compromise between these two considerations.

Optimal timing of insemination with sex-sorted semen may differ from conventional recommendations due to the reduced lifespan of sex-sorted sperm cells in the female reproductive tract (Maxwell et al., 2004), fewer sperm cells per insemination (DeJarnette et al., 2008), and increased incidence of precapacitation (Lu & Seidel, 2004). These factors may narrow the window of fertility with regard to timing of insemination relative to ovulation (Sales et al., 2011; Bombardelli et al., 2016). Research in this area has likewise explored this concept based on timing of ovulation and within FTAI protocols. The data obtained from these studies suggest that pregnancy rates to AI with sex-sorted semen are improved when animals are inseminated closer to the time of ovulation (Sales et al., 2011; Bombardelli et al., 2016). However, results have been mixed when delaying timing of FTAI with sex-sorted semen until later than typically recommended when using conventional semen. Some experiments have suggested modest improvements in P/AI with sex-sorted semen when timed AI is delayed (Sales et al., 2011; Oosthuizen et al., 2021) whereas others have observed no improvement (Hall et al., 2017; Drake et al., 2020; Ketchum et al., 2021; Oosthuizen et al., 2021).

Generally, the use of sex-sorted semen is not recommended for use in FTAI protocols due to reduced pregnancy rates, especially among females that fail to express estrus (Hall et al., 2010; Rhinehart et al., 2011; Sá Filho et al., 2012; Thomas et al., 2014a; Cooke et al., 2014). Regardless of semen type, pregnancy rates to FTAI are greater among females that express estrus (Richardson et al., 2016). This indicates the

importance of a high estrous response rate when performing timed AI. To overcome the potential reduction in pregnancy rates associated with performing FTAI rather than performing AI based on detected estrus, a FTAI timepoint must be identified that is late enough that a large proportion of females express estrus by the time of AI, yet early enough that insemination does not occur too late for those cattle that express estrus earliest in the synchronized group. In other words, the optimal timepoint for FTAI is the point that will result in the greatest number of pregnancies produced for an entire group of cattle rather than one individual. Furthermore, optimal timing of FTAI varies among estrus synchronization protocols due to differences in the distribution and synchrony of estrus expression.

A trial conducted in beef heifers that administered PG 7 days prior to 7-d CO-Synch + CIDR had greater P/AI when insemination with sex-sorted semen occurred at 72 h vs 54 h (Oosthuizen et al., 2021). In the same study, heifers treated with only 7-d CO-Synch + CIDR had similar conception rates to sex-sorted semen when timed AI occurred at 54 h and 72 h. Inducing luteolysis by PG injection will result in approximately 70% of cycling heifers, at random stages of their estrous cycle, to respond and express estrus in 3 days (Lauderdale et al., 1974; Louis et al., 1974). In order to respond to the GnRH injection administered at CIDR insertion, these heifers need to have a physiologically mature follicle (Prescott et al., 1992; Silcox et al., 1993). If expression of estrus occurs only 3 to 4 days prior to GnRH administration, there is likely a large proportion of females that do not have a dominant, LH-responsive follicle when GnRH is administered. Given the lack of control over stage of follicular development, optimal timing of FTAI following PG presynchronization prior to 7-d CO-Synch + CIDR may be later than what

is recommended following just the 7-d CO-Synch + CIDR protocol. The reduction in estrus expression by FTAI when PG presynchronization is used compared to heifers that only received the 7-d CO-Synch + CIDR treatment further illustrates this point (Oosthuizen et al., 2018; Oosthuizen et al., 2021). In several trials conducted using estrus synchronization protocols with a well-established optimal FTAI timepoint, later timing has not been shown to improve P/AI when using sex-sorted semen. This includes the 7-d CO-Synch + CIDR treatment group in the preceding trial where P/AI was not improved when insemination with sex-sorted semen was delayed from 54 h to 72 h (Oosthuizen et al., 2021). Another trial, conducted in lactating beef cows, indicated no improvement in P/AI when timed insemination with sex-sorted semen took place at a later timepoint following the 5-d CO-Synch + CIDR protocol (Hall et al., 2017). Results from the present experiment likewise do not indicate an improvement in P/AI with sex-sorted semen when later timing of FTAI is used following the 14-d CIDR-PG protocol. This aligns with results reported by Ketchum et al. (2021) indicating no improvement in P/AI among estrous heifers inseminated with sex-sorted semen at 72 h versus 66 h following the 14-d CIDR-PG protocol.

Several studies have cited a marked improvement in the proportion of females that have expressed estrus prior to FTAI when using a later timepoint for FTAI (Hall et al., 2017; Ketchum et al., 2021; Oosthuizen et al., 2021), as one would anticipate since more of the distribution of estrus expression is observed and the cumulative proportion of estrous females should therefore be greater. Results from this experiment likewise indicate that a greater proportion of heifers express estrus by the later timepoints; however, there is no improvement in conception rates among those estrous females

inseminated with sex-sorted semen. In fact, numerical conception rates observed raise questions as to whether the later timepoint for FTAI could be too late for a proportion of heifers that express estrus early following PG administration. Based on the mixed results across the published literature when using later timing of FTAI following varying estrus synchronization protocols, the degree to which delaying timing of FTAI may be advantageous or disadvantageous when using sex-sorted semen may be protocol dependent.

Using an estrus synchronization protocol that optimizes the proportion of females that express estrus and the degree of synchrony in the group is essential when combining timed AI with the use of sex-sorted semen. The recommendation to limit the use of sex-sorted semen to only those females that express estrus remains the most effective way to avoid greatly reduced pregnancy rates to timed AI with sex-sorted semen. When following this recommendation, using a later timepoint for FTAI following 14-d CIDR-PG does not result in improved pregnancy rates but does result in more total females being classified as estrous and therefore being inseminated with sex-sorted semen.

Conclusion

Currently, the most effective way to minimize the degree to which pregnancy rates to FTAI are reduced when using sex-sorted semen is to limit the use of sex-sorted semen to only heifers that express estrus prior to FTAI. These data indicate that delaying the time of FTAI following the 14-d CIDR-PG protocol allows a greater proportion of heifers to express estrus and to therefore be serviced with sex-sorted semen; however, extending the interval from PG administration to FTAI following the 14-d CIDR-PG

protocol did not improve pregnancy rates to AI among estrous heifers serviced with sex-sorted semen.

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Table 2.1. Reproductive tract score (RTS), body weight (BW), and interval from prostaglandin $F_{2\alpha}$ (PG) administration to artificial insemination (AI) by treatment.

Treatment[1]	N	RTS[2]	BW (kg)[3]	PG to AI interval (h)[4]
66 h	551	4.0 ± 1.0	326 ± 50	66.1 ± 1.1
70 h	542	4.0 ± 1.0	327 ± 50	69.9 ± 1.1
74 h	547	4.1 ± 1.0	326 ± 50	73.6 ± 0.9

Data presented as Mean ± SD.

[1]Following the 14-d CIDR-PG estrus synchronization protocol, FTAI was performed 66 h, 70 h, or 74 h after PG administration.

[2]Mean RTS (1-5 scale, 1=immature and 5= luteal phase) by treatment, determined on Day -33 at CIDR insertion.

[3]Mean BW by treatment, recorded on Day -33 at CIDR insertion.

[4]Time of PG administration and time of AI were recorded for each heifer to calculate PG to AI interval.

Table 2.2. Estrous response rate[1] by treatment.

Treatment[2]	66 h		70 h		74 h	
Location	Proportion	%	Proportion	%	Proportion	%
Location 1	61/72	85	61/73	84	63/72	88
Location 2	36/84	43	41/82	50	55/82	67
Location 3	50/65	77	50/64	78	46/63	73
Location 4	49/78	63	57/77	74	52/78	67
Location 5	79/114	69	83/110	75	90/119	76
Location 6	38/74	51	39/73	53	42/68	62
Location 7	27/64	42	30/63	48	40/65	62
Total	340/551	62[a]	361/542	67[ab]	388/547	71[b]

[1]The interval from PG administration to FTAI affected the proportion of heifers expressing estrus prior to FTAI (patch score = 0, 3, 4).

[2]See Figure 1 for treatment descriptions.

[ab]Values with different superscripts differ ($P = 0.01$).

Table 2.3. Pregnancy rates to AI[1] by treatment, location, and semen type.

