EVALUATION OF THE 7 & 7 SYNCH PROTOCOL FOR CONTROL OF THE
ESTROUS CYCLE AMONG BEEF COWS PRIOR TO FIXED-TIME ARTIFICIAL
INSEMINATION WITH SEX-SORTED OR CONVENTIONAL SEMEN

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by
CARSON ANDERSEN
Dr. Jordan Thomas, Thesis Supervisor

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

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Presented by Carson Andersen

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance

Dr. Jordan M. Thomas

Dr. Sofia Ortega

Dr. Scott E. Pooch

Dr. John B. Hall

Dedication

This thesis is dedicated to my friends and family, and in particular, my grandmother, Susan Fitzpatrick-Andersen. Friends and family, thank you for always cheering me on no matter where I am in the world. I could not have accomplished what I have without all of your love and encouragement. To my grandmother, Susan (Meemom), thank you for always instilling in me that I was capable of achieving anything and reminding me that no dream was too big. Thank you for always teaching me to be kind. I am a better person today because of you and hope to carry on your legacy by sharing lots of laughter, wisdom, and positivity to the people around me throughout my career and lifetime. I know you are cheering me on from above.

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List of Abbreviations

AI	Artificial insemination
BCS	Body Condition Score
CIDR	Controlled internal drug release
CL	Corpus Luteum
d	Day(s)
FSH	Follicle stimulating hormone
FTAI	Fixed-time Artificial insemination
g	Gram(s)
GnRH	Gonadotropin-releasing hormone
h	Hour(s)
LH	Luteinizing hormone
mg	Milligram(s)
MGA	Melengesterol acetate
ml	Milliliter(s)

mm	Millimeter(s)
PAPP-A	Pregnancy-associated plasma
P4	Progesterone
PG	Prostaglandin F _{2α}
Pregnancy Rate to AI	P/AI
SAS	Statistical Analysis System
μg	Microgram(s)

Abstract

Experiment 1 was conducted to compare 7 & 7 Synch and the 7-day CO-Synch + controlled internal drug release (CIDR[®]) protocols prior to fixed-time artificial insemination (FTAI) of beef cows with conventional or sex-sorted semen. Cows (n = 1,538) were blocked based on age and days postpartum (DPP) and randomly assigned to protocol and semen type. Cows assigned to 7-day CO-Synch + CIDR (n = 769) received administration of gonadotropin-releasing hormone (GnRH) and insertion of an intravaginal progesterone-releasing insert (CIDR) on Day -10, and administration of prostaglandin F_{2α} (PG) coincident with CIDR removal on Day -3. Cows assigned to 7 & 7 Synch (n = 769) received PG and insertion of CIDR on Day -17, GnRH on Day -10, and PG coincident with CIDR removal on Day -3. Estrus detection aids were applied to all cows. Cows received FTAI 66 h after CIDR removal with conventional (20×10^6 cells per unit) or sex-sorted (4×10^6 cells per unit) semen. Estrus expression was affected by protocol ($P = 0.01$) and by protocol $\times$ DPP ($P = 0.0004$), with 7 & 7 Synch (82%; 629/769) resulting in a greater proportion of cows expressing estrus (82% [629/769] versus 64% [492/769]), especially among cows with greater DPP. Pregnancy rates to FTAI were reduced ($P < 0.0001$) when using sex-sorted semen but greater among cows treated with 7 & 7 Synch (conventional semen: 72% [280/389]; sex-sorted semen: 52% [199/380]) compared with 7-day CO-Synch + CIDR (conventional semen: 61% [233/383]; sex-sorted semen: 44% [171/386]).

Experiment 2 was designed to evaluate later timing of fixed-time artificial insemination (FTAI) with sex-sorted semen among postpartum beef cows following the 7 & 7 Synch protocol, with the hypothesis that later timing would result in increased

pregnancy rates (P/AI) among cows that expressed estrus prior to FTAI. Beef cows (n=414) were blocked based on age and days postpartum (DPP) and randomly assigned to receive FTAI at 66 or 72 h after administration of prostaglandin $F_{2\alpha}$ (PG). Estrus was synchronized using the 7 & 7 Synch protocol, which consists of administration of PG (500 μ g cloprostenol) and insertion of an intravaginal progesterone-releasing insert (CIDR; 1.38 g progesterone) on Day 0, gonadotropin-releasing hormone (GnRH; 100 μ g gonadorelin) on Day 7, and PG coincident with CIDR removal on Day 14. Estrus detection aids (EstrojectTM) were applied to all cows on Day 14, and activation status was recorded at fixed-time artificial insemination (FTAI) on Day 17. All cows that expressed estrus prior to FTAI received sex-sorted semen (4×10^6 cells per unit; SexedULTRA 4MTM). The proportion of cows expressing estrus prior to FTAI did not differ between treatments at this power of test (66 h: 71% [146/205]; 72 h: 76% [158/209]). Additionally, P/AI of estrous cows inseminated with sex-sorted semen did not differ between treatments (66 h: 45% [68/146]; 72 h: 40% [63/158]). In conclusion, later timing of FTAI following the 7 & 7 Synch protocol failed to improve P/AI of estrous cows inseminated with sex-sorted semen.

Chapter 1
Review of Literature
Introduction

Reproductive efficiency is one of the most important components of a productive and profitable commercial cow-calf operation. Reproductive productivity can be achieved with a variety of management strategies; however, progress can be accelerated with the assistance of reproductive biotechnologies. Applicable technologies such as estrus synchronization and fixed-timed artificial insemination (FTAI) yield numerous benefits to increase productivity and profitability among an operation. Use of estrus synchronization can increase the proportion of females that conceive early in the breeding season, reduce the number of bulls required for natural service, shorten the calving season, and increase calf crop uniformity. When FTAI is performed, all cows or heifers are inseminated at a predetermined time, eliminating the need for estrus detection. Although not every animal will express estrus prior to FTAI, use of FTAI often results in pregnancy rates comparable to those obtained using traditional estrus detection protocols (Pursley et al., 1997). Use of natural service sires identified as elite on the basis of genomic-enhanced EPDs can accelerate the rate of genetic progress within a herd; however, combining use of these modern genetic technologies with use of estrus synchronization and FTAI provides a platform to improve both reproductive efficiency and genetic merit of animals in the operation (White et al., 2015).

Despite many advantages associated with use of these technologies, the beef industry has been slow to adopt estrus synchronization and AI. A 2017 survey from the

National Animal Health and Monitoring System reported that only 11.6% of beef operations in the United States utilize AI (USDA National Animal Health Monitoring System, 2020). In order to be readily adopted in the beef industry, estrus synchronization and FTAI protocols must be cost-effective while providing acceptable pregnancy rates and ease of application. Many short-term estrus synchronization protocols used in the beef industry have been designed with simplicity in mind, whereas protocols used in the dairy industry often include presynchronization treatments to enhance control over follicular development and luteal status (Wiltbank and Pursley, 2014). Due to females beginning estrus synchronization protocols in varying stages of follicular development, there is often a proportion of animals that do not respond to GnRH. In order to overcome the challenge of synchronizing follicular waves, presynchronization treatments provide a method to address the variation of stage in follicular development at the start of estrus synchronization protocols. Presynchronization methods have been widely adopted and applied in the dairy industry; however, the beef industry has not readily adopted methods of presynchronization (Wiltbank and Pursley, 2014).

Sexed semen is a reproductive technology that provides producers the opportunity to add merit to the cow-calf operation. Sexed semen gives producers the opportunity to generate calves of the desired sex by inseminating an animal with semen that has been sexed to contain either X- or Y- chromosome-bearing sperm. Although this biotechnology has the potential to increase profitability among a herd, it comes with limitations. Due to damage from the sexing procedure and subsequent cryopreservation, sexed sperm cells are presumed to have a shorter lifespan in the female reproductive tract. Across literature, sexed semen has been observed to result in decreased pregnancy

rates to AI compared to conventional semen. However, cows that express estrus prior to the time of FTAI are more likely to become pregnant to AI compared to cows that fail to express estrus (Richardson et al., 2016). Cows that fail to express estrus prior to FTAI have particularly decreased pregnancy rates to AI when sexed semen is used, perhaps due to the reduced sperm lifespan and suboptimal alignment of timing of insemination with timing of ovulation (Thomas et al., 2014). Estrus synchronization protocols that result in large proportion of cows expressing estrus prior to FTAI may provide an opportunity for sexed semen to be more readily adopted.

This review provides an overview of the literature relating to endocrinology and physiology of the bovine estrous cycle, ovarian follicular dynamics, the development of estrus synchronization, presynchronization as a method to improve synchrony among follicular waves, factors that influence success of estrus synchronization, and use of sexed semen as a reproductive technology in the beef industry.

Endocrinology of the Bovine Estrous Cycle

Introduction

The hypothalamic-pituitary-gonadal axis is constructed of neural and endocrine tissues that intercommunicate to regulate key reproductive events during the estrous cycle. The axis consists of negative and positive feedback loops that control the frequency and amplitude of hormone secretion. Moreover, endocrinological mechanisms regulate the hypothalamic-hypophyseal portal system, ovary, and the reproductive tract. The actions and interactions of hormones are essential for normal estrous cycle function and successful pregnancy establishment and maintenance.

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) is 10 amino acid peptide hormone that is an essential regulator in the hypothalamic-pituitary-axis. In 1971, GnRH was first successfully isolated from porcine hypothalami and identified as the factor stimulating release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Matsuo et al., 1971). The cell bodies of neurons releasing GnRH originate in the preoptic area and medial basal hypothalamus and project to the secretory zone of the median eminence (Clarke and Cummins, 1982; Clarke and Pompolo, 2005; Yin and Gore, 2010). The median eminence contains GnRH neuroterminals that are in close proximity to the capillary bed of the hypothalamic-hypophyseal portal system where GnRH is secreted into circulation (Page and Dovey-Hartman, 1984). The anatomical arrangement of the hypothalamic-hypophyseal portal system allows for efficient control of the gonadotropes by hypothalamic hormones. Synthesis and secretion of GnRH is regulated by positive and negative feedback from ovarian hormones, progesterone and estradiol (Downey, 1980; Maeda et al., 2010; Christensen et al., 2012). There are two distinct release modes of GnRH, the pulse mode and the surge mode. The two modes are controlled by two different centers in the hypothalamus: the tonic center and the surge center. The tonic center in the hypothalamus controls the basal release of GnRH and is primarily regulated by the negative feedback of progesterone and estradiol. When the tonic center is the primary regulator of GnRH release, GnRH is released

episodically in pulses, stimulating release of LH and FSH (Maeda et al., 2010). The surge center is activated upon via positive feedback from estradiol during the last follicular wave when progesterone concentrations have declined following luteolysis. The proliferation of granulosa cells from the growing dominant follicle increases steroidogenesis and secretion of estradiol during the proestrus period. The increase in estradiol concentrations upregulates GnRH receptor mRNA in gonadotrophs and subsequently enhances hypothalamic GnRH secretion (Schoenemann et al., 1985). The increased frequency and amplitude of GnRH secretion drives an increase in LH amplitude and frequency and eventually induces a LH surge (Rahe et al., 1980; Moenter et al., 1992; Maeda et al., 2010). Previous experiments in ovariectomized ewes demonstrated that the pulsatile pattern of LH secretion directly reflected the secretion of GnRH from the hypothalamus, revealing the coupled relationship of the hypothalamic-pituitary-gonadal system with respect to LH and GnRH release (Clarke and Cummins, 1982). In contrast, FSH secretion is not as coupled to GnRH secretion and is regulated by estradiol and inhibin through feedback mechanisms (Clarke et al., 1986; Li et al., 1989).

Luteinizing Hormone and Follicle Stimulating Hormone

Closely related glycoproteins, LH and FSH are synthesized in the anterior pituitary. To stimulate release of LH and FSH from the anterior pituitary, GnRH binds to its seven-transmembrane domain receptor on the cell surface of the gonadotrope (Sealfon et al., 1997). The pulsatile manner of LH release is not consistent and fluctuates during different phases of the estrous cycle depending on the amount and type of ovarian steroidal feedback (Rahe et al., 1980). For the majority of the estrous cycle, tonic LH

secretion is regulated by a negative feedback system involving the hypothalamic-pituitary-gonadal axis, estradiol and progesterone. During the luteal phase, negative feedback from progesterone and estrogen suppresses GnRH secretion from the hypothalamus, which in turn decreases LH pulse frequency (Hinshelwood et al., 1986; Karsch et al., 1973). As circulating progesterone concentrations decline following luteolysis, negative feedback of progesterone on the hypothalamus is removed, and LH pulse frequency increases. During the follicular phase, positive feedback from estradiol produced from the dominant follicle increases LH pulse frequency in preparation for the LH surge. During the ascending portion of the LH surge, LH frequency and amplitude is increased (Rahe et al., 1980).

In addition to LH having a critical role in promoting adequate growth and development of a dominant follicle, it is also the essential luteotropic hormone in CL formation (Milvae et al., 1996). The episodic pulses of LH during the preovulatory phase are critical for the final maturation of the preovulatory follicle and the transition into luteal tissue (Hunter, 2019; Smith et al., 1994). Inhibiting release of LH 2 days before the preovulatory surge until day 7 of the estrous cycle results in an inhibitory effect on CL development (Quintal-Franco et al., 1999). Although LH is needed for CL formation, it is not needed to sustain progesterone synthesis after day 12 of the bovine estrous cycle due to the maturation and proliferation of large luteal cells and their ability to maintain progesterone synthesis without luteotropic support (Peters et al., 1994).

In addition to its role in regulating LH release, GnRH acts on the anterior pituitary to regulate FSH release. Due to additional inhibition of FSH secretion by inhibin, the patterns of GnRH release and FSH release are not as tightly coupled (Clarke et al., 1986;

Padmanabhan et al., 2003). To allow for independent regulatory mechanisms, LH and FSH are stored in different secretory granules in the anterior pituitary (Mcneilly et al., 2003). At the beginning of each follicular wave, FSH initiates recruitment of gonadotropin-sensitive follicles from the follicular pool (Noseir, 2003; Sunderland et al., 1994). Sufficient levels of FSH secretion are critical for ensuring growth and development of follicles to 3-5 mm in diameter (Baird and McNeilly, 1981). By binding to receptors and activating target genes in granulosa cells, FSH promotes proliferation and differentiation of granulosa cells (Garverick et al., 2002; Hunzicker-Dunn and Maizels, 2006; Sasson et al., 2003). Inhibin and estradiol are the main negative feedback regulators on FSH (Clarke et al., 1986). In contrast, the ovarian-sourced peptide hormone activin has stimulatory effects on FSH secretion (Knight and Glister, 2001). Activin released from follicular fluid stimulates FSH biosynthesis and secretion from the anterior pituitary. In addition, activin upregulates the expression of GnRH receptors in the anterior pituitary and acts upon GnRH neurons in the hypothalamus to stimulate release of FSH and LH (Miller et al., 2012). Acting as an inhibitory hormone, inhibin binds to activin receptors and blocks the stimulatory effects of activin. The primary source of inhibin comes from granulosa cells in the dominant follicle. The dominant follicle secretes inhibin which starves subordinate follicles of gonadotropins needed for growth (Campbell et al., 1991; Knight and Glister, 2001; Padmanabhan et al., 1984). This inhibitory mechanism generally ensures only one follicle reaches dominance and ovulates, as cattle tend to be a single-ovulatory species with relatively infrequent rates of twinning.