Treatment[2]		66 h		70 h		74 h	
Location	Semen type[3]	Proportion	%	Proportion	%	Proportion	%
Location 1	Sex-sorted	34/61	56	34/61	56	37/63	59
	Conventional	3/11	27	3/12	25	4/9	44
Location 2	Sex-sorted	20/36	56	24/41	59	26/55	47
	Conventional	19/48	40	10/41	24	9/27	33
Location 3	Sex-sorted	34/50	68	29/50	58	32/46	70
	Conventional	7/15	47	3/14	21	10/17	59
Location 4	Sex-sorted	17/49	35	19/57	33	19/52	37
	Conventional	14/29	48	7/20	35	9/26	35
Location 5	Sex-sorted	47/79	59	47/83	57	50/90	56
	Conventional	12/35	34	8/27	30	7/29	24
Location 6	Sex-sorted	21/38	55	22/39	56	20/42	48
	Conventional	11/36	31	8/34	24	5/26	19
Location 7	Sex-sorted	16/27	59	17/30	57	22/40	55
	Conventional	13/37	35	7/33	21	5/25	20
Total	Sex-sorted	189/340	56	192/361	53	206/388	53
	Conventional	79/211	37[a]	46/181	25[b]	49/159	31[ab]

[1]Pregnancy rates to AI were determined by transrectal ultrasonography 75-90 days after FTAI.

[2]See Figure 2.1. for treatment descriptions.

[3]Heifers that expressed estrus (patch score = 0, 3, 4) were serviced with sex-sorted semen. Heifers that failed to express estrus (patch score = 1, 2) were serviced with conventional semen.

[ab]Values with different superscripts differ ($P = 0.02$).

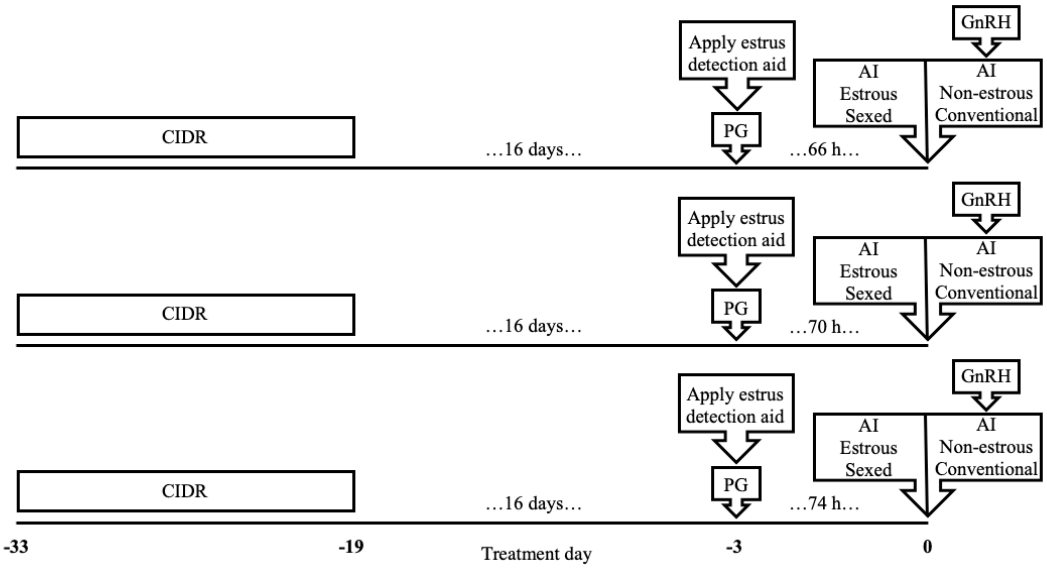

Figure 2.1. Treatment schedules for the 14-d CIDR-PG protocol. Heifers were treated with a 1.38 g progesterone-releasing intravaginal insert (CIDR) on Day -33 of the protocol, followed by removal of the device on Day -19, and by administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol sodium) on Day -3. Fixed-time AI was performed 66 h (n = 553), 70 h (n = 541), or 74 h (n = 546) after PG administration. Estrus detection aids (Estrotect) were applied at PG administration, with activation recorded at FTAI. Heifers that expressed estrus prior to FTAI were inseminated with sex-sorted semen (SexedULTRA 4M™). Heifers that failed to express estrus were inseminated with conventional semen. Gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered coincident with FTAI for heifers that failed to express estrus.

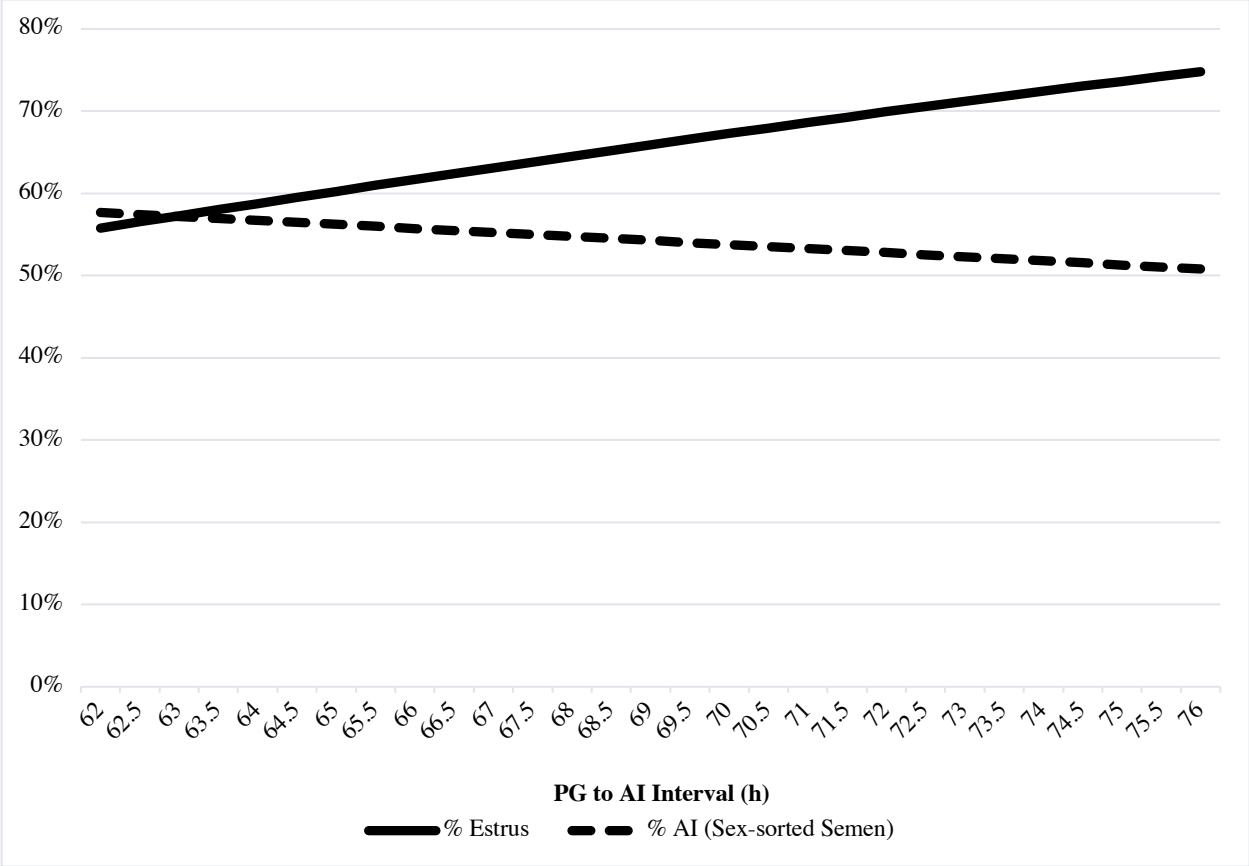

Figure 2.2. Mixed-model regression of the proportion of heifers expressing estrus prior to FTAI based on interval from PG administration to AI. Interval from PG administration to AI had an effect on the proportion of heifers expressing estrus prior to FTAI (P = 0.01). Mixed-model regression of the proportion of heifers serviced with sex-sorted semen that became pregnant to AI based on interval from PG administration to AI. Interval from PG administration to AI did not affect pregnancy rate to AI for estrous heifers inseminated with sex-sorted semen ($P = 0.35$).