Estradiol

Estradiol is an ovarian steroid hormone synthesized and secreted by the follicle. Estradiol is essential in regulating physiological processes throughout the estrous cycle such as estrus and ovulation. The “two-cell, two-gonadotropin model” explains the interactions of LH and FSH with granulosa and theca interna cells in the follicle wall to synthesize estradiol through paracrine signaling mechanisms (Fortune and Quirk, 1988). Neither theca nor granulosa cells from preovulatory follicles are capable of synthesizing estradiol independently (Liu and Hsueh, 1986). Furthermore, granulosa cells lack the ability to synthesize androgens, whereas theca cells lack the enzyme aromatase to synthesize estradiol from testosterone (Henderson and Swanson, 1978). FSH receptors are expressed exclusively in granulosa cells whereas LH receptors are expressed in theca cells; therefore, both gonadotropins are needed for production of estradiol (Camp et al., 1991). However, during the time of selection, expression of LH receptors increases in granulosa cells to increase the capacity of steroidogenesis (Bao and Garverick, 1998). Estradiol biosynthesis occurs through the Δ^5 pathway in the theca and granulosa cells of the follicle. LH promotes androgen production by binding to its receptors within the theca cells to stimulate conversion of cholesterol to pregnenolone. Granulosa cells also provide an additional source of pregnenolone as a substrate for theca cells, increasing the follicle’s capacity for estrogen synthesis and secretion (Fortune and Quirk, 1988). Pregnenolone is converted to progesterone by HSD3 β , and progesterone is converted to androstenedione by CYP17A1. Androstenedione is converted to testosterone by 17 β HSD, and testosterone is transferred to the adjacent granulosa cells to be aromatized. There, FSH promotes expression of aromatase which converts testosterone into estradiol-

17 β (Simpson and Davis, 2001). Prior to ovulation, the preovulatory follicle produces high intrafollicular levels of estradiol as a result of increased proliferation of granulosa cells and their ability to aromatize androgens (Baird, 1983; Baird and McNeilly, 1981). For the majority of the estrous cycle, estradiol exerts negative feedback on the tonic center in the hypothalamus, decreasing frequency of LH pulse release (Chongthammakun and Terasawa, 1993; Christensen et al., 2012; Evans et al., 1994). During the follicular phase, positive feedback from estradiol acts upon the hypothalamus to increase frequency of LH release, subsequently increasing circulating estradiol concentrations.

Progesterone

Progesterone is a steroid hormone synthesized and secreted by the CL and is essential for maintenance of pregnancy in the bovine. Progesterone exerts negative feedback actions on the hypothalamus and suppresses GnRH release during the luteal phase (Ireland and Roche, 1982; Quintal-Franco et al., 1999). Progesterone synthesis occurs within each luteal cell. Progesterone receptors are present 48 hours before the LH surge and progesterone begins to be synthesized in the preovulatory follicle (Cassar et al., 2002; Dieleman and Blankenstein, 1985). Following the LH surge, progesterone receptor expression is rapidly upregulated in the granulosa layer of the preovulatory follicle (Cassar et al., 2002). The theca and granulosa cells from the follicle begin to differentiate into small and large luteal cells, which are responsible for production of progesterone (Alila and Hansel, 1984; Fields and Fields, 1996). The majority of the CL is comprised of theca-derived cells, which persist throughout the majority of the estrous cycle (Alila and Hansel, 1984). Furthermore, the theca-derived luteal cells provide the majority of the

steroidogenic production. The CL reaches its mature size and maximum ability to secrete progesterone during the midluteal phase (Hafs and Armstrong, 1968). LH is essential in providing luteotropic support for the CL by driving progesterone biosynthesis. Sources of cholesterol that can be used as substrate in progesterone synthesis include high-density lipoprotein, low-density lipoprotein, and stores of cholesterol esters hydrolyzed by cholesterol esterase (Gwynne and Strauss, 1982). StAR transports cholesterol from the outer to the inner mitochondrial membrane of the luteal cell in order for cholesterol side-chain cleavage to occur (Lin et al., 1995). The cholesterol side-chain cleavage enzyme, or P450_{scc}, cleaves the sidechain of cholesterol to form the intermediate pregnenolone. Pregnenolone is transported into the smooth endoplasmic reticulum and converted to progesterone by 3 β -HSD, or 3 β -Hydroxysteroid dehydrogenase.

Prostaglandin F_{2 α}

Prostaglandin F_{2 α} (PG) is an arachidonic acid metabolism product of the prostaglandin synthetase pathway (Basu and Kindahl, 1987). The bovine endometrium has an abundance of arachidonic acid present and metabolizes polyunsaturated fatty acid into PG (Auletta and Flint, 1988). There are several prostaglandins involved in a variety of physiological processes; however, PG specifically is a uterine-origin luteolytic substance responsible for inducing luteolysis at the end of the luteal phase in ruminants (McCracken et al., 1981). Early experiments in sheep demonstrated that PG reaches the ovary by a local vascular countercurrent mechanism via the uterine-ovarian vein (Bonnin et al., 1999; McCracken et al., 1972). Pulsatile release of PG is regulated via oxytocin, which binds to oxytocin receptors expressed in the uterine endometrium (McCracken et

al., 1984). Results from two separate experiments demonstrated the role of oxytocin in luteolysis. Immunizing ewes against oxytocin prevented luteolysis and prolonged the lifespan of the CL (Sheldrick et al., 1980), while exogenous oxytocin administration induced luteolysis in cows (Armstrong and Hansel, 1959). Oxytocin and PG form a positive feedback loop to stimulate release of one another, with oxytocin stimulating secretion of PG from the uterus and PG stimulating release of oxytocin from the CL (Burns et al., 1997; Flint and Sheldrick, 1982)

Physiology of the Bovine Estrous Cycle

Introduction

The bovine estrous cycle is a complex series of events, in which hypothalamic-pituitary ovarian interactions regulate physiological processes that occur between successive periods of estrus. During the estrous cycle, a cascade of physiological events occurs that regulates follicular and luteal dynamics and results in differing hormonal profiles. These events include follicular growth and atresia, estrus expression, sexual receptivity, maturation and ovulation of a fertilizable oocyte, and luteolysis. Estrous cycles begin at the onset of puberty in heifers. Puberty in heifers is defined as the first ovulatory estrus followed by a luteal phase of normal duration (Atkins et al., 2013). Prior to the start of puberty, estradiol has an inhibitory effect on gonadotropin secretion. Sensitivity to estradiol will begin to decrease, which results in increased gonadotropin secretion, follicular growth, and eventually an LH surge and ovulation (Kinder et al., 1995). The age at which heifers attain puberty varies based on breed, body condition, and other factors (Hall et al., 1995; Kinder et al., 1995).

Cattle are a polyestrous species, with each estrous cycle an average of 21 days in length; however, cycle length can range from 17-24 days depending on the length of the luteal phase and the number of follicular waves (Ginther et al., 2001; Hansel and Echternkamp, 1972). The estrous cycle consists of two phases and four stages, defined by structural and hormonal changes. The two phases are the follicular phase and the luteal phase. The follicular phase consists of the proestrus and estrus stages, in which gonadotropin release from the anterior pituitary stimulates growth of the dominant follicle, subsequent sexual receptivity and ovulation. The follicular phase is relatively short, lasting approximately 4-6 days. The luteal phase consists of the metestrus and diestrus stage, in which CL formation occurs with a corresponding increase in progesterone production. The end of the luteal phase is considered to be luteolysis. The luteal phase makes up the majority of the estrous cycle and is approximately 14-18 days.

Proestrus

Proestrus initiates the follicular phase and is characterized by a short period of follicular growth and development that occurs prior to estrus. The physiological and endocrinological events during proestrus are critical in regulating the final growth and maturation of a preovulatory follicle. Toward the end of the luteal lifespan, oxytocin binds to oxytocin receptors on the uterine endometrium and stimulates secretion of PG in a pulsatile manner. PG acts upon the CL to induce luteolysis (Knickerbocker et al., 1988; Okuda et al., 2002). The CL undergoes functional and structural luteolysis, which in turn causes the steroidogenesis of progesterone to decline. Removal of the negative feedback of progesterone on the hypothalamus permits an increase in the synthesis and secretion of

GnRH, in turn resulting in increased release of LH and FSH. During proestrus, growth and maturation of the dominant follicle is regulated by high-frequency, low-amplitude pulses of LH (Rahe et al., 1980). The dominant follicle is dependent upon LH support; for example, in one experiment that involved suppression of LH, the dominant follicle did not grow beyond 9 mm in diameter and did not reach full physiological maturity (Gong et al., 1996). As follicular growth continues, expression of LH receptor increases on both theca and granulosa cells of the growing dominant follicle, resulting in a significant increase in estradiol synthesis and secretion (Bao and Garverick, 1998). The resulting increase in circulating estradiol causes a greater frequency of pulsatile GnRH and LH release (Walters and Schallenberger, 1984). Primary and secondary signs of behavioral estrus begin as estradiol reaches its peak level.

Estrus

As the follicular phase progresses, the frequency of GnRH pulse release increases due to further withdrawal from progesterone negative feedback and positive feedback of estradiol. It has been speculated that sustained exposure to increased levels of estrogen causes the switch from negative to positive feedback (Karsch et al., 1973; Moenter et al., 2009, 1990). The positive feedback relationship of estradiol acts upon the hypothalamus to increase frequency of LH release, which further increases circulating estradiol concentrations and ultimately results in stimulation of the surge center, leading to the ovulatory LH surge (Moenter et al., 1990). During the ascending portion of the LH surge, the amplitude of LH significantly increases until a threshold has been reached and the surge occurs (Rahe et al., 1980). The LH surge initiates the ovulatory event, in which the

dominant follicle ruptures and releases an oocyte for potential fertilization. The onset of estrus is coincident with a peak concentration of estradiol in the blood stream (Allrich, 1994). Estrus behavior is initiated once a threshold of estradiol has been reached (Allrich, 1994). To induce estrus behavior and subsequent sexual receptivity, estradiol self-amplifies its own production by stimulating estradiol receptor expression in the brain. Specifically, estradiol acts upon the arcuate nucleus, ventromedial nucleus, and preoptic areas of the hypothalamus to regulate estrus behavior (Molenda-Figueira et al., 2006; Pleim et al., 1989). The primary sign of estrus is when a heifer or cow stands to be mounted. This is colloquially referred to as standing estrus. Secondary signs of estrus include chin-resting, bellowing, restlessness or pacing, and vaginal mucus. The duration of standing estrus lasts on average 12 hours but can vary among each individual animal (Wiltbank et al., 1967). Ovulation occurs approximately 24-32 hours after the onset of estrus (Brewster and Cole, 1941; Walker et al., 1996).

The LH surge also initiates a series of follicular morphological and steroidogenic changes, in which follicular residual cells undergo transformation into a luteal structure (Smith et al., 1994). This transformation involves the luteinization and differentiation of theca and granulosa cells into small and large luteal cells, as well as a change in enzyme production within these cells to shift steroidogenic pathways toward progesterone production (Alila and Hansel, 1984; Fields and Fields, 1996). The LH surge also activates a variety of transcription factors that regulate genes with critical roles in inflammatory responses and tissue remodeling (Duffy et al., 2019). As ovulation approaches, secretion of several proteolytic enzymes aids in degradation of the follicle wall (Reich et al., 1991).

Additionally, the follicle undergoes morphological, enzymatic, inflammatory changes in preparation for release of an oocyte (Espey, 1994, 1974).

Metestrus

Following estrus and the end of the follicular phase, metestrus begins. This marks the start of the luteal phase. The CL is a heterogeneous, transient organ composed of small and large luteal cells, vascular endothelial cells, fibrocytes and immune cells (O'Shea et al., 1989). During the luteal phase of the estrous cycle, the primary function of the CL is to secrete progesterone. The majority of the CL is comprised of theca-derived cells that are responsible for the majority of steroidogenic activity within the CL. During luteogenesis, the CL undergoes rapid angiogenesis and vascularization, which facilitates the exchange of large quantities of progesterone produced by the luteal cells to the circulatory system (Reynolds et al., 1992). During the early luteal phase when progesterone levels are low, LH is secreted at high-frequency and low-amplitude pulses (Rahe et al., 1980).

Diestrus

Diestrus makes up the majority of the luteal phase and is characterized by an increase in progesterone secretion from the CL until luteolysis. The CL reaches its mature size and peak level of progesterone secretion during diestrus (Hafs and Armstrong, 1968). For the majority of diestrus, estradiol and oxytocin receptor expression in the uterine endometrium is blocked by progesterone (Spencer et al., 1995). However, prolonged exposure of the uterus to progesterone for approximately 9-10 days downregulates

progesterone receptors in the endometrial luminal epithelial. Following the downregulation of progesterone receptor expression in the endometrium, estradiol receptor expression will increase, followed by oxytocin receptor expression (Spencer et al., 1995; Spencer and Bazer, 2004). During luteolysis, oxytocin binds to endometrial oxytocin receptors and stimulates episodic secretion of uterine PG. After release, PG is transported through a vascular countercurrent transfer to reach the ovary, initiating the luteolytic cascade previously described. The CL will begin to regress between days 16 and 19 of the estrous cycle once PG is secreted. Regression of the CL consists of functional luteolysis, a decline of steroidogenic production of progesterone, structural luteolysis, and the involution of the CL (McCracken et al., 1999).

Follicular Dynamics

Introduction

Folliculogenesis refers to the dynamic events of the formation, growth and maturation of an ovarian follicle. These events include the progression of a primordial follicle into a primary follicle, secondary follicle, pre-ovulatory follicle and ultimately into a follicle with full ovulatory capacity. During embryogenesis, primordial germ cells originate in the epithelium of the yolk sac and migrate to the genital ridge several weeks after fertilization (Smitz and Cortvrindt, 2002). During fetal life, the primordial germ cells within the ovary form into primordial follicles, which all house an individual oocyte arrested in the first prophase of meiosis (Fortune, 1994). The pool of primordial follicles remain in a resting period until recruited for continued follicular growth (Fortune et al., 2000). Primordial follicles are recruited into the growing follicle pool continuously

throughout the cycle but are not recruited into the follicular wave until acted upon by FSH. The fate of each follicle is determined by endocrine and paracrine factors throughout the follicular wave.