Chapter 3

Inducing luteolysis prior to atresia of the first follicular wave by altering the interval to prostaglandin $F_{2\alpha}$ administration after long-term progestin presynchronization

Abstract

A series of experiments was designed to evaluate treatment schedules for control of the estrous cycle in which luteolysis is induced prior to atresia of the first follicular wave following a long-term progestin presynchronization treatment in beef heifers. The overarching hypothesis was that the proportion of heifers undergoing luteolysis and expressing estrus would be affected by the duration of treatment with an intravaginal progesterone-releasing insert (CIDR®; 1.38 g progesterone) and/or by the interval from CIDR removal to prostaglandin $F_{2\alpha}$ (PG; 500 µg cloprostenol) administration. In Experiment 1, heifers (n = 91) were blocked by reproductive tract score (RTS) and body weight (BW) and were assigned randomly within block to one of four treatments in a 2 x 2 factorial design: presynchronization via CIDR treatment for either 14 d (Treatments 14-8 and 14-9) or 18 d (Treatments 18-8 and 18-9) with administration of PG either 8 d (Treatment 14-8 and 18-8) or 9 d (Treatment 14-9 and 18-9) following CIDR removal. In Experiment 2, heifers (n = 63) were blocked by RTS and BW and randomly assigned to receive administration of PG either 9 d or 10 d following a 14 d CIDR presynchronization. In Experiment 3, heifers (n = 83) were blocked by RTS and BW and randomly assigned to receive administration of PG either 9 d or 16 d following a 14 d CIDR presynchronization. In all three experiments, estrus detection aids (Estrotect) were applied at the time of PG administration. In Experiments 1 and 2, heifers were observed

for expression of estrus three times daily for 4 d following PG administration and received artificial insemination (AI) based on estrous response. For heifers that failed to express estrus by 96 h after PG administration, timed AI was performed and gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered. In Experiment 3, Estrotect patch activation was recorded at 66 h, and heifers that expressed estrus by 66 h were inseminated at that timepoint. Heifers that failed to express estrus by 66 h were inseminated at 90 h. For heifers that failed to express estrus by 90 h, GnRH was administered at timed AI. In all three experiments, blood samples were collected at the time of PG administration and, for heifers that failed to express estrus, at timed AI to determine serum progesterone concentrations via radioimmunoassay. In Experiment 1, serum estradiol concentrations were also determined via radioimmunoassay. Transrectal ovarian ultrasonography was performed to determine CL status and to measure largest follicle diameter (LFD) at PG administration and, for heifers that failed to express estrus, at timed AI. Transrectal ultrasonography was performed 60-80 d after AI to determine pregnancy status. In Experiment 1, there was a tendency for a greater proportion of heifers to express estrus by 96 h after PG administration when PG administration occurred 9 d after CIDR removal versus 8 d after CIDR removal ($P = 0.07$; 8 d: 57% [26/46]; 9 d: 76% [34/45]). Additionally, serum E2 concentrations were greater at the time of PG administration when PG was administered 9 d after CIDR removal versus 8 d after CIDR removal following an 18-d CIDR treatment ($P = 0.006$; 18-8: 5.7 ± 1.0 pg/mL; 18-9: 9.6 ± 0.9 pg/mL). In Experiment 2, pregnancy rate to AI (P/AI) was greater among heifers receiving PG administration 9 d after CIDR removal versus 10 d after CIDR removal ($P = 0.03$; 14-9: 58% [18/31]; 14-10: 28% [9/32]). There was a tendency

for LFD to be greater among non-estrous heifers in treatment 14-10 versus non-estrous heifers in treatment 14-9 when measured at timed AI ($P < 0.08$; 14-9: 11.9 ± 1.6; 14-10: 13.6 ± 1.3). In Experiment 3, there was a tendency for serum progesterone concentrations at the time of PG administration to be greater among heifers in treatment 14-16 versus heifers in treatment 14-9 ($P = 0.07$; 14-9: 1.2 ± 0.2 pg/mL; 14-16: 1.8 ± 0.2 pg/mL). These results provide a preliminary evaluation of the feasibility of inducing luteolysis prior to atresia of the first follicular wave following long-term progestin presynchronization.

Introduction

Long-term progestin-based presynchronization can serve as an effective method to induce cyclicity among peripubertal heifers (Zimbelman & Smith, 1966; Gonzalez-Padilla et al., 1975; Short et al., 1976; Sheffield & Ellicott, 1982; Patterson et al., 1990; Anderson et al., 1996). Ovarian response of pubertal heifers to long-term progestin treatment varies based on stage of the estrous cycle at the start of progestin treatment (Sirois & Fortune, 1990). In pubertal heifers, regression of the CL will likely occur during a long-term progestin treatment, as luteolysis is not inhibited by treatment with exogenous progestins. If the CL undergoes luteolysis, endogenous production of progesterone will decline, and the exogenous progestin provided at a sub-luteal dose will prevent atresia of the dominant follicle due to inadequate negative feedback on GnRH and LH pulsatility at the level of the hypothalamus (Beal et al., 1988; Patterson et al., 1989; Lucy et al., 1990; Sirois & Fortune, 1990; Savio et al., 1993). Sub-luteal concentrations of progesterone will maintain an LH pulse frequency sufficient for

continued follicular development and dominant follicle maintenance (Kojima et al., 1995). Thus, a persistent follicle can develop as a result of progestin treatment. This is a follicle that remains in dominance for an extended period of time and generally reaches a larger size than is typical in a normal estrous cycle. Prolonging the period of dominance may result in premature maturation of the oocyte due to the progression of meiosis to metaphase II before the LH surge (Mihm et al., 1994; Revah & Butler, 1996). This can reduce oocyte quality and negatively impact fertility of the first synchronized estrus following long-term progestin treatments (Ahmad et al., 1995; Kinder et al., 1996).

Despite this reduction in fertility of the aged oocyte, a high degree of synchrony of estrus and ovulation can be attained via long-term progestin treatment. Rather than breeding at the time of the first synchronized estrus following progestogen treatment, protocols have been developed using long-term progestin treatment for presynchronization, with AI performed at the second rather than first estrus following progestin treatment. These protocols involve progestin administration for an extended period of time (i.e. 14 d) and administration of PG 16 to 19 d after removal of the progestin (Brown et al., 1988; Kojima et al., 2004; Schafer et al., 2006). The rationale for the initial development of this approach was that PG would be administered when cattle are in the late luteal phase, which had proven to be more effective for generating estrus expression than when PG is administered in the early luteal phase (King et al., 1982; Tanabe & Hann, 1984; Watts & Fuquay, 1985).

Progestin treatment for an extended duration results in a large proportion of pubertal heifers undergoing luteolysis and therefore having a preovulatory follicle at the time of progestin removal. Cessation of progestin treatment will remove the negative

feedback on GnRH secretion, allowing for estrus and ovulation of the preovulatory follicle to occur. This effectively presynchronizes estrus among heifers prior to PG administration (Brown et al., 1988; Leitman et al., 2009b; Mallory et al., 2010). Synchronous expression of estrus following progestin removal results in less variability among heifers in stage of follicular development at subsequent time points in the treatment schedule, as synchronized ovulation will also result in synchronous recruitment of the next follicular wave. If luteolysis does not occur during progestin treatment, however, the CL will maintain endogenous progesterone concentrations and prevent expression of estrus and ovulation until luteolysis occurs. This reduces the degree of synchrony among females in the group, as heifers with a CL that fails to undergo luteolysis will be behind in terms of recruitment and development of the next follicular wave. With a 14-d progestin treatment, as used in the 14-d CIDR-PG protocol, there will be a small proportion of heifers that have a CL that does not undergo luteolysis during treatment. In an attempt to allow a greater proportion of treated heifers to undergo luteolysis prior to progestin removal, Knickmeyer et al. (2019) and Christenson et al. (2022) evaluated extending duration of progestin treatment from 14 d to 18 d within the 14 d CIDR-PG protocol and the 14 d MGA-PG protocol, respectively. Neither study resulted in an improvement in estrus synchrony following induced luteolysis.

In the 14-d CIDR-PG protocol, luteolysis is induced 16 d after progestin removal. This is approximately 12 to 14 d after the expression of estrus that follows progestin removal. By this time, the first follicular wave of the cycle has become atretic and the second follicular wave has emerged. Biological variation likely exists with respect to timing of atresia of the first follicular wave and recruitment of the second follicular wave

due to animal-to-animal differences in the feedback mechanisms associated with the hypothalamic-pituitary-gonadal axis and the number of follicular waves per cycle; thus, induction of luteolysis prior to atresia of the first follicular wave could conceivably result in reduced variance in stage of follicular maturity at a timed AI.

Heifers treated with PG early in the estrous cycle (Day 5 to 7) have been shown to express estrus earlier following administration of PG as compared with heifers treated with PG later in their estrous cycle (Day 8 to 15) (King et al., 1982; Tanabe & Hann, 1984; Watts & Fuquay, 1985). Heifers in early diestrus would have lesser serum progesterone concentrations compared to those in mid and late diestrus; however, rate of serum progesterone decline following PG administration is similar among these groups (King et al., 1982). Serum progesterone concentrations influence follicular dynamics, with the dominant follicle that develops during the first follicular wave reaching a greater diameter than the dominant follicles that develop in later waves of the estrous cycle (Sirois & Fortune, 1988; Ginther et al., 1989a; Adams, Matteri, & Ginther, 1992c). Dominant follicle size is correlated to estradiol output and also influences the amount of luteal tissue that develops following ovulation (Vasconcelos et al., 2001). These factors influence pregnancy rates (Perry et al., 2007).