Each follicular wave consists of a recruited cohort of follicles undergoing selection that leads to the maturation of a single dominant follicle. Development of ultrasonographic imaging provided the opportunity to characterize follicular populations and growth throughout the estrous cycle in cattle. Follicular growth occurs in wave-like patterns and involves the synchronous development of a group of follicles until one becomes dominant and suppresses further growth of smaller subordinate follicles (Pierson and Ginther, 1987). The phases of follicular growth during each follicular wave include recruitment, selection, and dominance. There are typically 2-3 follicular waves throughout each estrous cycle (Sirois and Fortune, 1988).

Follicular waves do not occur exclusively in cycling animals. Anovulatory follicular waves that mirror follicular growth patterns in mature cows have been observed in prepubertal heifers. In prepubertal heifers, a dominant follicle never reaches ovulatory capacity and becomes atretic at the end of each wave (Evans et al., 1994). Anovulatory follicular waves have also been observed throughout the majority of the gestational period in cows (Adams, 1999).

Recruitment

Follicular recruitment refers to the event in which a cohort of follicles are synchronously recruited from the follicular pool in response to pituitary gonadotropic stimulation. FSH receptors are expressed shortly after formation of the granulosa cell

layer of primordial follicles (Bao and Garverick, 1998). During recruitment, FSH secreted from the anterior pituitary acts upon FSH receptors located within the granulosa cells of the follicles to initiate this synchronous recruitment (Xu et al., 1995). After ovulation, a transient rise in circulating FSH acts upon the pool of primordial follicles to initiate the first follicular wave (Lucy, 2007). Concentrations of FSH increase, followed by emergence of a new follicular wave. A second rise of FSH occurs when the first-wave dominant follicle undergoes atresia, and this transient rise in FSH initiates a second follicular wave. If a third wave is present, a third and final rise of FSH will initiate the last wave following atresia of the dominant follicle from the second wave. The increase in circulating FSH initiates expression of P450_{scc} and P450_{arom} mRNAs in the granulosa cells of the recruited follicles (Bao et al., 1997). These enzymes are necessary for steroidogenesis and promote continued follicular growth (Xu et al., 1995).

Selection

Endocrine and cellular mechanisms regulate selection, the event in which a follicle is selected for eventual dominance from among the cohort of synchronously growing follicles. Selection is defined as a deviation in growth rate between the dominant follicle and the largest subordinate follicle. Selection is associated with decreased FSH release by the pituitary, increased expression of LH receptor within granulosa cells, and increased estradiol production by the dominant follicle (Sartori et al., 2001). Subordinate follicles not selected for continued growth ultimately become atretic. An increase in expression of P450_{scc} and P450_{arom} mRNA occurs in granulosa cells of the selected follicle to drive estradiol synthesis and secretion (Bao et al., 1997).

Two models have been proposed for how deviation of the largest follicle occurs: the Missouri model and the Cornell model. The Missouri model proposed that the first follicle to acquire LH receptor expression acquires follicular dominance by producing inhibitory hormones, inhibin and estradiol, which starve subordinate follicles of gonadotropins (Lucy, 2007; Xu et al., 1995). In the Missouri model, LH is the driving force of selection, and FSH is nonessential in the selection process due to selection occurring in a low FSH environment. The Cornell model proposed that, within any one follicular wave, there is one follicle that has a slight developmental advantage over subordinate follicles at the beginning of the follicular wave (Fortune et al., 2004). In the Cornell model, a follicle acquires pregnancy-associated plasma protein-A (PAPP-A) as soon as dominance is achieved. Acquisition of PAPP-A leads to a decrease in IGFBP-4 and -5 and increase in free IGF-1. IGF-1 and FSH act synergistically to increase estradiol production. The resultant increase in estradiol inhibits FSH and starves the FSH-dependent subordinate follicles. The process of selection is likely a mixture of both the Missouri and Cornell models and includes mechanisms involving LH, FSH and IGF-1 (Beg and Ginther, 2006).

Dominance

During dominance, the selected follicle controls the fate of subordinate follicles and regulates hormonal changes in the hypothalamus, pituitary and ovaries. The LH-dependent dominant follicle continues growing while subordinate follicles become atretic. The dominant follicle synthesizes and secretes estradiol and inhibin, which starves subordinate follicles of gonadotropic support (Clarke et al., 1986; Sunderland et

al., 1994; Taya et al., 1996). A follicle reaches dominance within each follicular wave of the estrous cycle; however, the fate of the dominant follicle varies among each wave. A follicle that is not exposed to the LH surge will undergo atresia. The first-wave dominant follicle of a two-wave cycle and the first-wave and second-wave dominant follicle of a three-wave cycle do not experience an ovulatory LH surge and therefore undergo atresia. During the last follicular wave, luteolysis occurs and circulating progesterone concentrations decrease, allowing the frequency of LH pulse release to increase (Rahe et al., 1980). The increase in LH frequency will drive final maturation processes of the dominant follicle. In order to ovulate in response to the LH surge, a follicle must acquire LH receptor expression on granulosa cells in addition to theca cells (Bao et al., 1997). The increase in LH frequency causes LH receptor expression to increase rapidly in granulosa cells to further increase LH secretion and subsequent estradiol production (Bao and Garverick, 1998).

Estrus Synchronization Products

Introduction

Immense research efforts have gone into evaluating and understanding the physiology of the estrous cycle, both for basic and translational purposes. Advancements in understanding the follicular and luteal phases of the estrous cycle provided the opportunity to develop estrus synchronization. Estrus synchronization yields numerous benefits for beef cattle operations, such as shortening the breeding season and subsequent calving season, thereby increasing the uniformity of the calf crop (Patterson et al., 2011). Estrus synchronization is also commonly employed to facilitate use of other reproductive

technologies such as AI. Over the course of the last half century, different hormonal compounds have been used to mimic and manipulate the natural events that occur during the estrous cycle in order to synchronize estrus. Some of the earliest research on exogenous control of the estrous cycle in ruminants involved the discovery that injecting pituitary-extract gonadotropins resulted in ovulation of an ovarian follicle (Casida et al., 1944). Injecting cattle with progesterone solubilized in corn oil was observed to inhibit CL formation (Ulberg et al., 1951). Progression in estrus synchronization research led to the use of various hormonal compounds in combination with one another to more effectively control the estrous cycle.

Progestins

Early research evaluating hormonal control of the estrous cycle began with utilizing progestins to artificially extend the luteal phase. Progestin synchronization products imitate the actions of progesterone produced by the CL during the luteal phase of the estrous cycle. The CL will still spontaneously regress following the end of its luteal lifespan even in the presence of exogenous progesterone; however, treatment with a progestin at a sufficient level can suppress ovulation and behavioral estrus in the absence of a CL. Administering progesterone at an effective dose inhibits estrus and ovulation by acting on the hypothalamic-pituitary axis to suppress LH secretion (Nellor and Cole, 1956; Trimberger and Hansel, 1955; Ulberg et al., 1951).

When progestins are administered when a CL is present, LH pulse frequency remains low and is similar to the pulsatile pattern during the luteal phase (Kojima et al., 1995). Progestin treatment at fairly low doses has little effect on follicular development.

When treating with a subluteal concentration of progesterone sufficient to inhibit ovulation during the follicular phase, however, the frequency of LH pulse release may still be sufficiently elevated as to allow the dominant follicle to be maintained for a longer period of time before becoming atretic (Imakawa et al., 1986; Mihm et al., 1994; Sirois and Fortune, 1990). This phenomenon is referred to as the formation of a persistent follicle, or a follicle that persists in dominance for a longer period of time and often reaches a larger size than would be typical in a normal estrous cycle (Kinder et al., 1996). The extended exposure to increased frequency LH pulse release affects the gap junctions between the mural granulosa and cumulus cells, causing the oocyte to prematurely resume meiosis prior to the LH surge (Revah and Butler, 1996). Ovulation of an oocyte that has undergone prolonged follicular development results in reduced fertility (Mihm et al., 1994).

In addition to preventing behavioral estrus, use of progestins in estrus synchronization protocols have been found to benefit prepubertal heifers and anestrous cows by inducing cyclicity among a proportion of non-cycling females (Lucy et al., 2001). During anestrus, the GnRH “pulse generator” is inhibited due to an increased sensitivity of the hypothalamus to negative feedback of estradiol (Acosta et al., 1983). Postpartum cows often undergo a short luteal phase following the first ovulation after parturition (Perry et al., 1991; Werth et al., 1996). The progesterone exposure from the short luteal phase acts upon the hypothalamic-pituitary axis to initiate resumption of normal estrous cycles. Progesterone reduces the concentration of estradiol receptors in the hypothalamus, decreasing the sensitivity to the negative feedback of estrogen (Day and Anderson, 1998). Treatment of postpartum cows with progesterone has been

observed to stimulate expression of LH receptor genes in the dominant follicle (Garcia-Winder et al., 1987; Rhodes et al., 2001). Based on findings in prepubertal heifers, exposure to progesterone reduces the concentration of estradiol receptors in the hypothalamus, lessening the negative feedback on GnRH release (Day and Anderson, 1998). The increase in expression of LH receptor leads to an increase in synthesis and secretion of estradiol from the follicle. This ultimately establishes the positive feedback relationship of estradiol and LH within the hypothalamic-pituitary-gonadal axis, which is critical for attainment of puberty. Similarly, for postpartum anestrous cows, exposure to progesterone for a short period of time can reestablish normal estrous cyclicity by mimicking the short luteal phase after the first ovulation (Perry et al., 1991). The efficacy of short-term progestin exposure to initiate resumption of estrous cycles varies among postpartum cows based on the depth of anestrus, which is influenced by several factors. Cows that are in poor body condition score and are deficient in essential nutrients often have a longer period of postpartum anestrus compared to cows in proper body condition score (Short et al., 1990). Additionally, presence of a suckling calf will prolong the period of anestrus compared to a cow who has had a calf removed (Acosta et al., 1983).

Currently, only one commercially available progestin product is FDA-approved for use in estrus synchronization among both multiparous cows and nulliparous heifers (Food and Drug Administration, and Center for Veterinary Medicine, 2019). The EAZI-Breed controlled internal drug release (CIDR) is a progesterone-impregnated intravaginal device containing 1.38 g of progesterone. This product is a T-shaped silicone insert, which is inserted directly into the vagina for immediate release of progesterone. This product results in a rapid elevation of progesterone concentration and maintains a

concentration above 2 ng/ml until removal (Rathbone and Burke, 2013). EAZI-Breed CIDR devices were developed and formulated to be used in a 12-14 day treatment period but are also commonly used in 5-7 day protocols.

Melengesterol acetate (MGA) is an orally active progestin feed additive that is FDA-approved for suppression of behavioral estrus in heifers. There are two MGA products currently marketed by Zoetis and Elanco. MGA was originally used in heifers as a feed additive for growth promoting effects and its ability to suppress estrus (Bloss et al., 1966). It is also used in some protocols for estrus synchronization, such as the commonly used MGA-PG protocol, which involves feeding MGA for 14 consecutive days (Zimbelman and Smith, 1966). Estrus expression among heifers will begin 2 to 3 days after MGA withdrawal; however, estrus expression will occur over 6 to 7 days (Zimbelman and Smith, 1966). It is recommended that heifers consume MGA at a rate of 0.5 mg per day in order to maintain levels of progesterone capable of suppressing behavioral estrus. Although previous studies have observed MGA being effective for use in estrus synchronization among cows, it is solely FDA-approved for use in heifers.

Prostaglandins

The next advancement in estrus synchronization involved evaluating the effect of prostaglandins on the estrous cycle. In 1972, PG was discovered to be a luteolytic factor in cattle when injected intrauterine, intramuscular or subcutaneously (Lauderdale, 1972). Specifically, PG was found to induce luteolysis when administered between days 6 and 16 of the estrous cycle, but not when administered before day 5. Therefore, stage of the luteal phase is a major determinant of the efficacy of PG to induce luteolysis. A large-

scale trial identified the effective dose to induce luteolysis was 25 mg PG injected intramuscularly. Early estrus synchronization programs involved injecting PG twice at 10-12 day intervals due to the CL not responding to PG on days 0-5 of the estrous cycle. Because PG is not effective among non-cycling animals, PG is often used in combination with other hormones.

Prostaglandin products mimic PG that is naturally released from the uterus. These products are either produced from biological prostaglandin or a prostaglandin analogue (Lauderdale and Enterprises, 2015). Lutalyse[®] was the first prostaglandin estrus synchronization product that was FDA approved. Lutalyse[®] was approved by the FDA first in 1979, for use in treatment schedule involving two administrations of PG at a 11-14 day interval, and later in 1981 for use as a single administration of PG. Presently, there are five FDA-approved prostaglandin products available for use in estrus synchronization, with four of the five FDA-approved for use in conjunction with another product for estrus synchronization (Food and Drug Administration, and Center for Veterinary Medicine, 2019). The four PG products commercially available for use in conjunction with another synchronization product include Lutalyse[®] or Lutalyse[®] HighCon (dinoprost tromethamine), Estrumate[®] (cloprostenol), and estoPLAN[®] (cloprostenol sodium). ProstaMate[™] (dinoprost tromethamine) is a commercially available PG product that is not approved for use in conjunction with other synchronization products.

Gonadotropin-Releasing Hormone

Intramuscular and intracarotid injections of GnRH were observed to stimulate release of LH and FSH in cattle (Kaltenbach et al., 1974). Development of ultrasonography made it possible to observe follicular response to GnRH administration throughout the estrous cycle. Administering exogenous GnRH was discovered to induce ovulation when a dominant follicle was present on the ovary at the time of administration (Schmitt et al., 1996). Since a dominant follicle is not present on all days of the estrous cycle, the efficacy of GnRH administration for inducing ovulation is therefore highly dependent on day of cycle and the corresponding stage of follicular development. Administration of GnRH at a random stage of the estrous cycle has been observed to induce ovulation with variable success rates, with GnRH-induced ovulation occurring among approximately 65% of cows on average (Geary et al., 2000). Cows that fail to ovulate in response to administration of GnRH are in a stage of follicular development that lacks a physiologically mature follicle capable of ovulating in response to the LH surge induced via GnRH administration.