With this knowledge, a series of experiments were designed to evaluate the feasibility of inducing luteolysis prior to atresia of the first follicular wave following progestin presynchronization. The hypothesis was that the proportion of heifers undergoing luteolysis and expressing estrus would be affected by duration of progestin presynchronization and/or by timing of luteolysis relative to progestin removal.

Materials & Methods

Animals and Estrus Synchronization

For each of the three experiments, a randomized complete block design was used to block heifers based on individual animal body weight (BW) and reproductive tract score (RTS; Scale 1-5; 1 = infantile and 5 = presence of a CL; Andersen et al., 1991; Rosenkrans & Hardin, 2003; Holm et al., 2009) recorded at the first animal handling event of the experiment.

In Experiment 1, estrus was synchronized for 91 beef heifers at one location in the spring of 2021. Within block, heifers were randomly assigned to one of four treatments (Figure 1) in a 2 x 2 factorial design: treatment with an Eazi-Breed CIDR insert (1.38 g progesterone controlled internal drug release insert; Zoetis, Madison, NJ) for either 14 d (treatment 14-8 and 14-9) or 18 d (treatment 18-8 and 18-9) with administration of prostaglandin $F_{2\alpha}$ (PG; 25 mg dinoprost, i.m.; Lutalyse; Zoetis, Madison, NJ) either 8 d (treatment 14-8 and 18-8) or 9 d (treatment 14-9 and 18-9) following CIDR removal. Treatments were scheduled so that PG administration occurred at the same time on the same day for all four treatments.

In Experiment 2, estrus was synchronized for 64 beef heifers in one location in the fall of 2021. Within block, heifers were assigned to one of two treatments (Figure 2): administration of PG either 9 d (Treatment 14-9) or 10 d (Treatment 14-10) following a 14 d CIDR presynchronization. The treatment schedules were designed so that PG administration occurred at the same time on the same day for both treatments.

In Experiment 3, estrus was synchronized for 93 beef heifers in one location in the spring of 2022. Within block, heifers were randomly assigned to one of two

treatments (Figure 3): administration of PG either 9 d (Treatment 14-9) or 16 d (Treatment 14-16) following a 14 d CIDR presynchronization. The treatment schedules were designed so that PG administration occurred at the same time on the same day for both treatments.

Estrus Detection and Artificial Insemination

For Experiments 1 and 2, estrus detection aids (Estrotect, Rockway Inc, Spring Valley, WI) were applied at the time of CIDR removal and at the time of PG administration. Heifers were observed for expression of estrus three times daily for 4 d following CIDR removal and following PG administration. Heifers were considered to have expressed estrus when 50% or greater of the estrus detection aid was activated or when standing estrus was observed. Time of observed standing estrus or Estrotect patch activation was recorded. Heifers that expressed estrus following PG administration received artificial insemination (AI) 12-18 h after onset of estrus. For heifers that failed to express estrus by 96 h after PG administration, timed AI was performed and gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin acetate, i.m.; Fertagyl; Merck Animal Health, Madison, NJ) was administered concurrently. Within experiment, one technician performed all inseminations, and semen from a single sire was used for all AI services.

In Experiment 3, estrus detection aids were applied at the time of PG administration on Day -4, and activation was recorded on Day -1 and/or Day 0, at the time of STAI. Estrus detection aid activation was scored on a scale of 0-4 (0 = missing patch; 1 = 0-25% activated; 2 = 25-50% activated; 3 = 50-75% activated; 4 = 75-100%

activated; Pohler et al., 2016b). Heifers were considered to have expressed estrus when 50% or greater of the estrus detection aid was activated (patch score 3 or 4). Heifers were considered to have failed to express estrus if less than 50% of their patch was activated (patch score = 1 or 2). No heifers lost their estrus detection aid between application and AI. Heifers that expressed estrus by 66 h were inseminated at that time. Heifers that failed to express estrus by 66 h were inseminated 24 h later and GnRH was administered concurrently with AI to heifers failing to express estrus by 90 h. One technician performed all inseminations, and semen from a single sire was used for all AI services.

Blood Sampling and Radioimmunoassay

For all three experiments, blood samples were collected by jugular venipuncture at the time of PG administration (Day -4) and, for heifers that failed to express estrus, at timed AI (Day 0). Blood samples were allowed to clot and stored at 4 °C for 24 h. Blood serum was obtained by centrifugation and stored at -20 °C. Samples collected at PG and at timed AI were analyzed for serum progesterone and, in Experiment 1, serum estradiol concentrations.

For all three experiments, circulating concentrations of progesterone were determined for blood samples collected on Day -4 and Day 0 in duplicate (100 μl of serum per tube) by double antibody radioimmunoassay (RIA; MP Biomedicals, California, USA) as previously described (Pohler et al., 2016a). The sensitivity of the assay was 0.05 ng/mL. Intraassay coefficients of variation (CV) for Experiment 1, 2, and 3 were 1.35%, 1.37%, and 1.92% respectively. Interassay CV for Experiment 1 was 5%.

In Experiment 1, circulating concentrations of estradiol were determined for blood samples collected on Day -4 and Day 0 in duplicate (300 μl of serum per tube) by RIA as previously described (Kirby et al., 1997). Sensitivity of the assay was 0.5 pg/mL, and the intraassay CV was 3.9% respectively.

Ovarian Ultrasonography and Pregnancy Diagnosis

In all three experiments, transrectal ovarian ultrasonography was used to determine large follicle diameter (LFD) and CL presence or absence at PG (Day -4) for all heifers and at timed AI (Day 0) for heifers that failed to express estrus. Electronic calipers were used to determine follicle height and width, which were averaged to determine LFD. Pregnancy rate to AI was determined by transrectal ultrasonography 60-80 d after timed AI. Pregnancies resulting from first-service AI were determined based on fetal size.

Statistical Analyses

For all three experiments, mixed models (PROC GLIMMIX of SAS; SAS 9.4 Inst. Inc., Cary, NC) were used to evaluate the continuous variables of serum progesterone concentrations and LFD at the time of PG administration and, for heifers that failed to express estrus, at timed AI. In Experiment 1, mixed models (PROC GLIMMIX of SAS) were also used to evaluate serum estradiol concentrations at the time of PG administration and, for heifers that failed to express estrus, at timed AI. Mixed models (PROC GLIMMIX of SAS) using the binomial distribution link logit function were used to evaluate the proportion of heifers expressing estrus following CIDR

removal in Experiment 1, expression of estrus following PG administration in all three experiments, and pregnancy rate to AI in all three experiments. Fixed effects tested for inclusion in the mixed models were RTS, BW, RTS x treatment, and BW x treatment.

In Experiment 1, given the factorial design, treatments were pooled for analysis when appropriate based on portions of the treatment schedule that were identical. To test for effects of progestin treatment duration, treatments 14-8 and 14-9 were pooled and compared with 18-8 and 18-9 for analysis of the proportion of heifers expressing estrus following CIDR removal and PG administration, serum progesterone and estradiol concentrations, as well as LFD at PG administration and, for heifers that failed to express estrus, at timed AI. Likewise, to test for effects length of the interval from CIDR removal to PG administration, treatments 14-8 and 18-8 were pooled and compared with 14-9 and 18-9 for analysis of the proportion of heifers expressing estrus following PG administration, serum progesterone and estradiol concentrations, as well as LFD at PG administration and, for heifers that failed to express estrus, at timed AI.

Results

Location summary

Data for RTS and BW by treatment for Experiments 1, 2, and 3 are shown in Tables 3.1., 3.2., and 3.3. respectively. Within each of the three experiments, there were no differences in RTS and BW among treatments.

Estrus Expression

In Experiment 1, the proportion of heifers that expressed estrus by 108 hours after progestin removal did not differ based on the duration of progestin treatment ($P = 0.47$; 14 d: 87% [40/46]; 18 d: 80% [36/45]; Table 3.4.). The proportion of heifers that expressed estrus by 96 hours after PG administration did not differ based on the duration of progestin treatment ($P = 0.43$; 14 d: 70% [32/46]; 18 d: 62% [28/45]), but there was a tendency for the proportion of heifers that expressed estrus by 96 hours after PG administration to be greater among heifers administered PG 9 d after CIDR removal versus 8 d after CIDR removal ($P = 0.07$; 8 d: 57% [26/46]; 9 d: 76% [34/45]; Table 3.5.).