Estrus synchronization products in the gonadorelin class mimic GnRH released from the hypothalamus and are used to induce ovulation. The first GnRH product approved by the FDA was Cystorelin[®] (gonadorelin diacetate tetrahydrate). Cystorelin[®] was first marketed for the treatment of ovarian follicular cysts in dairy cattle and is now commonly used in estrus synchronization protocols to induce ovulation and initiate a new follicular wave. Presently, there are five FDA-approved gonadorelin products available to treat ovarian cysts, and four out of the five are also FDA-approved for use in estrus synchronization in conjunction with prostaglandin products (Food and Drug

Administration, and Center for Veterinary Medicine, 2019). The four GnRH products approved for use in estrus synchronization include Fertagyl[®] (gonadorelin acetate), Cystorelin[®] (gonadorelin), GONAbreed[®] (gonadorelin acetate), and Factrel[®] (gonadorelin hydrochloride). OvaCyst[®] is another FDA-approved GnRH product but is only label-approved for treatment of cystic follicles.

Development of Estrus Synchronization Protocols

Introduction

The ideal estrus synchronization program minimizes the number of animal handlings, provides ease of application and optimizes cost. However, many factors influence the success of a FTAI program from a physiological basis, including the degree to which the synchronization protocol effectively controls follicular wave development, timing of estrus expression and ovulation, and steroidogenic capacity of follicles and corpora lutea. Effective control of both the follicular and luteal phase is critical in order to synchronize the timing of estrus expression among a group of animals. Technologies such as ultrasonography were not available during the early phases of estrus synchronization research; therefore, methods to manipulate the estrous cycle were developed in phases. During each developmental phase, the efficacy of individual hormones or combinations of hormones to control the estrous cycle were assessed. Additionally, research was performed to evaluate the proportion of cows expressing estrus prior to FTAI, timing intervals to the onset of estrus after synchronization, and pregnancy rates of estrus synchronization protocols.

Progesterone Phase

During the early phases of estrus synchronization development, technology for ultrasonography had not yet been developed, and the dynamics of follicular waves were not clearly understood. The estrous cycle was initially thought to be controlled by the CL due to the observation that administering exogenous progesterone inhibited ovulation (Nellor and Cole, 1956; Ulberg et al., 1951). Progesterone was hypothesized to block the actions of gonadotropins LH and FSH and therefore affect follicular growth. With this understanding, the first efforts to synchronize estrus were based solely on manipulation of the luteal phase. The first phase of estrus synchronization development focused on administering exogenous progesterone to create an artificial luteal phase that would extend beyond the normal lifespan of the CL. During this phase, experiments assessed the amount of progesterone needed to effectively inhibit estrus (Trimberger and Hansel, 1955; Ulberg et al., 1951). These trials also evaluated the time intervals from progesterone removal to timing of estrus expression. The transition into the next phase of estrus synchronization development began after the discovery of PG and its role as a luteolytic factor.

Progesterone-PG Phase

Progesterone-PG synchronization regimens resulted in improved rates of synchronized estrus and acceptable pregnancy rates to AI among heifers and cows compared to PG alone or no hormonal treatment (Lucy et al., 2001; Smith et al., 1984). Additionally, one-shot PG protocols were also developed as a method to synchronize estrus (Lauderdale, 2009); however, PG is not effective in anestrus cows or prepubertal

heifers due to the fact that only cycling animals have CL able to undergo luteolysis in response to PG. Estrus synchronization with PG alone is ineffective in producing successful pregnancy rates to FTAI due to the wide variation in the resulting timing of estrus expression, stemming from differences among females with respect to stage of follicular development at the time of PG administration (Berardinelli and Adair, 1989). Protocols involving single or repeated administration of PG are successful if females receive AI on the basis of detected estrus; however, data has shown that animals were detected in estrus over a 10-day period, which increases labor requirements and decreases convenience (Macmillan and Henderson, 1984). The variation in the interval from prostaglandin to estrus is dependent upon the capacity of the CL to undergo luteolysis in response to PG and maturity of the largest follicle present at the time of PG administration (Macmillan, 1978; Macmillan and Henderson, 1984). Therefore, further efforts were made to develop protocols that would result in greater synchrony of the timing of estrus expression among a group of animals.

Prior to ultrasound, slaughter studies were often the only method available to observe follicular dynamics by studying morphological characteristics of the ovary once it was removed. The development of ultrasonography led to an in-depth understanding of follicular dynamics including recruitment, selection and dominance as well as CL function. This provided an understanding of why progesterone and PG protocols did not result in a greater synchrony of estrus since progesterone and PG-based protocols only mimicked or controlled luteal function and not follicular dynamics.

GnRH Phase

Experiments using ultrasonography led to the understanding that administering exogenous GnRH induces ovulation of a dominant follicle and subsequent luteinization (Bao and Garverick, 1998; Sartori et al., 2001). Through ultrasonography and experiments undertaken to understand follicular and luteal dynamics, it is now understood that the structures present on the ovary on a particular day of the estrous cycle influence the efficacy of exogenous hormones. For example, exogenous GnRH is observed to only be effective in inducing ovulation when a follicle of sufficient physiological maturity is present on the ovary. While it is the physiological maturity not size that determines this responsiveness, this corresponds to a follicle size of approximately 10 mm or greater in diameter (Perry et al., 2007; Sartori et al., 2001). A follicle that is physiologically mature has acquired LH receptors on both granulosa and theca cells and can ovulate in response to a LH surge (Bao and Garverick, 1998). With a better understanding of both follicular and luteal function, the next phase in estrus synchronization development involved GnRH-PG protocols to manipulate follicular dynamics and luteal function in order to increase the synchrony of the timing of estrus expression among a group of animals.

GnRH-PG Phase

In 1995, the Ovsynch protocol was developed and evaluated among lactating dairy cows and resulted in pregnancy rates to AI similar to results generated from using traditional heat detection protocols (Pursley et al., 1997). The treatment schedule includes a combination of exogenous hormones to manipulate both the follicular and luteal phase

to increase the precision of synchronizing the timing of estrus expression among a group of cows. The initial GnRH administration on Day 0 of the protocol induces ovulation and subsequent CL formation among a large proportion of cows that have a LH-responsive follicle. When PG is administered on Day 7, corpora lutea that resulted from the initial GnRH administration will have had sufficient time to gain the capacity to undergo luteolysis in response to PG. The time period between the two GnRH administrations is sufficient time to permit a new follicle to be recruited and develop to a preovulatory size, so as to be responsive to the final GnRH administration given 48 hours after PG. Ovsynch is proven to not be an effective method in synchronizing estrus among heifers, perhaps due to a reduced ovulatory response to GnRH (Pursley et al., 1997). The Ovsynch protocol continues to be a widely used estrus synchronization protocol among lactating dairy cows (Rabiee et al., 2005).

Recent efforts to improve estrus synchronization protocols have largely consisted of additional steps or small modifications to the treatment schedule of existing protocols based off hypothesized improvements. Variations of the Ovsynch protocol, Select Synch and CO-Synch, were developed and evaluated among postpartum beef cows. These protocols are variations of the same estrus synchronization protocol but each include variations in the treatment schedule. The treatment schedule of Select Synch involves administering GnRH on Day 0 and PG on Day 7. Due to the small percentage of cows that exhibit estrus prior to the PG administration, it is recommended that heat detection begins 24-30 hours prior to PG (Geary et al., 2000). The majority of cows have been shown to express estrus by 120 hours after PG. Although estrus detection requires more labor than a FTAI program, performing AI on the basis of detected estrus provides the

opportunity to optimize the timing of insemination relative to ovulation for each individual animal. Select Synch is effective among anestrous and cyclic cows and produces successful pregnancy rates to AI. The CO-Synch protocol differs from Ovsynch with respect to one alteration to the protocol schedule: when using the CO-Synch protocol, cows are inseminated at the time of the final GnRH administration. As a result, CO-Synch requires three total animal handlings instead of four yet resulted in pregnancy rates to AI comparable to Ovsynch (Geary et al., 2001).

GnRH-Progesterone-PG Phase

One of the biggest challenges in estrus synchronization is the proportion of animals that are not cycling at the start of the protocol. Incorporating a progesterone product in an estrus synchronization program has been shown to induce cyclicity among a proportion of non-cycling animals whether that be anestrous cows or prepubertal heifers. The next phase of developing estrus synchronization protocols included combinations of a progestin and PG. The efficacy of synchronizing estrus with a CIDR and PG was evaluated in a large field trial (Lucy et al., 2001). This trial was evaluated in 851 primiparous and multiparous postpartum beef cows across multiple states. Animals were randomly assigned to one of three treatments and estrus response and pregnancy rates were evaluated. The three treatments included 1) untreated control 2) a single injection of PG; and 3) 7-Day CIDR + PG. Cows that received the 7-Day CIDR + PG had the CIDR inserted for 7 days and received administration PG on day 6 of the CIDR insertion. Treatment with the 7-Day CIDR + PG was found to be effective in synchronizing estrus among cyclic and acyclic animals and increased estrus response and

pregnancy rates compared to cows that were not synchronized or received a single injection of PG (Lucy et al., 2001). To further evaluate the effectiveness of progesterone in an estrus synchronization program, a large trial was conducted among approximately 2,600 postpartum beef cows to evaluate the success of the CO-Synch FTAI protocol with or without a CIDR. Results showed that synchronizing estrus with the CO-Synch + CIDR increased pregnancy rates to timed AI. This protocol is now known as the 7-day CO-Synch + CIDR. Additional research was performed evaluating the timing of FTAI following the 7-day CO-Synch + CIDR, with results pointing to 66 hours as the optimal time to perform FTAI after PG administration (Busch et al., 2008). Investigators have also observed acceptable pregnancy rates to AI when performing FTAI at 48 and 60 hours after PG administration (Lamb et al., 2001; Larson et al., 2006). Presently, 60-66 hours is recommended as the optimal time to perform FTAI after PG administration.

Factors Affecting Success in Fixed-Time AI Programs

Compared to the dairy industry, the beef industry has been slow to adopt estrus synchronization and artificial insemination technologies. In order to be readily adopted by producers, estrus synchronization protocols need to be inexpensive, convenient, and result in acceptable pregnancy rates. Due to the convenience and acceptable success rates, many operations use FTAI protocols. Optimized pregnancy rates to FTAI are achieved when using a protocol that affords greater control over the estrous cycle, as this minimizes the variation among cows with respect to timing of estrus expression and ultimately ovulation. Cows may vary in days postpartum, body condition, and parity, which are all factors that can impact the ability of a protocol to synchronize the timing of

estrus expression. A FTAI protocol that addresses the variation in stage of cycle among cows at the start of the protocol may increase synchrony in timing of estrus expression and potentially result in increased pregnancy rates to FTAI.

As stated previously, the proportion of cows that have not resumed having normal estrous cycles at the beginning of the estrus synchronization protocol often affect the success of the program. Anestrus is characterized by a period of ovarian quiescence before the first estrous cycle after calving. During anestrus, GnRH release is suppressed due to an increased sensitivity of the hypothalamus to negative feedback of estradiol (Acosta et al., 1983). Although a short period of anestrus is common after calving, prolonged periods of anestrus can negatively impact reproductive efficiency. Several weeks are needed for a cow to undergo uterine involution, for the hypothalamic-pituitary-ovarian axis to recover from the gestational period, and for normal follicular growth to resume. During anestrus, the functional competence of the hypothalamus and pituitary is reduced for a period after calving (Short et al., 1990). During this time, LH is secreted infrequently and in lesser amounts as a result of the negative feedback of estradiol (Short et al., 1990, 1972). Follicular dynamics mirror that of the luteal phase, in which follicular growth occurs in wave-like patterns but the follicles become atretic and never ovulate.

There are many factors that work together or individually to control the length of anestrus. Presence and suckling of a calf, days postpartum, poor body condition, nutritional deficiencies, and parity are all factors that can contribute to prolonged anestrus. Cows that are nursing a calf have a longer mean interval from calving to resumption of normal estrous cycles than cows that have a calf removed (Short et al., 1972). Suckling of a calf reduces the frequency and amplitude of LH pulsatility by

increasing the sensitivity of the hypothalamus to the negative feedback of estradiol (Acosta et al., 1983). There is a positive correlation between the number of days postpartum and the likelihood of resuming normal estrous cycles; as days postpartum increases, cows are more likely to have resumed normal estrous cycles compared with cows with shorter days postpartum (Stevenson et al., 2002). Nutrients for maintenance requirements, basic energy reserves, activity, and growth are prioritized over reproduction (Short et al., 1990). Only when a cow has sufficient energy and protein intake will she be able to resume estrous cycles. In regard to the effect of parity, primiparous cows have increased nutrient demand due to their additional growth requirements, resulting in prioritization of nutrient use for growth rather than reproduction. The low physiological prioritization of reproductive processes increases the length of anestrus and generally results in a reduced proportion of primiparous cows that have resumed normal estrous cyclicity at the start of the breeding season in comparison with multiparous cows (Randel, 1990; Short et al., 1990). Multiparous cows do not have the same growth requirements and therefore have more nutrients available for initiation of estrous cycles.

Although FTAI provides convenience by enabling producers to service all cows at one predetermined time without the labor and time associated with estrus detection, not every cow will express estrus prior to the time of FTAI. Additionally, not all cows that express estrus will be inseminated at the optimal time point relative to ovulation.

Previous investigations have observed a greater pregnancy rate to FTAI among cows that express estrus near the time of FTAI compared to cows that fail to express estrus (Galvão et al., 2004; Pereira et al., 2016; Pohler et al., 2016). Animals that express estrus within

24 hours of FTAI have increased diameter dominant follicles and increased serum estradiol concentrations, corresponding to an increase in pregnancy success (Perry et al., 2007). Estradiol produced by the dominant follicle is critical for regulation of physiological processes that aid in establishment and maintenance of pregnancy in cattle (Jinks et al., 2013). Estradiol causes expression of estrus and plays a critical role in sperm transport within the female reproductive tract, preparation of follicular cells for luteinization, and expression of estradiol and progesterone receptors in the endometrium (Hawk, 1987; Ing and Belen Tornesi, 1997; Jinks et al., 2013). With this understanding, maximizing the number of animals that express estrus or at least present with a physiologically mature preovulatory follicle at the time of FTAI is critical (Atkins et al., 2010).

Ineffective control of follicular and luteal dynamics early in the protocol can result in poor pregnancy rates when FTAI is performed. Induced ovulation of a physiologically immature follicle at the time of FTAI can lead to reduced fertility (Pohler et al., 2012). An estrus synchronization protocol needs to include exogenous hormones that manipulate both luteal and follicular dynamics in order to increase the synchrony of the timing of estrus expression. Many short-term estrus synchronization protocols rely on exogenous administration of GnRH to induce ovulation of a dominant follicle and initiate recruitment of a new follicular wave (Bo et al., 1995). As previously discussed, the efficacy of GnRH to reset a follicular wave is dependent on the stage of the cycle. A follicle has to be of sufficient physiological maturity in order to respond to the GnRH administration. Cows that fail to ovulate in response to administration of exogenous GnRH are in a stage of follicular development that lacks a dominant follicle capable of

responding. The resulting lack of uniformity among cows in subsequent stage of the estrous cycle constitutes a potential limitation to pregnancy rates achieved among cows when performing FTAI, as resultant variation in follicular maturity, estrus expression, and timing of ovulation at the end of the protocol can contribute to suboptimal fertility when FTAI is performed. For example, the 7-day CO-Synch + CIDR protocol is a widely used protocol for estrus synchronization in the beef industry. However, the protocol begins with an initial administration of GnRH; therefore, only cows that happen to have an LH-responsive follicle at the initiation of the protocol will ovulate in response to GnRH. In previous experiments, approximately 35% of the cows were observed to not ovulate in response to this administration of GnRH (Geary et al., 2000; Vasconcelos et al., 1999). This variation among cows in response to GnRH effectively creates two subpopulations of cows that varying follicular development at the end of the protocol, resulting in potentially reduced proportions of cows expressing estrus and/or greater variance in timing of estrus onset. Additionally, cows that do not have a LH-responsive follicle present and no CL present may develop a persistent follicle, which is defined as a dominant follicle that is stimulated to persist beyond the normal period of dominance as the result of treatment with progesterone at a subluteal concentration after luteolysis (Bridges and Fortune, 2003). Fertility has been observed to be reduced among cows in which dominance of the follicle is prolonged via subluteal progesterone treatment for more than four days (Mihm et al., 1994). Second, cows that have an LH-responsive follicle and a CL may ovulate and form an accessory CL. Elevated levels of progesterone during follicular development have been observed to reduce follicular development and result in ovulation of a smaller follicle (Adams et al., 1992). Modestly elevated

progesterone concentrations may not be detrimental for the underlying fertility of the oocyte; however, variability among cows with respect to circulating progesterone concentrations could conceivably result in greater variability in timing of estrus expression and ovulation, reducing pregnancy rates when performing FTAI rather than AI following detected estrus. To address the challenge of cows beginning the protocol at varying stages of the estrous cycle, presynchronization treatments can be implemented prior to the start of a synchronization protocol to achieve a more uniform response.

Presynchronization in the Industry

In Ovsynch, a commonly used protocol in the dairy industry, the treatment schedule begins with an initial administration of GnRH to induce ovulation and reset the follicular wave. However, only cows with LH-responsive follicles will ovulate in response to GnRH. Previous research has shown that the ideal time to initiate Ovsynch among lactating dairy cows is during days 5-12 of the estrous cycle, when an LH-responsive follicle is present (Vasconcelos et al., 1999). Cows that begin the treatment schedule during the late luteal phase on days 13-17 of the estrous cycle and fail to ovulate in response to GnRH may undergo premature regression of the CL and express estrus and ovulate prior to the second administration of GnRH. Additionally, cows that begin the protocol during the metestrus phase of the estrous cycle (d 1-4) may have a follicle that has not reached physiological maturity and therefore will not ovulate in response to GnRH, resulting in no functional control of stage of ovarian follicular wave among this sub-population of animals. These subsets of animals that begin the treatment schedule on days outside of 5-12 of the estrous cycle often have reduced fertility to timed-AI due to

variation in timing of estrus expression and follicular maturity (Moreira et al., 2000). Therefore, presynchronization methods were developed to increase the proportion of dairy cows that present with an LH responsive follicle at the first administration of GnRH in Ovsynch.

Presynch-Ovsynch, G6G, and Double Ovsynch are modified versions of the Ovsynch protocol that incorporate presynchronization prior to the start of the protocol. These presynchronization treatment schedules involve administering PG or PG and GnRH to manage stage of cycle in advance of the first GnRH administration of the Ovsynch protocol. The objective of these presynchronization strategies is to manage stage of cycle prior to the start of Ovsynch, so that a greater proportion of cows go on to have a dominant follicle present on the ovary at the time of the final GnRH administration in Ovsynch. (Bello et al., 2006; Navanukraw et al., 2004; Souza et al., 2008; Wiltbank and Pursley, 2014). Presynch-Ovsynch is a presynchronization approach evaluated in postpartum dairy cows that resulted in an increase in pregnancy rates compared to results observed among cows receiving Ovsynch with no presynchronization. The treatment schedule of Presynch-Ovsynch involves administration of PG at two timepoints 14 days apart. Several versions of Presynch-Ovsynch have been developed and evaluated, with modifications to the number of days from the second administration of PG to the start of Ovsynch. The original treatment schedule of Presynch-Ovsynch involved starting Ovsynch 14 days after the last PG administration (Navanukraw et al., 2004). The Presynch-Ovsynch schedule that has resulted in the greatest pregnancy rates to AI begins Ovsynch 11 days after the final PG. Based on the dynamics of follicular waves, beginning Ovsynch 11 days after the

presynchronization treatment has the greatest likelihood of generating an ovulatory response to GnRH based on follicular maturity. Although this is relatively inexpensive method to improve control of the estrous cycle, there is often a proportion of cows that are anestrus at the start of the protocol. Inducing luteolysis with administration of PG is only effective among PG-responsive CL and anestrus cows lack this structure and therefore are unaffected by this presynchronization approach. Another presynchronization method, G6G, was evaluated in dairy cows and resulted in a greater proportion of cows ovulating in response to the initial GnRH administration in Ovsynch. The G6G treatment schedule begins with administration of PG followed by administration of GnRH two days later. Ovsynch is initiated 6 days after the GnRH administration. The hypothesized mechanism for G6G is that a large proportion of cows would ovulate in response to administration of GnRH and a new follicular wave would be recruited. The 6-day interval between the GnRH of G6G and the initial GnRH of Ovsynch provides adequate time for a follicle to reach physiological maturity and be LH-responsive, and a large proportion of cows are on what would be analogous to day 6 of the estrous cycle at the initiation of Ovsynch. Additionally, G6G was found effective in a proportion of anestrus cows. Administering GnRH has been observed to induce an LH surge in anestrus cows. Depending on the depth of anestrus and presence of a preovulatory sized follicle, there may be sufficient GnRH receptor expression and releasable LH present in the pituitaries at the time of GnRH administration (McDougall et al., 1995). Overall, use of presynchronization treatments have proven to increase the proportion of dairy cows ovulating in response to exogenous GnRH, decrease variation in

the timing of estrus, and improve pregnancy rates to AI compared to results with no presynchronization.

In contrast, the beef industry has not readily adopted methods of presynchronization, perhaps due to the greater number of handling events required and the additional cost of pharmaceutical products and labor. Previous research performed with the 5-Day CO-Synch + CIDR protocol demonstrated that administering PG 3 days prior to CIDR insertion and GnRH administration increased the proportion of cows that ovulated in response to GnRH (Perry et al., 2012). Consequently, an increase in pregnancy rates was also observed among the cows that received presynchronization prior to the protocol, suggesting greater control of the estrous cycle and increased synchrony of the timing of estrus expression was obtained from the increased ovulatory response to GnRH. A similar experiment was performed using heifers to evaluate the effectiveness of presynchronization. Heifers that received administration of PG 3 days prior to GnRH administration and insertion of a CIDR had a greater proportion respond to the GnRH compared to heifers that did not receive PG 3 days prior (Dargatz et al., 2004). However, administering PG prior to synchronization is perhaps not a strategy that would be broadly effective for heifers given that there is often a fairly high proportion of prepuberal heifers within a group. As mentioned previously, only cycling animals have luteal structure present on the ovary, and a PG-responsive CL needs to be present in order for PG to be effective.

Other presynchronization strategies that have been evaluated include administering GnRH and treating with a CIDR prior to the start of a typical short-term protocol. Giles et al. (2013) evaluated the efficacy of GnRH to presynchronize a group of

cows prior to the start of the 5-Day CO-Synch + CIDR protocol (Giles et al., 2013).

Cows received administration of GnRH coincident with insertion of a CIDR 9 days prior to the start of the 5-Day CO-Synch + CIDR protocol. Cows that received presynchronization had greater pregnancy rates to FTAI compared to cows that did not receive presynchronization. Another approach that has been evaluated to increase the proportion of cows that ovulate in response to GnRH is to induce luteolysis prior to GnRH administration. Perry et al. (2012) observed that administering PG 3 days prior to GnRH administration increased the proportion of cows ovulating in response to GnRH compared to cows that did not receive PG.

A presynchronization treatment with subluteal levels of progesterone has been observed to increase the size of the dominant follicle prior to GnRH. This method increased the proportion of cows ovulating in response to GnRH but did not improve pregnancy rates to AI (Small et al., 2009). Inducing intentional follicular persistence through use of subluteal progesterone is an effective method to enhance follicular maturity. Bonacker et al. performed an experiment evaluating treatments to promote follicular maturity in advance of administration of exogenous GnRH administration for increased control of the estrous cycle (Bonacker et al., 2020b). The experiment was designed to evaluate the physiology behind inducing a persistent follicle as a presynchronization strategy. In this approach, later termed 7 & 7 Synch (Bonacker et al., 2020a), PG is administered on Day 0 concurrent with CIDR insertion, GnRH is administered on Day 7 and PG is administered on Day 14 concurrent with CIDR removal. Administering PG concurrent with CIDR insertion induces luteolysis among cows with PG-responsive CL, resulting in circulating concentrations of progesterone to

subluteal concentrations of CIDR-source. Subluteal progesterone permits enough LH pulsatility to maintain follicular growth while inhibiting atresia, resulting in an extended period of dominance by the preovulatory follicle. Although formation of persistent follicle is generally detrimental due to premature resumption of meiosis of the oocyte (Kinder et al., 1996), intentional formation of persistent follicles is advantageous in this context as a means to enhance follicular maturity and thereby improve likelihood of ovulatory response to GnRH administration and subsequent emergence of a new synchronized follicular wave. Based on the hypothesis that the observed improvements in control over follicular wave development would result in increased synchrony among cows in timing of estrus expression, a subsequent large field trial was conducted to evaluate use of the 7 & 7 Synch protocol for control of the estrous cycle among recipient beef cows prior to embryo transfer (Bonacker et al., 2020a). A greater proportion of cows treated with 7 & 7 Synch expressed estrus compared to cows treated with the 7-Day CO-Synch + CIDR protocol. With a greater proportion of cows expressing estrus following synchronization, 7 & 7 Synch gives the potential to result in greater pregnancy rates to AI with both conventional and sexed semen in a FTAI program.

Efficacy of Sexed Semen in Estrus Synchronization Programs

Introduction

Through natural mating or use of conventional semen, the sex ratio of the offspring produced in a breeding program has a fixed probability of 51:49, with a slight increase in the probability of male calves (Vishwanath and Moreno, 2018). Sex of the calf is one of the most economically relevant traits. The use of sexed semen allows

producers to selectively produce offspring of the desired sex from a particular mating. This can reduce generation interval, potentially increasing the rate of genetic progress for traits of interest, and also can enable more strategic production of terminal progeny. Presently, there are two methods commercially available to produce sexed semen; however, the oldest and most widely used method is based on flow-cytometric measurements of sperm DNA content (Seidel, 2007).

Current Sexing Technologies

Flow cytometry-based sexing was the first technology commercially available to successfully sort sperm (Gledhill et al., 1982; Pinkel et al., 1982). Over the last several decades, an immense number of research efforts have gone into improving the efficiency of the sorting process with flow cytometry-based sexing (Garner and Seidel, 2008). Flow cytometry quantifies cellular DNA content at thousands of sperm per second. As the sperm pass through, droplets form with each sperm cell and each droplet is given a positive or negative charge based on X- or Y-chromosome bearing sperm. The droplets pass through a pair of electrodes and are then sorted into different holding tubes based on charge (Garner et al., 1983; Vishwanath and Moreno, 2018). Sperm are stained with the DNA-binding dye, Hoechst 33342, which makes it possible to observe detectable differences in DNA content in order to differentiate X- and Y-chromosome-bearing sperm due to X-chromosome-bearing sperm having approximately 3-5% more DNA content than Y-chromosome-bearing sperm (Garner et al., 1983; Seidel, 2007). SexedULTRA 4MTM is a commercially available sexed product for both beef and dairy sires produced and marketed by ST Genetics and by other various companies through licensing agreements (Vishwanath and Moreno, 2018). These sexed products are

produced using a flow cytometry-based technology. A recently developed method to sex sperm is based off a laser-ablation technique, in which sperm that are carrying the undesired chromosome are selectively destroyed. With the laser ablation process, Hoechst 33342 dye is also used to quantify DNA differences among sperm. Sexcel™ is a recently developed sexing technology produced by ABS Global (Genus plc) using their proprietary IntelliGen™ technology (Perry et al., 2020). Sexcel™ is currently marketed for X-sorted semen for a variety of beef and dairy breeds. Both of these sorting methods produce sexed semen products that are marketed with 90% accuracy (Perry et al., 2020; Vishwanath and Moreno, 2018). However, for both sexed products, sexed semen is generally observed to result in lower pregnancy rates to AI compared to conventional semen (Perry et al., 2020; Thomas et al., 2017).

Limitations of Sexed Semen

Sperm are damaged during the sorting procedure and subsequent cryopreservation, leading to compromised viability and fertility. During the sex-sorting procedure, sperm are exposed to DNA-binding dye Hoechst 33342, electromagnetic energy, mechanical damage, and post-sorting centrifugation and cryopreservation (Carvalho et al., 2010; Seidel and Schenk, 2008). Upon being deposited in the female reproductive tract, sperm must undergo a series of acrosomal changes to acquire fertilization capacity. However, the sexing procedure induces alterations to the sperm membrane that prematurely initiates capacitation and acrosome reaction processes of sperm after cryopreservation. Acceleration of capacitation and acrosome reaction processes of sperm subsequently reduce the fertile lifespan of sperm in the female reproductive tract (Carvalho et al., 2010; Mocé et al., 2006). Due to the damage and

alterations that sex-sorted sperm undergo, pregnancy rates to artificial insemination with sexed semen are often reduced compared to pregnancy rates to AI with conventional semen (Hall and Glaze, 2013; Seidel and Schenk, 2008; Thomas et al., 2017). Given the decreased fertile lifespan of sexed sperm, some investigators have suggested that sexed semen is more sensitive to the timing of insemination in relation to ovulation compared to non-sexed sperm (Bombardelli et al., 2016; Sales et al., 2011). The optimal timing of AI with sexed semen may not correspond to the timing traditionally recommended as optimal for conventional semen, and it has been hypothesized that decreasing the time interval from insemination to ovulation may improve conception rates with sexed semen (Sales et al., 2011). Sales et al., reported an increase in pregnancy rates to sexed semen when increasing the interval from progesterone removal to insemination (Sales et al., 2011). Other observations have suggested that inseminating closer to the timing of ovulation with sexed semen increases conception rates to AI (Bombardelli et al., 2016; Sá Filho et al., 2013). When FTAI is performed, cows and heifers that fail to express estrus prior to FTAI have particularly decreased pregnancy rates when sexed semen is used, perhaps due to the reduced sperm lifespan and timing of ovulation not being optimally aligned. With this understanding, pregnancy rates to FTAI with sexed semen are optimized among the proportion of females that express estrus prior to FTAI (Seidel, 2007; Thomas et al., 2019). Timing of insemination clearly affects fertility, with some indication in the literature that insemination closer to ovulation is particularly critical for some bulls (Dalton et al., 2001; Saacke et al., 2000; Trimberger, 1948). This may create further challenges for sexed semen when considering sire differences. In addition, it may simply not be possible to produce fertile sexed semen from a bull, even if the same bull's

ejaculate may be successfully collected and cryopreserved as conventional semen. Sexed semen is typically packaged at lower concentrations such as 2×10^6 or 4×10^6 live cells per straw (Garner and Seidel, 2008; Hall and Glaze, 2013; Thomas et al., 2019). Several investigators (DeJarnette et al., 2011, 2008) have noted no difference among pregnancy rates to artificial insemination when different concentrations of sexed semen were used. The lower concentration of sperm per straw might contribute to the lower pregnancy rates to AI when compared to conventional semen which is typically packaged at 20×10^6 sperm per straw.