In Experiment 2, the proportion of heifers expressing estrus by 96 h after PG administration did not differ between treatment groups ($P = 0.17$; 14-9: 77% [24/31]; 14-10: 66% [21/32]; Table 3.8.).

In Experiment 3, the proportion of heifers expressing estrus by 66 h after PG administration did not differ between treatments 14-9 and 14-16 ($P = 0.63$; 14-9: 56% [23/41]; 14-16: 52% [22/42]; Table 3.9.). Likewise, the cumulative proportion of heifers expressing estrus by 90 h after PG administration did not differ between treatments 14-9 and 14-16 ($P = 0.42$; 14-9: 63% [26/41]; 14-16: 76% [32/42]; Table 3.9.).

Blood Sampling and Radioimmunoassay

In Experiment 1, there were no detectable differences based on treatment with respect to serum progesterone concentrations (Table 3.7.) at the time of PG administration ($P = 0.43$; 14-8: 2.4 ± 0.3 ng/ml; 14-9: 2.6 ± 0.3 ng/ml; 18-8: 2.4 ± 0.3

ng/ml; 18-9: 3.0 ± 0.4 ng/ml) or, among non-estrous heifers, at timed AI (P = 0.85; 14-8: 1.4 ± 0.4 ng/ml; 14-9: 0.7 ± 0.2 ng/ml; 18-8: 1.7 ± 0.4 ng/ml; 18-9: 1.5 ± 0.3 ng/ml). Serum estradiol concentrations (Table 3.6.) at the time of PG administration were greater among heifers in treatment 18-9 compared to those in treatment 18-8 (P = 0.0057; 18-8: 5.7 ± 1.0 pg/ml; 18-9: 9.6 ± 0.9 pg/mL). Additionally, serum estradiol concentrations at the time of PG administration were greater among heifers in treatment 18-9 versus heifers in treatment 14-9 (P = 0.03; 14-9: 6.5 ± 1.0 pg/ml; 18-9: 9.6 ± 0.9 pg/ml). Serum estradiol concentrations among non-estrous heifers at timed AI did not differ based on treatment at this power of test (P = 0.91; 14-8: 10.1 ± 2.2 pg/ml; 14-9: 7.0 ± 1.9 pg/ml; 18-8: 9.9 ± 1.6 pg/ml; 18-9: 6.6 ± 2.2 pg/ml).

In Experiment 2, serum progesterone concentrations (Table 3.8.) did not differ between treatments at the time of PG administration (P = 0.12; 14-9: 0.9 ± 0.2 ng/mL; 14-10: 0.7 ± 0.2 ng/mL) or, among non-estrous heifers, at timed AI (P = 0.22; 14-9: 0.5 ± 0.2 ng/mL; 14-10: 0.2 ± 0.1 ng/mL).

In Experiment 3, serum progesterone concentrations (Table 3.11.) at the time of PG administration tended to be greater among heifers receiving PG administration 16 d after CIDR removal versus 9 d after CIDR removal (P = 0.07; 14-9: 1.2 ± 0.2 pg/mL; 14-16: 1.8 ± 0.2 pg/mL). Serum progesterone concentrations among non-estrous heifers at timed AI did not differ between treatments (P = 0.63; 14-9: 0.7 ± 0.1 ng/mL; 14-16: 0.6 ± 0.2 ng/mL).

Ovarian Ultrasonography and Pregnancy Diagnosis

In Experiment 1, no differences were observed in LFD (Table 3.7.) at the time of PG administration ($P = 0.43$; 14-8: 13.1 ± 0.7 mm; 14-9: 12.3 ± 0.3 mm; 18-8: 12.3 ± 0.3 mm; 18-9: 12.7 ± 0.4 mm) or, among non-estrous heifers, at timed AI ($P = 0.85$; 14-8: 13.5 ± 1.2 mm; 14-9: 13.3 ± 0.6 mm; 18-8: 14.6 ± 0.7 mm; 18-9: 14.1 ± 0.5 mm). Pregnancy rate to AI (Table 3.6.) did not differ among treatments ($P = 0.77$; 14-8: 57% [13/23]; 14-9: 43% [10/23]; 18-8: 43% [10/23]; 18-9: 55% [12/22]).

In Experiment 2, large follicle diameter (Table 3.8.) at the time of PG did not differ between treatments ($P = 0.62$; 14-9: 11.9 ± 0.5 mm; 14-10: 11.9 ± 0.3 mm), but there was a tendency for LFD of non-estrous heifers at timed AI to be greater among heifers receiving PG administration 10 d after CIDR removal versus 9 d after CIDR removal ($P = 0.08$; 14-9: 11.9 ± 1.6; 14-10: 13.6 ± 1.3). Pregnancy rate to AI (Table 3.9.) was greater among heifers receiving treatment 14-9 than among heifers receiving treatment 14-10 ($P = 0.03$; 14-9: 58% [18/31]; 14-10: 28% [9/32]).

In Experiment 3, LFD (Table 3.11.) at the time of PG did not differ between treatments ($P = 0.18$; 14-9: 11.1 ± 0.2 mm; 14-16: 10.8 ± 0.2 mm). Largest follicle diameter among non-estrous heifers at timed AI also did not differ between treatments ($P = 0.37$; 14-9: 12.1 ± 0.9 mm; 14-16: 12.5 ± 0.9 mm). Pregnancy rate to AI (Table 3.10.) did not differ between treatments at this power of test ($P = 0.12$; 14-9: 44% [18/41]; 14-16: 62% [26/42]).

Discussion

In Experiment 1, the proportion of heifers that expressed estrus following CIDR removal did not differ among treatment groups that received a 14-d or 18-d CIDR. There was also no effect of duration of CIDR treatment on the proportion of heifers expressing estrus following PG administration. These data align with findings by Knickmeyer et al. (2019) and Christenson et al. (2022), who observed no improvement in synchrony of estrus when increasing duration of progestin treatment from 14 d to 18 d. Knickmeyer et al. (2019) evaluated extending CIDR treatment duration to 18 d and compared this to the 14-d CIDR treatment. In both treatment groups, PG was administered to heifers 16 d after CIDR removal. The proportion of females expressing estrus prior to timed AI did not differ between the two treatment groups, nor did P/AI. In a similar study, extending duration of melengestrol acetate (MGA) treatment from 14 d to 18 d was evaluated. In both treatment groups, PG was administered 19 d after MGA removal. Duration of MGA treatment did not affect timing of or the proportion of heifers expressing estrus following MGA withdrawal or PG administration (Christenson et al., 2022). Progestin treatment duration was evaluated again in this study due to the earlier induction of luteolysis; however, no improvement in synchrony of estrus resulted from extending the duration of progestin treatment. In the present experiment, serum estradiol concentrations at the time of PG administration were greater among heifers in the 18-9 treatment group versus heifers in the 14-9 treatment group. This difference in estradiol concentrations may be correlated to follicular maturity at the time of PG administration; however, greater concentrations of serum estradiol at the time of PG administration did not result in a

greater proportion of heifers expressing estrus following PG administration or conceiving to AI.

In Experiment 1, there was a tendency for a greater proportion of heifers to express estrus following PG administration when it occurred 9 d rather than 8 d after CIDR removal. This raises the question as to whether an 8 d interval from progestin removal to PG administration is sufficient for all heifers to express estrus, ovulate, and develop a CL capable of responding to PG. The CL does not acquire the ability to respond to exogenous PG administration until about Day 5 of development (Lauderdale, 1972; Liehr et al., 1972; Louis et al., 1972, 1974), meaning heifers that do not ovulate within 3 d of CIDR removal may not acquire a PG-responsive CL by the time of PG administration if using this treatment schedule. Additionally, serum estradiol concentrations were greater at the time of PG administration when PG was administered 9 d after CIDR removal versus 8 d after CIDR removal following an 18-d CIDR treatment, suggesting the longer period from CIDR removal to PG administration may simply provide more time for follicular development. Together, these results suggest PG administration 8 d after CIDR removal may be too early for a proportion of heifers if seeking to induce luteolysis prior to atresia of the first follicular wave following progestin-based presynchronization.

In Experiment 2, P/AI was greater among those heifers administered PG 9 d after CIDR removal compared to heifers administered PG 10 d after CIDR removal. There was a tendency for LFD to be greater among non-estrous heifers that received treatment 14-10 versus non-estrous heifers that received treatment 14-9. It is possible that PG administration occurred too late for a proportion of heifers in the 14-10 treatment group

and that the dominant follicle of the first follicular wave had already lost functional dominance by the time of luteolysis. This would mean the largest follicle measured at timed AI for the non-estrous heifers originated from the second follicular wave. This may indicate that use of a 10-d interval from CIDR removal to PG administration could be too long if seeking to induce luteolysis prior to atresia of the first follicular wave following progestin-based presynchronization.