Application in the Industry

From an economic standpoint, one sex of calf, male or female, has more value over another as a result of several potential factors. Value differences between sex of the calf can stem from differences in overall market value (i.e., market price of steer vs heifer progeny) or from genetic and other operation-specific considerations (i.e., production of bulls vs replacement heifers). Over the last decade, sexed semen has been readily incorporated into breeding programs in the dairy industry. In the dairy industry, dairy-influenced steer calves are discounted in the market, making sexed semen a valuable tool to increase profitability (Weigel, 2004). Additionally, use of sexed semen and genomic-enhanced EPDs in a breeding program gives dairy producers the opportunity to reduce the generation interval and accelerate the rate of genetic progress by producing genetically superior replacement heifers (De Vries et al., 2008; Weigel, 2004). Due to the growth of use of sexed semen for heifer production, conventional beef semen is being used on genetically poorer dairy cows in the herd (De Vries et al., 2008). Presently, approximately 15% of the total US beef supply is produced from the dairy industry.

Speculatively, sexed semen may also provide some profitable alternatives to conventional breeding methods by using beef sexed semen on dairy cows to increase efficiency of the emerging “beef on dairy” chain. X-sorted semen from dairy bulls can be used on genetically superior females to generate quality replacement heifers, and Y-sorted semen from beef bulls with complementary genetics to improve growth and carcass merit could be used on less genetically superior females.

The primary consideration for commercial producers is the economic return potential associated with use of sexed semen. Across the literature, sexed semen often results in reduced pregnancy rates to AI compared to conventional semen (Hall et al., 2017; Seidel, 2007; Thomas et al., 2017, 2019). Sexed semen also costs more per unit than conventional semen, with prices dependent on the sire used. Due to the cost and risk associated with use of sexed semen, the beef industry has not as readily adopted this technology. In recent years, however, extensive research with sexed semen has provided insight on the limitations and opportunities of sexed semen in beef cattle operations (Hall and Glaze, 2013). As is the case for dairy operations, the use of sexed semen provides the opportunity to increase profitability among beef operations due to the market value differences between male and female progeny. A cow-calf operation with a goal of generating replacement females can inseminate with X-sorted semen to shift the sex ratio to majority female progeny (Hall and Glaze, 2013). In addition, producers can choose to inseminate all of their heifers with X-sorted semen to produce much of the next generation of replacement females out of the current genetically superior replacement heifers. In addition to using a proven sire with calving ease EPDs, inseminating heifers with X-sorted semen can further reduce incidence of dystocia (Hall and Glaze, 2013). Y-

sorted semen can be used in a specific mating to produce progeny with genetics for suited for terminal performance. Moreover, Y-sorted semen can be used to generate a greater proportion of steer calf progeny that are half-siblings and more uniform in size and weight, potentially resulting in price premiums when marketed as feeder calves.

Summary

Improving effective control of the estrous cycle continues to be an important area of research. Although the dairy industry has readily adopted methods of presynchronization to improve control of the estrous cycle among a group of cows prior to FTAI, the beef industry has not had simple presynchronization options available. Despite substantial progress in the processing of sexed semen, poor pregnancy rates to FTAI with sexed semen are a barrier to adoption of sexed semen. Cows that express estrus prior to FTAI are more likely to become pregnant to FTAI than cows that fail to express estrus, and this is particularly the case when sexed semen is used for FTAI. An estrus synchronization protocol that results in a greater proportion of cows expressing estrus prior to FTAI could mitigate the reduction in pregnancy rate typically observed with sexed semen and lead to increased adoption. These considerations form the basis for the research presented in this thesis.

Chapter 2

Evaluation of the 7 & 7 Synch and 7-day CO-Synch + CIDR protocols for control of the estrous cycle among beef cows prior to fixed-time artificial insemination with conventional or sex-sorted semen

Abstract

An experiment was conducted to compare 7 & 7 Synch and the 7-day CO-Synch + controlled internal drug release (CIDR[®]) protocols prior to fixed-time artificial insemination (FTAI) of beef cows with conventional or sex-sorted semen. Cows (n = 1,538) were blocked based on age and days postpartum (DPP) and randomly assigned to protocol and semen type. Cows assigned to 7-day CO-Synch + CIDR (n = 769) received administration of gonadotropin-releasing hormone (GnRH) and insertion of an intravaginal progesterone-releasing insert (CIDR) on Day -10, and administration of prostaglandin F_{2α} (PG) coincident with CIDR removal on Day -3. Cows assigned to 7 & 7 Synch (n = 769) received PG and insertion of CIDR on Day -17, GnRH on Day -10, and PG coincident with CIDR removal on Day -3. Estrus detection aids were applied to all cows. Cows received FTAI 66 h after CIDR removal with conventional (20×10^6 cells per unit) or sex-sorted (4×10^6 cells per unit) semen. Estrus expression was affected by protocol ($P = 0.01$) and by protocol $\times$ DPP ($P = 0.0004$), with 7 & 7 Synch (82%; 629/769) resulting in a greater proportion of cows expressing estrus (82% [629/769] versus 64% [492/769]), especially among cows with greater DPP. Pregnancy rates to FTAI were reduced ($P < 0.0001$) when using sex-sorted semen but greater among cows treated with 7 & 7 Synch (conventional semen: 72% [280/389]; sex-sorted semen: 52%

[199/380]) compared with 7-day CO-Synch + CIDR (conventional semen: 61% [233/383]; sex-sorted semen: 44% [171/386]).

Introduction

Many short-term estrus synchronization protocols, such as the 7-day CO-Synch + CIDR[®] (controlled internal drug releasing device), utilize exogenous gonadotropin-releasing hormone (GnRH) at the start of the protocol to induce ovulation of a dominant follicle and initiate a new follicular wave. However, stage of follicular development is a major determinant of an animal's ability to respond to exogenous hormone treatment during an estrus synchronization protocol (Bo et al., 1995), and GnRH is only effective for inducing ovulation of a dominant follicle of sufficient physiological maturity.

Administration of GnRH at a random stage of the estrous cycle has been observed to induce ovulation with variable success rates, resulting in ovulation in approximately 65% of cows on average (Geary et al., 2000) and considerably poorer rates possible in any one group. Cows that fail to ovulate in response to GnRH are in a stage of follicular development that lacks a dominant follicle capable of responding to the surge of luteinizing hormone (LH) induced via GnRH administration. The resulting lack of uniformity among cows in subsequent stages of the estrous cycle constitutes a potential limitation to pregnancy rates achieved among cows when performing fixed-time artificial insemination (FTAI), as variation in follicular maturity, estrus expression, and timing of ovulation at the end of the protocol can contribute to suboptimal fertility.

Presynchronization treatments to manage stage of cycle in advance of the start of the protocol are one method to address the problem of variable response to GnRH. Presynchronization treatments now widely adopted in the dairy industry include

administering prostaglandin $F_{2\alpha}$ (PG) or administering a combination of GnRH and PG in order to manage stage of the estrous cycle prior to administering GnRH (Wiltbank and Pursley, 2014). These methods of presynchronization have proven to reduce variation among cows in stage of follicular development and increase the proportion of cows ovulating in response to GnRH (Souza et al., 2008; Wiltbank and Pursley, 2014). However, the beef industry has not readily adopted methods of presynchronization, perhaps due to the greater number of handling events required and the additional cost of pharmaceutical products and labor. Results from previous experiments have suggested that administering PG prior to administering GnRH increases the ovulatory response to GnRH by increasing the number of animals presenting with a GnRH responsive follicle (Grant et al., 2011; Perry et al., 2012). In addition, this method of presynchronization decreased the variation in follicle size at CIDR removal, suggesting a greater degree of uniformity among cows in stage of follicular development.

Another approach to potentially address the challenge of variable ovulatory response to GnRH among beef cows may be to induce formation of a persistent follicle prior to GnRH administration. By administering PG and placing a CIDR in advance of GnRH administration, an increased proportion of cows present with a physiologically mature, LH-responsive follicle at the time of GnRH administration (Bonacker et al., 2020a; French et al., 2013; Small et al., 2009). This approach is of practical significance as a simple, one-step method for presynchronization, and early results with the 7 & 7 Synch protocol that incorporates this presynchronization approach have suggested improved control of the estrous cycle (Bonacker et al., 2020a, 2020b).

In comparison with cows receiving the 7-day CO-Synch + CIDR protocol, cows receiving 7 & 7 Synch presented with significantly greater largest follicle diameter at the time of GnRH administration, with subsequent CL status and estrus expression suggesting a high ovulatory response to GnRH (Bonacker et al., 2020b). In addition, a subsequent embryo transfer trial observed a greater proportion of recipient cows expressed estrus and became pregnant to embryo transfer when treated with 7 & 7 Synch (Bonacker et al., 2020a). Based on these observations, the following experiment was designed to evaluate expression of estrus and pregnancy outcomes among multiple herds when using the 7 & 7 Synch protocol prior to FTAI. We hypothesized that the 7 & 7 Synch protocol would result in an increased proportion of cows expressing estrus prior to FTAI, as well as increased pregnancy rates to FTAI with conventional and/or sex-sorted semen when compared with the 7-day CO-Synch + CIDR protocol.

Materials and Methods

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee (Protocol #9432).

Animals and Estrus Synchronization

The 7-day CO-Synch + CIDR and 7 & 7 Synch protocols (Figure 2.1) were used to synchronize estrus among suckled beef cows ($n = 1,538$) of varying age. The experiment was conducted across multiple locations ($n = 11$) in Missouri and South Dakota in the spring and fall breeding seasons of 2019. Within each location, cows were blocked based on age, days postpartum (DPP), and body condition score (BCS; 1 to 9

scale; 1 = emaciated and 9 = obese) (Richards et al., 1986) and randomly assigned to treatment within block. Cows treated with the 7-day CO-Synch + CIDR protocol (n = 769) received administration of 100 µg gonadorelin acetate (GnRH; Fertagyl, Merck Animal Health, Madison, NJ) and insertion of a 1.38 g progesterone-releasing intravaginal insert (EAZI-Breed CIDR[®] Zoetis, Madison, NJ) on Day -10, and administration of 500 µg cloprostenol sodium (PG; Estrumate, Merck Animal Health, Madison, NJ) coincident with removal of CIDR on Day -3. Cows treated with the 7 & 7 Synch protocol (n = 769) received administration of PG and insertion of CIDR on Day -17, administration of GnRH on Day -10, and administration of PG coincident with removal of CIDR on Day -3. For both protocols, all cows received administration of GnRH at the time of FTAI, which was performed on Day 0 at 66 h after CIDR removal and PG administration.

Estrus Detection

Estrus detection aids (EstroTECT[™], Rockway Inc, Spring Valley, WI) were applied to all cows on Day -3 at the time of PG administration and CIDR removal, and estrus detection aid activation was recorded at the time of FTAI on Day 0. Estrus detection aid activation was scored on a scale of 0 to 4 (0 = missing patch; 1 = 0-25% activated; 2 = 25-50% activated; 3 = 50-75% activated; and 4 = 75-100% activated) (Pohler et al., 2016). Estrus was defined as 50% or greater of the estrus detection aid activated or a missing estrus detection aid (patch score 0, 3, 4).

Artificial Insemination

Within protocol in each location, cows were blocked based on age, DPP, and BCS and pre-assigned randomly within block to receive either conventional or sex-sorted semen at FTAI (Figure 2.1). To minimize potential confounding of treatment effects in locations where FTAI was performed by two technicians, cows were also blocked on these criteria within protocol and semen type and pre-assigned to technician. Animals in Locations 1, 2, 4, 6, and 10 were inseminated by a single technician. Semen was collected from eight commercially available AI bulls, and units of sex-sorted and conventional semen were produced from contemporaneous ejaculates. Both conventional and sex-sorted semen from each bull passed the standard quality control criteria used for the respective semen types. Units of conventional semen were generated with 20.0×10^6 live cells per 0.25 mL straw prior to freezing. Units of sex-sorted semen were produced using the SexedULTRA™ Genesis III sorting technology (Sexing Technologies, Navasota, TX) with 4.0×10^6 live cells per 0.25 mL straw prior to freezing with a marketed level of > 90% accuracy for the desired sex. Sex-sorted units were sorted to either contain X (Bulls A, B, C, D, F, and G) or Y-bearing chromosomes (Bulls E and H). Bulls varied between locations, but, within location, all treatments used semen from the same bull or bulls. Semen from two bulls was used within Location 1, while semen from only a single bull was used within Locations 2-11. In Location 1 where semen from two bulls was used, semen from each bull was used in equal proportion across treatments, with cows blocked based on age, BCS, and DPP and pre-assigned randomly to bull within block. In all locations, clean-up bulls were exposed to the cows 14 days after AI was performed.

Pregnancy Diagnosis

Pregnancy per artificial insemination (P/AI) was determined 75 to 90 days after artificial insemination by transrectal ultrasonography, using an Aloka 500V equipped with a 5.0-MHz linear-array transducer (Aloka, Wallingford, CT) in Locations 1-6 and a SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer (SonoSite, Inc., Bothwell, WA) in Locations 7-11.