In Experiment 3, a similar proportion of heifers in each treatment group expressed estrus by 66 h and 90 h after PG administration. Pregnancy rate to AI also did not differ between these two treatment groups at this power of test. There was a tendency for serum progesterone concentrations at the time of PG administration to be greater among heifers in treatment group 14-16 versus treatment group 14-9. This aligns with data collected by King et al. (1982), in which circulating progesterone concentrations were greater in mid to late diestrus compared with early diestrus. At this power of test, dominant follicle size did not differ due to lower circulating progesterone concentrations during follicular development. However, it is possible that lower circulating progesterone concentrations may have influenced when heifers expressed estrus following PG administration (King et al., 1982). Figure 4 illustrates the distribution of estrus for the 14-9 treatment group based on data collected in Experiments 1 and 2. Distribution of estrus was not directly compared between treatment groups in Experiment 3, however.

Conclusion

Based on the preceding results, use of a 9 d interval to PG following a 14 d CIDR presynchronization may merit further research as a potential treatment schedule.

However, inducing luteolysis prior to atresia of the first follicular wave following progestin-based presynchronization would ultimately only be a viable strategy if (1) all heifers have a PG-responsive CL that undergoes complete luteolysis following PG administration and (2) all heifers still have a first-wave dominant follicle that has not become atretic. If, among a proportion of the heifers, ovulation is occurring too late following progestin removal and the resulting CL has not acquired the ability to respond to exogenous PG administration, attempting to induce luteolysis prior to atresia of the first follicular wave would be likely to reduce net pregnancy rates resulting from timed AI. Thus, further research with greater animal numbers is needed.

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Table 3.1. Experiment 1: Reproductive tract score (RTS) and body weight (BW) by treatment.

Treatment[1]	N	RTS[2]	BW[3] (kg)
14-8	23	4.4 ± 0.9	358 ± 25
14-9	23	4.3 ± 1.0	357 ± 24
18-8	23	4.4 ± 0.9	359 ± 25
18-9	22	4.5 ± 0.8	358 ± 24

Data presented as Mean ± SD

[1]Heifers were treated with an intravaginal progesterone-releasing insert (CIDR) for either 14 d (treatment 14-8 and 14-9) or 18 d (treatment 18-8 and 18-9) with administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol) either 8 d (treatment 14-8 and 18-8) or 9 d (treatment 14-9 and 18-9) following CIDR removal.

[2]Average RTS (1-5 scale, 1=immature and 5= luteal phase) by treatment, determined on Day -30.

[3]Average BW by treatment, recorded on Day -30.

Table 3.2. Experiment 2: Reproductive tract score (RTS) and body weight (BW) by treatment.

Treatment[1]	N	RTS[2]	BW[3] (kg)
14-9	31	4.3 ± 1.0	315 ± 25
14-10	32	4.3 ± 1.0	312 ± 23

Data presented as Mean ± SD

[1]Heifers were treated with an intravaginal progesterone-releasing insert (CIDR) for 14 d with administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol) either 9 d (treatment 14-9) or 10 d (treatment 14-10) following CIDR removal.

[2]Average RTS (1-5 scale, 1=immature and 5= luteal phase) by treatment, determined on Day -28.

[3]Average BW by treatment, recorded on Day -28.

Table 3.3 Reproductive tract score (RTS) and body weight (BW) by treatment.

Treatment[1]	N	RTS[2]	BW (kg)[3]
14-9	31	4.0 ± 1.1	368 ± 36
14-16	32	4.0 ± 1.2	376 ± 36

Data presented as Mean ± SD

[1]Heifers were treated with an intravaginal progesterone-releasing insert (CIDR) for 14 d with administration of prostaglandin $F_{2\alpha}$ (PG; 500 μg cloprostenol) either 9 d (treatment 14-9) or 16 d (treatment 14-16) following CIDR removal.

[2]Average RTS (1-5 scale, 1=immature and 5= luteal phase) by treatment, determined on Day -34.

[3]Average BW by treatment, recorded on Day -34.

Table 3.4. Experiment 1: Proportion of heifers expressing estrus following CIDR removal by duration of progestin treatment.

CIDR treatment duration[1]	Expression of estrus following CIDR removal[2]	
	Proportion	%
14 d	40/46	87
18 d	36/45	80

[1]CIDR treatment duration did not affect the proportion of heifers expressing estrus following CIDR removal ($P = 0.47$).

[2]Heifers were observed for expression of estrus 3 times daily for 4 days following CIDR removal.

Table 3.5. Experiment 1: The proportion of heifers expressing estrus following PG administration by treatment and interval from progestin removal to PG administration.

Treatment	Proportion	%	Treatment	Proportion	%
14-8	14/23	61	14-9	18/23	78
18-8	12/23	52	18-9	16/22	73
Total[1]	26/46	57[a]	Total	34/45	76[A]

[1]There was a tendency for a greater proportion of heifers that received PG administration 9 d after CIDR removal versus 8 d after CIDR removal to express estrus following PG administration ($P = 0.07$).

[aA]Values with different superscripts tend to differ ($P = 0.07$).

Table 3.6. Experiment 1: Pregnancy rate to AI (P/AI) by treatment.

Treatment[1]	P/AI[2]	
	Proportion	%
14-8	13/23	57
18-8	10/23	43
14-9	10/23	43
18-9	12/22	55

[1]See Figure 3.1. for treatment descriptions.
[2]Pregnancy rate to AI did not differ among treatments (P = 0.77).

Table 3.7. Experiment 1: Largest follicle diameter (LFD), serum progesterone concentrations (P_4), and serum estradiol concentrations (E_2) at the time of prostaglandin $F_{2\alpha}$ (PG) administration and at timed AI.

Treatment[1]	LFD (mm)[2]		P_4 (ng/mL)[3]		E_2 (pg/mL)[4]	
	PG[5]	NR[6]	PG	NR	PG	NR
14-8	13.1 ± 0.7	13.5 ± 1.2	2.4 ± 0.3	1.4 ± 0.4	6.8 ± 1.1[a]	10.1 ± 2.2
14-9	12.3 ± 0.3	14.6 ± 0.7	2.6 ± 0.3	0.7 ± 0.2	6.5 ± 1.0[a]	7.0 ± 1.9
18-8	12.3 ± 0.3	13.3 ± 0.6	2.4 ± 0.3	1.7 ± 0.4	5.7 ± 1.0[a]	9.9 ± 1.6
18-9	12.7 ± 0.4	14.1 ± 0.5	3.0 ± 0.4	1.5 ± 0.3	9.6 ± 0.9[b]	6.6 ± 2.2

Data presented as Mean ± SE
[1]See Figure 3.1 for treatment descriptions.
[2]LFD (largest follicle diameter) was measured by transrectal ovarian ultrasonography.
[3]Blood samples were collected and serum progesterone concentrations (P_4) were measured via radioimmunoassay.
[4]Blood samples were collected and serum estradiol concentrations (E_2) were measured via radioimmunoassay.
[5]Data was collected at the time of prostaglandin $F_{2\alpha}$ (PG) administration for all heifers.
[6]Data was collected at timed AI for heifers that failed to express estrus (non-responders; NR).
[ab]Values with different superscripts differ ($P = 0.03$).

Table 3.8. Experiment 2: Largest follicle diameter (LFD) and serum progesterone concentrations (P_4) at the time of prostaglandin $F_{2\alpha}$ (PG) administration and at timed AI.

Treatment[1]	LFD (mm)[2]		P_4 (ng/mL)[3]	
	PG[4]	NR[5]	PG	NR
14-9	11.9 ± 0.5	11.9 ± 1.6[a]	0.9 ± 0.2	0.5 ± 0.2
14-10	11.9 ± 0.3	13.6 ± 1.3[A]	0.7 ± 0.2	0.2 ± 0.1

Data presented as Mean ± SE.

[1]See Figure 3.2 for treatment descriptions.

[2]LFD (large follicle diameter) was measured by transrectal ovarian ultrasonography.

[3]Blood samples were collected and serum progesterone concentrations were measured via radioimmunoassay.

[4]Data was collected at the time of prostaglandin $F_{2\alpha}$ (PG) administration for all heifers

[5]Data was collected at timed AI for heifers that failed to express estrus (non-responders; NR).

[aA]Values with different superscripts tend to differ ($P = 0.08$).

Table 3.9. Experiment 2: The proportion of heifers expressing estrus following prostaglandin $F_{2\alpha}$ (PG) administration and the proportion of heifers that conceived to AI by treatment.

Treatment[1]	Expression of estrus following PG administration[2]		P/AI[3]	
	Proportion	%	Proportion	%
14-9	24/31	77	18/31	58[a]
14-10	21/32	66	9/32	28[b]

[1]See Figure 3.2 for treatment descriptions.

[2]Proportion of heifers expressing estrus following PG administration did not differ between treatments ($P = 0.17$).

[3]Pregnancy rates to AI (P/AI) were determined by transrectal ultrasonography 60-80 days after AI.

[ab]Values with different superscripts differ ($P = 0.03$).

Table 3.10. Experiment 3: The proportion of heifers expressing estrus by 66 h and 90 h after prostaglandin $F_{2\alpha}$ (PG) administration and the proportion of heifers that conceived to AI by treatment.

Treatment[1]	Estrus by 66 h[2]		Estrus by 90 h[3]		P/AI[4]	
	Proportion	%	Proportion	%	Proportion	%
14-9	23/41	56	26/41	63	18/41	44
14-16	22/42	52	32/42	76	26/42	62

[1]See Figure 3.3 for treatment descriptions.
[2]Proportion of heifers expressing estrus by 66 h after PG administration did not differ between treatments ($P = 0.63$).
[3]Proportion of heifers expressing estrus by 90 h after PG administration did not differ between treatments ($P = 0.42$).
[4]Pregnancy rate to AI (P/AI) did not differ between treatments ($P = 0.12$).

Table 3.11. Experiment 3: Largest follicle diameter (LFD) and serum progesterone concentrations (P_4) at the time of prostaglandin $F_{2\alpha}$ (PG) administration and timed AI.

Treatment[1]	LFD (mm)[2]		P4 (ng/mL)[3]	
	PG[4]	NR[5]	PG	NR
14-9	11.1 ± 0.2	12.1 ± 0.9	1.2 ± 0.2[a]	0.7 ± 0.1
14-16	10.8 ± 0.2	12.5 ± 0.9	1.8 ± 0.2[A]	0.6 ± 0.2

Data presented as Mean ± SE.
[1]See Figure 3.2 for treatment descriptions.
[2]Largest follicle diameter (LFD) was measured by transrectal ovarian ultrasonography.
[3]Blood samples were collected and serum progesterone concentrations were measured via radioimmunoassay.
[4]Data was collected at the time of prostaglandin $F_{2\alpha}$ (PG) administration for all heifers.
[5]Data was collected at timed AI for heifers that failed to express estrus (non-responders; NR).
[aA]Values with different superscripts tend to differ ($P = 0.07$).

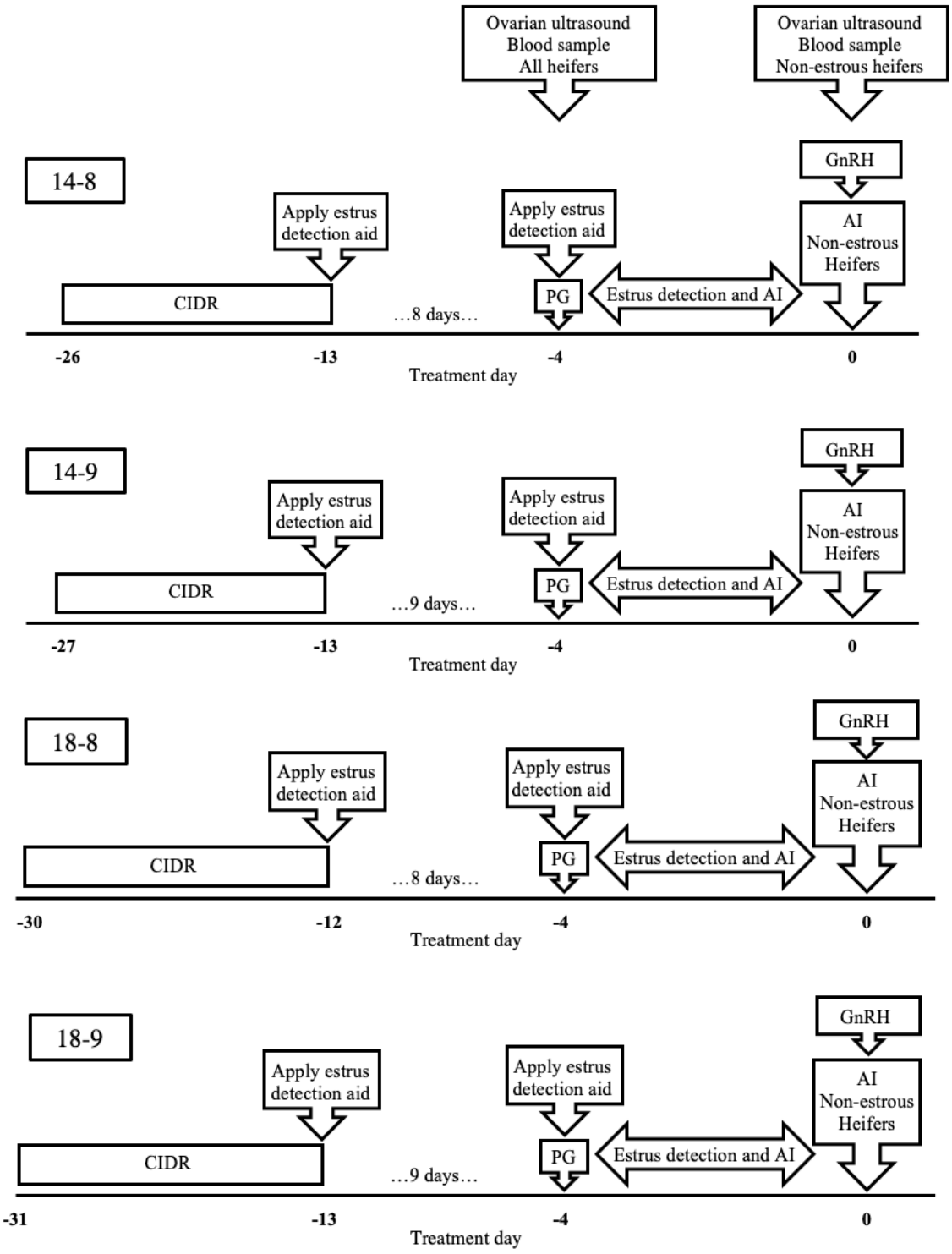

Figure 3.1. Treatment schedules for heifers in Experiment 1. This included presynchronization via treatment with an intravaginal progesterone-releasing insert (CIDR) for either 14 d (Treatments 14-8 and 14-9) or 18 d (Treatments 18-8 and 18-9) with administration of prostaglandin $F_{2\alpha}$ (PG) either 8 d (Treatment 14-8 and 18-8) or 9 d (Treatment 14-9 and 18-9) following CIDR removal. Estrus detection aids were applied

at the time of CIDR removal and at the time of PG administration. Heifers were observed for expression of estrus 3 times daily for 4 d following CIDR removal and PG administration. Heifers expressing estrus following PG administration received artificial insemination (AI) 12 to 18 h after observed estrus. For heifers that failed to express estrus by 96 h after PG administration, timed AI was performed and gonadotropin-releasing hormone (GnRH; 100 μg gonadorelin) was administered. Blood samples were collected and transrectal ovarian ultrasonography was performed at PG administration and, for heifers that failed to express estrus, at timed AI.