Statistical Analysis

Statistical analyses were performed using SAS (SAS 9.4 Inst. Inc., Cary, NC). A general linear model (PROC GLM) was used to confirm balance across treatments with respect to DPP, BCS, and age of cows. A mixed model (PROC GLIMMIX) using the binomial distribution link logit function was used to evaluate the proportion of cows expressing estrus prior to FTAI and P/AI. Variables tested for inclusion in the model were protocol (7 & 7 Synch or the 7-day CO-Synch + CIDR), age, BCS, DPP, and all two-way interactions. Location was included as a random effect. To visualize the protocol $\times$ DPP interaction, regression equation coefficient estimates from the GLIMMIX procedure of SAS (SAS 9.4 Inst. Inc., Cary, NC) were used to model the effect of protocol across the DPP range. For this purpose, regression equation coefficient estimates were used modeling the effect of protocol $\times$ DPP for cows of the average BCS (5.7) and age (4.6).

A mixed model (PROC GLIMMIX) using the binomial distribution link logit function was used to evaluate the proportion of cows becoming pregnant as a result of AI (P/AI). Variables tested for inclusion in the model were protocol, semen type, age, BCS,

DPP, and all two-way interactions. Bull, technician, location, and location $\times$ protocol $\times$ semen were included as random effects. Interactions not observed to have an effect ($P > 0.10$) were removed from the models using backwards elimination, with the exception of the protocol $\times$ semen interaction central to the experiment. The final mixed model for P/AI included fixed effects of protocol, semen, age, BCS, DPP, and protocol $\times$ semen as well as the random effects of bull, technician, location, and location $\times$ protocol $\times$ semen. The three-way interaction of location $\times$ protocol $\times$ semen was included in the random statement to provide an appropriate analysis of P/AI, as protocol $\times$ location interaction is the most conservative error term for tests of potential treatment differences in multi-location experiments.

Because expression of estrus prior to FTAI was a dependent variable that was hypothesized to vary between protocols, expression of estrus was not included as an independent variable in the model for P/AI. Expression of estrus prior to FTAI has a significant effect on P/AI across the literature, and all models for P/AI were therefore also analyzed independently for cows that expressed estrus prior to FTAI as well as for cows that failed to express estrus prior to FTAI. Across treatments and within each treatment, Chi-square was used for comparisons of P/AI obtained among cows that expressed estrus versus cows that failed to express estrus.

Results

Location Summary

Age, DPP, BCS, and the proportion of cows expressing estrus prior to FTAI by treatment are summarized in Table 2.1. Age, DPP, and BCS did not differ between

treatments within each location or across all locations. Individualized cow age was not known in Location 10, which consisted exclusively of mature cows seven years of age and older.

Estrus Expression

The proportion of cows expressing estrus prior to FTAI is summarized in Table 3.1. The proportion of cows expressing estrus was increased ($P = 0.01$) among cows treated with 7 & 7 Synch (82%; 630/769) compared with the 7-day CO-Synch + CIDR protocol (64%; 492/769). Estrus expression was also affected by a protocol $\times$ DPP interaction ($P = 0.0004$), with 7 & 7 Synch resulting in a greater magnitude increase in the proportion of cows expressing estrus prior to FTAI among cows with greater DPP. Figure 2.2 represents modeled likelihood of estrus expression assuming the average body condition score (5.7) and age (4.6) across cows in the experiment. The proportion of cows expressing estrus prior to FTAI was also affected by BCS ($P = 0.05$), with an increased proportion of cows in greater BCS expressing estrus. Additionally, estrus expression tended to be affected by a protocol $\times$ age interaction ($P = 0.08$), with 7 & 7 Synch resulting in a greater magnitude increase among older cows with respect to the proportion of cows expressing estrus prior to FTAI.

Pregnancy Rate

Pregnancy outcomes are summarized by location in Table 2.2. P/AI were affected by protocol ($P = 0.001$), semen type ($P < 0.0001$), age ($P = 0.04$), and DPP ($P = 0.02$). Regardless of semen type used for AI, P/AI was increased ($P = 0.001$) among cows treated with 7 & 7 Synch (conventional semen: 72% [280/389]; sex-sorted semen: 52%

[199/380]) compared with 7-day CO-Synch + CIDR (conventional semen: 61% [233/383]; sex-sorted semen: 44% [171/386]). Irrespective of treatment, sex-sorted semen resulted in decreased ($P < 0.0001$) P/AI compared with conventional semen. Pregnancy results based on protocol, semen type, and expression of estrus prior to FTAI are presented in Table 2.3. Across treatments and within each treatment, cows that expressed estrus prior to FTAI achieved greater P/AI ($P < 0.001$). In comparison with conventional semen, sex-sorted semen resulted in reduced P/AI both among cows that expressed estrus ($P < 0.0001$) and among cows that failed to express estrus ($P = 0.001$) prior to FTAI. Specifically, among cows that expressed estrus prior to FTAI, greater P/AI ($P = 0.01$) was achieved among cows receiving 7 & 7 Synch in comparison with cows receiving the 7-day CO-Synch + CIDR protocol. Among cows that failed to express estrus prior to FTAI, P/AI did not differ significantly ($P = 0.14$) between protocols at this power of test.

Discussion

Short-term estrus synchronization protocols such as the 7-day CO-synch + CIDR rely on administration of exogenous GnRH at the beginning of the protocol to induce formation of a new follicular wave. However, as a consequence of animals beginning an estrus synchronization protocol at varying stages of the estrous cycle, a proportion of animals are in a stage of follicular development in which a physiologically mature dominant follicle capable of responding to the GnRH-induced LH surge is simply not present. As a result, a proportion of females that began the treatment late in the luteal phase undergo luteolysis during the course of CIDR treatment and therefore can develop

a persistent, subfertile follicle that is maintained until CIDR removal (Bigelow and Fortune, 1998; Sirois and Fortune, 1990). Although these follicles will appear physiologically mature, the oocyte is functionally compromised due to a prolonged period of increased frequency of LH pulses resulting in premature resumption of meiosis (Mihm et al., 1994). The single-step approach of combining a progestin and PG at the beginning of 7 & 7 Synch induces persistent follicle formation among a large proportion of cows in advance of GnRH administration (Bonacker et al., 2020b). However, the intentional formation of persistent follicles is advantageous in this context as a means to enhance follicular maturity on Day -10 (Bonacker et al., 2020b), thereby improving likelihood of ovulatory response to GnRH administration and synchronized emergence of a new follicular wave.

In the present experiment, the increase observed in the proportion of cows that expressed estrus prior to FTAI following 7 & 7 Synch is attributed to the greater degree of uniformity previously observed among cows using this treatment schedule (Bonacker et al., 2020b). By improving follicular maturity in advance of GnRH administration, variation among cows is reduced later in the protocol. At the time of PG administration on Day -3, a greater proportion of cows were observed to have a single CL following treatment with 7 & 7 Synch, whereas a greater proportion of cows were observed to have no CL or a CL and an accessory CL following treatment with the 7-day CO-Synch + CIDR (Bonacker et al., 2020b). Recognizing that 7 & 7 Synch induces luteolysis among the majority of cyclic cows via PG administration on Day 0, the observation that the majority of cows presented with a single CL on Day -3 indicates a successful response to

GnRH administration on Day -10 and an enhanced degree of uniformity among the synchronized group.

The proportion of cows expressing estrus prior to FTAI was greatly increased among cows treated with the 7 & 7 Synch protocol. Results from this field trial emphasize the importance of estrus expression prior to FTAI, as the published literature consistently notes that P/AI is increased among cows that express estrus compared with cows that fail to express estrus prior to FTAI (Richardson et al., 2016). In the present study, 82% (630/769) of cows treated with the 7 & 7 Synch expressed estrus prior to FTAI while 64% (492/769) of the cows treated with the 7-day CO-Synch + CIDR expressed estrus prior to FTAI. Correspondingly, P/AI was increased among cows that expressed estrus prior to FTAI compared with cows that failed to express estrus at FTAI. Among cows treated with 7 & 7 Synch, cows that expressed estrus had an increased pregnancy rate (conventional semen: 75% [243/322]; sex-sorted semen: 55% [171/308]) compared with non-estrous cows (conventional semen: 55% [37/67]; sex-sorted semen: 38% [28/72]). Likewise, among cows treated with the 7-day CO-Synch + CIDR, cows that expressed estrus also obtained increased P/AI (conventional semen: 67% [170/255]; sex-sorted semen: 51% [120/237]) compared with non-estrous cows (conventional semen: 49% [63/128]; sex-sorted semen: 34% [50/149]). While estrus expression was associated with greater pregnancy rates in both protocols, a greater proportion of cows expressed estrus prior to FTAI following 7 & 7 Synch, resulting in greater overall P/AI.

Moreover, when the analysis was restricted only to cows that expressed estrus prior to FTAI, greater P/AI was achieved among estrous cows receiving 7 & 7 Synch compared with estrous cows receiving the 7-day CO-Synch + CIDR protocol. This merits

further investigation, as this observation suggests an amelioration of subfertility observed among cows that express estrus prior to FTAI following the 7-day CO-Synch + CIDR protocol. In the 7-day CO-Synch + CIDR protocol, subfertility may result among cows that failed to ovulate in response to the initial administration of GnRH and underwent luteolysis prior to CIDR removal. This subpopulation of cows ovulates a persistent follicle, resulting in subfertility despite an otherwise normal display of standing estrus (Bigelow and Fortune, 1998; Mihm et al., 1994; Sirois and Fortune, 1990). In theory, considering the treatment schedule of 7 & 7 Synch, potential for subfertility stemming from ovulation of a persistent follicle could be reduced or even eliminated. An alternative explanation for improved fertility observed specifically among estrous cows could stem from optimized timing of ovulation relative to FTAI following 7 & 7 Synch. In a previous large multi-location embryo transfer field trial, 7 & 7 Synch resulted in a greater proportion of cows expressing estrus earlier in the distribution of estrus onset (Bonacker et al., 2020a). This observation suggests FTAI would be occurring later relative to onset of estrus on average and therefore closer to the timing of ovulation.

Although pregnancy rates were increased among cows treated with 7 & 7 Synch across semen types, use of sex-sorted semen resulted in reduced pregnancy rates in comparison with conventional semen irrespective of protocol. Despite the numerous hypothetical benefits that progeny sex selection offers in the beef industry, adoption of sex-sorted semen has been limited by the increased cost per unit of sex-sorted semen and the reduced pregnancy rates in FTAI programs observed in comparison to conventional semen (Sá Filho et al., 2012; Seidel, 2007; Thomas et al., 2014). Some investigators have suggested that sex-sorted semen has increased sensitivity to the timing of insemination in

relation to ovulation and onset of estrus (Sales et al., 2011). Cows that fail to express estrus prior to FTAI have particularly decreased P/AI when sexed semen is used, perhaps due to the reduced sperm lifespan and timing of ovulation not being optimally aligned (Thomas et al., 2014). With this understanding, P/AI with sex-sorted semen is optimized in females when estrus is expressed prior to FTAI (Seidel, 2007; Thomas et al., 2017, 2019). An estrus synchronization protocol that optimizes estrus expression among females prior to FTAI may increase utilization of sex-sorted semen across operations in the beef industry.

Another factor that can alter success of synchronization and P/AI in postpartum beef cows is the proportion of cows that are anestrus at the start of the breeding season (Stevenson et al., 2002). Factors that influence the proportion of cows that resume estrous cyclicity prior the beginning of the breeding season include DPP, parity and BCS (Short et al., 1990). Across protocols, BCS had a significant effect on the proportion of cows expressing estrus, with a greater proportion of cows in greater body condition expressing estrus prior to FTAI. Cows that are in body condition scores less than 5 have an increased potential for an extended period of anestrus when compared with cows in greater BCS (Short et al., 1990).

Several observations from the present study suggest the enhanced P/AI observed following 7 & 7 Synch is primarily due to an effect specific to cows that are presumed to be cycling at the start of the protocol. For example, there was a significant effect of DPP $\times$ protocol (Figure 2.2) on the proportion of cows expressing estrus prior to FTAI, with 7 & 7 Synch resulting in a greater magnitude of improvement among cows with greater DPP. There is a positive correlation between DPP and cyclicity; as DPP increases, cows

are more likely to have resumed normal estrous cycles compared with cows with shorter DPP (Stevenson et al., 2002). Likewise, the proportion of cows expressing estrus prior to FTAI tended to be affected by age $\times$ protocol, with 7 & 7 synch resulting in a greater magnitude of improvement among cows of greater age. This supports the hypothesis that improvements resulting from 7 & 7 Synch stem primarily from an effect observed among cows that are cyclic at the initiation of the protocol. Primiparous cows have increased nutrient demand due to their additional growth requirements, resulting in prioritization of nutrient use for growth rather than reproduction. The low physiological prioritization of reproductive processes increases the length of anestrus and limits the proportion of primiparous cows that have resumed normal estrous cyclicity at the start of the breeding season (Randel, 1990; Short et al., 1990). The interactions of protocol and age, and protocol and DPP observed in this experiment supports the hypothesis that the treatment schedule of 7 & 7 Synch is primarily advantageous for cows that have resumed estrous cyclicity prior to initiation of treatment.

Conclusion

When compared with the 7-day CO-Synch + CIDR protocol, 7 & 7 Synch resulted in a greater proportion of cows expressing estrus prior to FTAI and enhanced P/AI to both conventional and sex-sorted semen. Resultantly, 7 & 7 Synch provides a promising opportunity to increase utilization of sex-sorted semen in the beef industry as well as improve success rates with FTAI regardless of semen type. The increased proportion of cows expressing estrus prior to FTAI is associated with greater P/AI. Additionally, even specifically among cows that expressed estrus prior to FTAI, 7 & 7

Synch resulted in a significant increase in P/AI in comparison with the 7-day CO-Synch + CIDR protocol. This merits further mechanistic investigation. The significant effect of protocol on the proportion of cows expressing estrus as well as on P/AI was observed across cows of varying age, DPP, and BCS. However, the magnitude of improvement observed in the proportion of cows expressing estrus was greater among cows in greater DPP ranges and tended to be greater among cows of increased age. Given the relationship of age and DPP with estrous cyclicity, this observation supports the hypothesized mechanism for improved control of the estrous cycle using 7 & 7 Synch. With only one additional handling of animals and one additional week in added length of the protocol compared with the 7-day CO-Synch + CIDR, 7 & 7 Synch offers much potential as a platform to improve success with FTAI among postpartum beef cows.

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Table 2.1. Cow age, days postpartum (DPP), body condition score (BCS), and proportion expressing estrus prior to fixed-time artificial insemination (FTAI) by treatment and location.