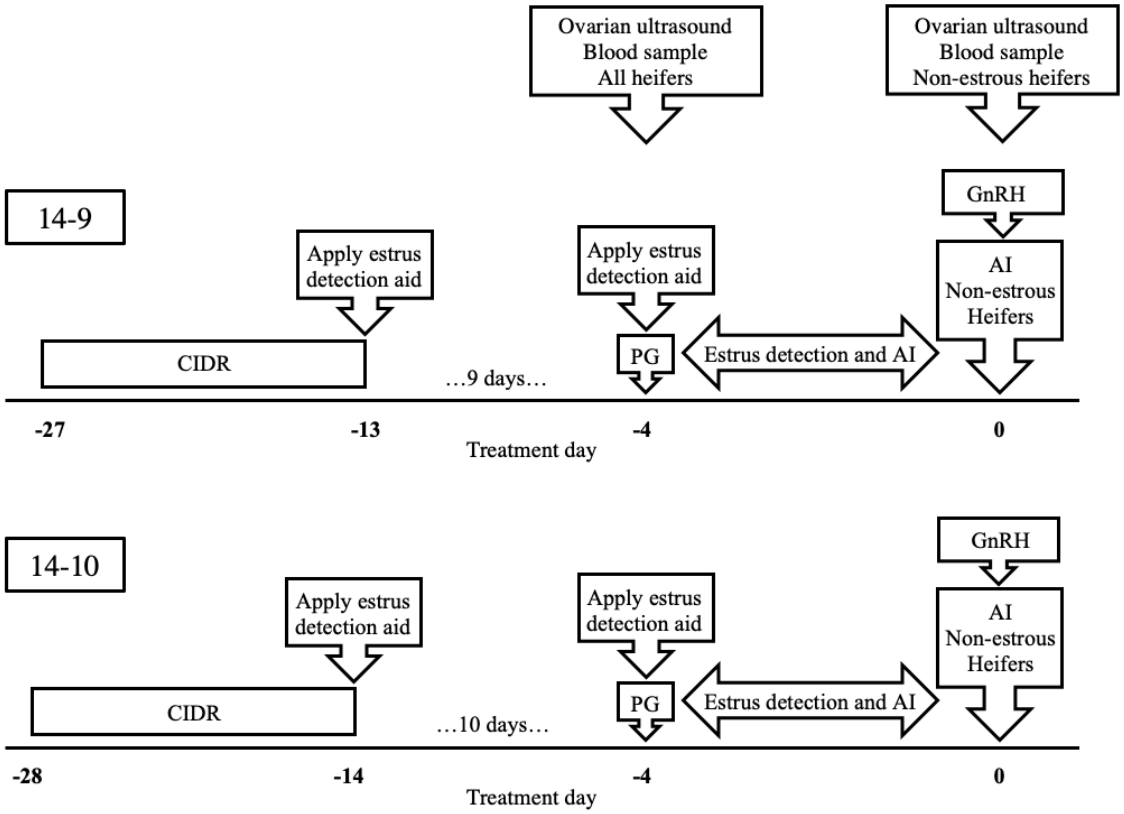

Figure 3.2. Treatment schedules for heifers in Experiment 2. Prostaglandin $F_{2\alpha}$ (PG) was administered to heifers either 9 d or 10 d following presynchronization with an intravaginal progesterone releasing insert (CIDR) for 14 d. Estrus detection aids were applied at the time of CIDR removal and at the time of PG administration. Heifers were observed for expression of estrus 3 times daily for 4 d following CIDR removal and PG administration. Heifers expressing estrus following PG administration received artificial insemination (AI) 12 to 18 h after observed estrus. For heifers that failed to express estrus by 96 h after PG administration, timed AI was performed and gonadotropin-releasing hormone (GnRH; 100 µg gonadorelin) was administered. Blood samples were collected and transrectal ovarian ultrasonography was performed at PG administration and, for heifers that failed to express estrus, at timed AI.

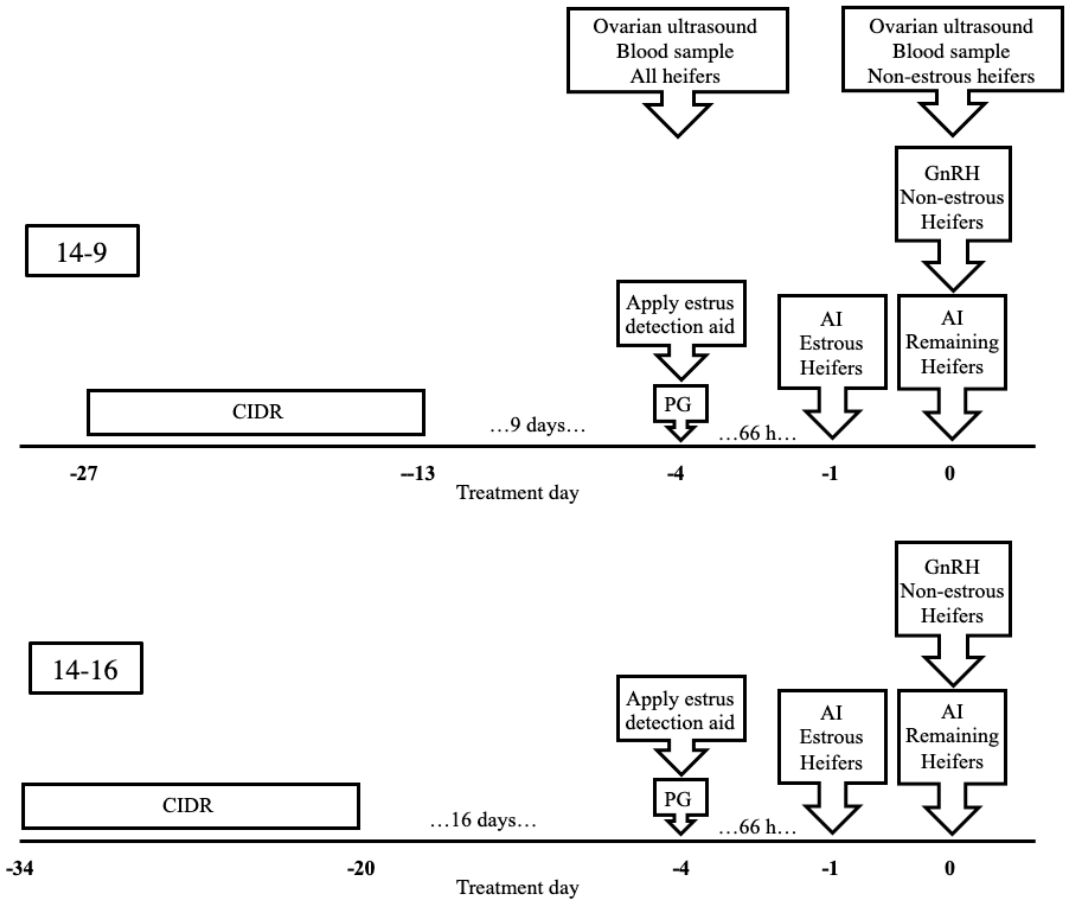

Figure 3.3. Treatment schedules for heifers in Experiment 3. Prostaglandin $F_{2\alpha}$ (PG) was administered to heifers either 9 d or 16 d following presynchronization with an intravaginal progesterone-releasing insert (CIDR) for 14 d. Estrus detection aids were applied at the time of PG administration. Estrotect patch activation was recorded at 66 h, and heifers that expressed estrus by 66 h were inseminated at that timepoint. Heifers that failed to express estrus by 66 h were inseminated at 90 h. For heifers that failed to express estrus by 90 h, gonadotropin-releasing hormone (GnRH; 100 µg gonadorelin) was administered concurrently with timed AI. Blood samples were collected and transrectal ovarian ultrasonography was performed at PG administration and, for heifers that failed to express estrus, at timed AI.

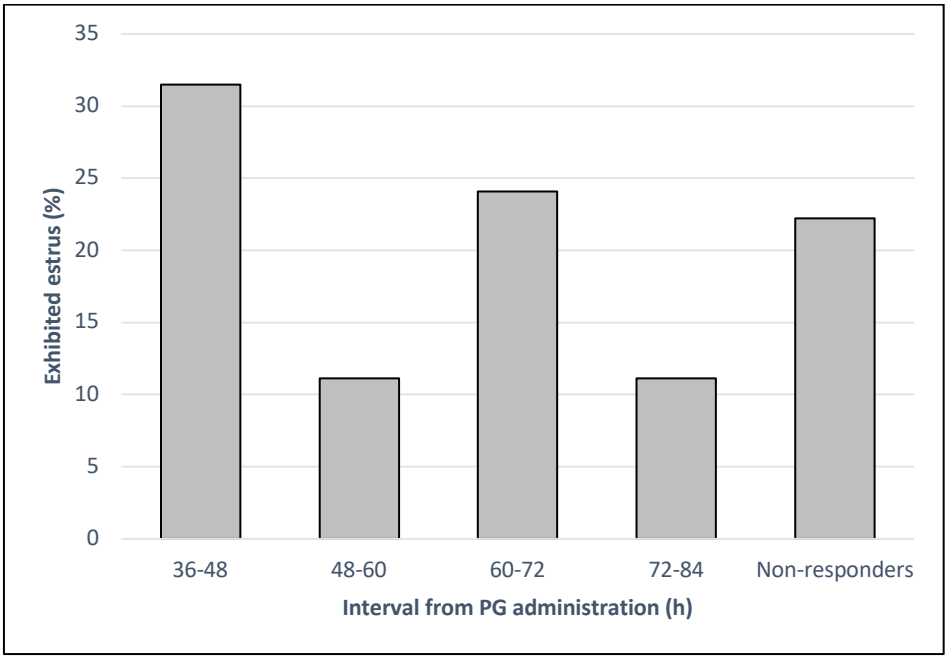

Figure 3.4. Proportion of heifers expressing estrus by hours after prostaglandin $F_{2\alpha}$ (PG) administration for heifers treated with a 14 d CIDR and PG administered 9 d following CIDR removal (Experiments 1 and 2).

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