Treatment ¹					Estrus expression prior to FTAI ⁴	
Location	N	Age	DPP ²	BCS ³	Proportion	%
7-day CO-Synch + CIDR	769	4.6 ± 1.8	80.2 ± 19.3	5.7 ± 0.5	492/769	64 ^b
Location 1	43	3.5 ± 1.7	98.8 ± 18.8	5.5 ± 0.5	31/43	72
Location 2	72	4.1 ± 1.6	75.0 ± 11.8	5.7 ± 0.4	52/72	72
Location 3	78	4.1 ± 0.4	98.3 ± 26.7	5.8 ± 0.1	59/78	76
Location 4	59	4.8 ± 1.7	63.8 ± 17.1	5.8 ± 0.5	47/59	80
Location 5	82	3.0 ± 1.6	76.2 ± 17.2	5.5 ± 0.4	56/82	68
Location 6	94	5.3 ± 2.0	74.2 ± 11.8	5.9 ± 0.5	69/94	73
Location 7	84	4.3 ± 1.2	66.6 ± 11.5	5.5 ± 0.4	35/84	42
Location 8	58	5.1 ± 2.7	88.4 ± 16.1	5.9 ± 0.6	39/58	67
Location 9	78	5.0 ± 0.0	82.0 ± 12.6	5.8 ± 0.6	41/78	53
Location 10	62	-	87.1 ± 12.9	5.8 ± 0.4	32/62	52
Location 11	59	6.0 ± 0.0	78.0 ± 13.9	5.7 ± 0.4	31/59	53
7 & 7 Synch	769	4.6 ± 1.9	80.2 ± 19.1	5.8 ± 0.6	630/769	82 ^a
Location 1	46	3.9 ± 1.8	95.2 ± 20.0	5.6 ± 0.6	41/46	89
Location 2	71	4.1 ± 1.4	73.4 ± 12.9	5.6 ± 0.4	56/71	79
Location 3	91	4.8 ± 2.1	94.0 ± 26.1	5.8 ± 0.4	85/91	93
Location 4	58	4.5 ± 1.6	64.2 ± 16.8	6.1 ± 0.7	49/58	85
Location 5	77	3.0 ± 1.6	76.3 ± 17.2	5.5 ± 0.4	56/77	73
Location 6	96	5.3 ± 1.9	74.2 ± 11.8	5.9 ± 0.5	84/96	88
Location 7	71	4.3 ± 1.2	66.6 ± 11.5	5.5 ± 0.4	47/71	66
Location 8	61	5.1 ± 2.7	88.4 ± 16.1	5.9 ± 0.6	55/61	90
Location 9	74	5.0 ± 0.0	82.0 ± 12.7	5.8 ± 0.6	54/74	73
Location 10	63	-	87.1 ± 12.9	5.7 ± 0.4	52/63	83
Location 11	61	6.0 ± 0.0	78.8 ± 14.0	5.7 ± 0.4	51/61	84

Data presented as mean value (± standard deviation of mean)

¹See Figure 2.1 for treatment descriptions.

²DPP calculated from calving date to FTAI date

³BCS of cows recorded on Day -17 using a scale of 1-9 (1 = emaciated and 9 = obese)

⁴Proportion of cows expressing estrus prior to FTAI, based on ≥ 50% of the patch coating rubbed off or a missing patch

^{ab}Values with different superscripts differ ($P = 0.01$)

Table 2.2. Pregnancy rates (P/AI)¹ to fixed-time artificial insemination²

Treatment ³	P/AI Conventional Semen		P/AI Sex-Sorted Semen	
	Proportion	%	Proportion	%
7-day CO-Synch + CIDR	233/383	61 ^b	171/386	44 ^d
Location 1	15/21	71	15/22	68
Location 2	24/37	65	13/35	37
Location 3	24/40	60	17/38	45
Location 4	18/30	60	14/29	48
Location 5	20/40	50	14/42	33
Location 6	29/47	62	18/47	38
Location 7	24/40	60	20/44	46
Location 8	19/30	63	15/28	54
Location 9	22/37	59	22/41	54
Location 10	19/31	61	13/31	42
Location 11	19/30	63	10/29	35
7 & 7 Synch	280/389	72 ^a	199/380	52 ^c
Location 1	19/25	76	13/21	62
Location 2	26/36	72	20/35	57
Location 3	38/45	84	25/46	54
Location 4	22/29	76	11/29	38
Location 5	27/39	69	17/38	45
Location 6	30/48	63	28/48	58
Location 7	22/36	61	16/35	46
Location 8	23/31	74	20/30	67
Location 9	26/37	70	21/37	57
Location 10	25/31	81	12/32	38
Location 11	22/32	69	16/29	55

¹P/AI was determined via transrectal ultrasonography 80-85 days after AI²Fixed-time artificial insemination was performed 66 hours after administration of PG³See Figure 2.1 for treatment descriptions^{abcd}Values with different superscripts differ ($P = 0.001$)

Table 2.3. Pregnancy rates to fixed-time artificial insemination (FTAI) based on treatment, semen type, and expression of estrus prior to FTAI

Treatment ¹	Conventional Semen				Sex-Sorted Semen				Across Semen Types			
	P/AI		P/AI		P/AI		P/AI		P/AI		P/AI	
	Estrous ⁴ Cows		Non-Estrous ⁵ Cows		Estrous Cows		Non-Estrous Cows		Estrous Cows		Non-Estrous Cows	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%	Proportion	%	Proportion	%
7-day CO-Synch + CIDR	170/255	67	63/128	49	120/237	51	50/149	34	290/492	59 ^b	113/277	41 ^c
7 & 7 Synch	243/322	75	37/67	55	171/308	55	28/72	38	414/630	66 ^a	65/139	47 ^c

¹See Figure 2.1 for treatment descriptions

²Pregnancy rate to AI was determined via transrectal ultrasonography 80-85 days after FTAI

³FTAI was performed 66 hours after administration of PG (Day -17) by a pre-assigned technician

⁴Estrous cows based on $\geq 50\%$ of the patch coating rubbed off or a missing patch at FTAI

⁵Non-estrous was recorded as having $< 50\%$ of the patch coating rubbed off at FTAI

^{abcd}Values with different superscripts differ ($P = 0.01$)

Figure 2.1. Treatment schedules for the 7-day CO-Synch + CIDR protocol with fixed-time AI (FTAI) and the 7 & 7 Synch protocol with FTAI. Cows treated with the 7-day CO-Synch + CIDR protocol (n = 769) received administration of 100 µg gonadotropin-releasing hormone (GnRH) and insertion of a 1.38 g intravaginal progesterone releasing insert (CIDR) on Day -10, and administration of 500 µg prostaglandin F2α (PG) coincident with CIDR removal on Day -3. Cows treated with the 7 & 7 Synch protocol (n = 769) received administration of PG and insertion of CIDR on Day -17, administration of GnRH on Day -10, and administration of PG coincident with CIDR removal on Day -3. Estrotect™ estrus detection aids were applied to all cows on Day -3 and patch activation score were recorded at the time of FTAI on Day 0.

Figure 2.2. Modeled proportion of cows expressing estrus prior to fixed-time artificial insemination (FTAI) based on days postpartum (DPP) and protocol. Estrus expression was affected by a protocol $\times$ DPP interaction ($P = 0.0004$), with 7 & 7 Synch resulting in a greater increase in the proportion of cows expressing estrus prior to FTAI among cows with greater DPP. A mixed model was generated using regression equation coefficient estimates from the GLIMMIX procedure of SAS (SAS 9.4 Inst. Inc., Cary, NC). The model contained effects of protocol, DPP, body condition score, age and DPP $\times$ protocol. Figure 2.2 represents modeled likelihood of estrus expression assuming the average body condition score (5.7) and age (4.6) across cows in the experiment.

Chapter 3

Evaluation of later timing of fixed-time artificial insemination for beef cows when using sex-sorted semen following the 7 & 7 Synch protocol

Abstract

An experiment was designed to evaluate later timing of fixed-time artificial insemination (FTAI) with sex-sorted semen among postpartum beef cows following the 7 & 7 Synch protocol, with the hypothesis that later timing would result in increased pregnancy rates (P/AI) among cows that expressed estrus prior to FTAI. Beef cows (n=414) were blocked based on age and days postpartum (DPP) and randomly assigned to receive FTAI at 66 or 72 h after administration of prostaglandin F_{2α} (PG). Estrus was synchronized using the 7 & 7 Synch protocol, which consists of administration of PG (500 µg cloprostenol) and insertion of an intravaginal progesterone-releasing insert (CIDR; 1.38 g progesterone) on Day 0, gonadotropin-releasing hormone (GnRH; 100 µg gonadorelin) on Day 7, and PG coincident with CIDR removal on Day 14. Estrus detection aids (Estrotect™) were applied to all cows on Day 14, and activation status was recorded at fixed-time artificial insemination (FTAI) on Day 17. All cows that expressed estrus prior to FTAI received sex-sorted semen (4×10^6 cells per unit; SexedULTRA 4M™). The proportion of cows expressing estrus prior to FTAI did not differ between treatments at this power of test (66 h: 71% [146/205]; 72 h: 76% [158/209]). Additionally, P/AI of estrous cows inseminated with sex-sorted semen did not differ between treatments (66 h: 45% [68/146]; 72 h: 40% [63/158]). In conclusion,

later timing of FTAI following the 7 & 7 Synch protocol failed to improve P/AI of estrous cows inseminated with sex-sorted semen.

Introduction

Sex of the calf is one of the most economically relevant traits. Use of sexed semen, especially when coupled with use of genomic-enhanced EPDs for sire selection, can facilitate reduced generation intervals and a rapid rate of genetic progress for traits of interest. Use of sexed semen also provides the opportunity to selectively produce offspring of the desired sex from a particular mating. Although sexed semen is a valuable technology that has the potential to shift the sex ratio of a calf crop to the desired sex, it comes with risks and limitations in achieving success. Across the literature, sexed semen has been observed to result in reduced pregnancy rates to AI (P/AI) in comparison to conventional semen (Hall et al., 2017; Perry et al., 2020; Seidel et al., 1999; Thomas et al., 2017, 2019). The reduction in P/AI is partly the result of damage to sperm cells from the sexing procedure and subsequent cryopreservation. During the sexing procedure, sperm are exposed to electromagnetic energy, pressure, mechanical damage, post-sorting centrifugation and cryopreservation (Seidel, 2007). The sexing procedure and subsequent cryopreservation modifies membrane stability, sperm motility, and acrosome integrity (Gosálvez et al., 2011). As a result, these modifications to the sperm cells result in reduced fertilization capacity (Mocé et al., 2006). The reduced P/AI often observed with sexed semen is one of the main reasons that the beef industry has been slow to use and adopt this technology. Although improvements to the sexing procedure have

compensated for some of the cellular damage, multiple efforts have still noted reductions in P/AI using sexed semen compared to results achieved using conventional semen.

Due to the damage that sexed sperm undergo, it is hypothesized that sexed semen has a reduced lifespan after deposition in the female reproductive tract compared to conventional semen (Schenk et al., 2009). Results from multiple experiments suggest that delaying the time of insemination until closer to ovulation results in more favorable P/AI with sexed semen (Bombardelli et al., 2016; Sales et al., 2011; Thomas et al., 2014). Inseminating closer to the time of ovulation with sexed semen is hypothesized to result in a greater number of viable sperm in the female reproductive tract when ovulation occurs.

Cows that express estrus prior to FTAI are more likely to become pregnant to FTAI with sexed semen compared to cows that fail to express estrus. Estrus synchronization protocols differ with respect to the distribution of estrus activity produced, and a protocol in which a greater proportion of cows express estrus early in the distribution may provide an opportunity to increase P/AI with sexed semen. 7 & 7 Synch is an estrus synchronization protocol that uses a presynchronization approach to tighten the distribution of the timing of estrus expression among a group of cows (Bonacker et al., 2020b, 2020a). When compared to the 7-Day CO- Synch + CIDR protocol, 7 & 7 Synch resulted in a greater proportion of cows expressing estrus prior to FTAI with improved P/AI using either conventional and sexed semen (Andersen et al., 2020). The 7 & 7 Synch protocol results in a large proportion of cows expressing estrus within a narrow window of time; however, no research efforts to date have evaluated alternative timepoints for fixed-time AI when sexed semen is used following this protocol. 7 & 7 Synch may provide a platform by which timing-related improvements with sex-sorted

semen can be made. We hypothesized that delaying timing of insemination from 66 h to 72 h with 7 & 7 Synch would increase the proportion of cows expressing estrus prior to FTAI as well as P/AI to sexed semen

Materials and Methods

2.1 Animals and Estrus Synchronization

The 7 & 7 Synch protocol (Figure 3.1) was used to synchronize estrus among postpartum beef cows (n = 414) of varying age, body condition score (BCS), and days postpartum (DPP). The experiment was conducted across multiple locations (n = 4) in Missouri in the spring and fall breeding seasons of 2020. Cows received administration of 500 µg cloprostenol sodium (PG; Estrumate, Merck Animal Health, Madison, NJ) and insertion of 1.38 g progesterone-releasing intravaginal insert (CIDR[®], Zoetis, Madison, NJ) on Day 0, administration of 100 µg gonadorelin acetate (GnRH; Fertagyl, Merck Animal Health, Madison, NJ) on Day 7, and administration of PG coincident with removal of CIDR on Day 14. Within each location, cows were blocked based on age, DPP and BCS (Richards et al., 1986); 1 to 9 scale; 1 = emaciated and 9 = obese) and randomly assigned to receive FTAI on Day 17 at 66 h or 72 h after CIDR removal and PG administration.

2.2 Estrus Detection

Estrus detection aids (Estrotect[™], Rockway Inc, Spring Valley, WI) were applied to all cows on Day 14 at the time of PG administration and CIDR removal, and estrus detection aid activation was recorded at the time of FTAI on Day 17. Estrus detection aid activation was scored (Pohler et al., 2016) on a scale of 0 to 4 (0 = missing patch; 1 = 0-25%

activated; 2 = 25-50% activated; 3 = 50-75% activated; and 4 = 75-100% activated).

Expression of estrus prior to FTAI was defined as 50% or greater of the estrus detection aid activated or a missing estrus detection aid (patch score 0, 3, 4).

2.3 Artificial Insemination

Within each location, cows were blocked based on age, DPP, and BCS and preassigned randomly within block to receive FTAI either at 66 h or 72 h after CIDR removal and PG administration. To minimize potential confounding in locations in which FTAI was performed by two technicians, cows were also blocked on these criteria and pre-assigned to technician. Semen was collected from two commercially available AI bulls, and units of sex-sorted and conventional semen were produced from contemporaneous ejaculates. Sexed semen from each bull passed the standard quality control criteria. Units of sex-sorted semen were produced using the SexedULTRATM Genesis III sorting technology (Sexing Technologies, Navasota, TX) with 4.0×10^6 live cells per 0.25 mL straw prior to freezing with a marketed level of > 90% accuracy for the desired sex. Sex-sorted units were sorted to either contain X (Bulls A, B) or Y-bearing chromosomes (Bull A). Bulls varied between locations, but, within location, all treatments used semen from the same bull or bulls. Cows that expressed estrus prior to FTAI were inseminated with sexed semen whereas cows that failed to express estrus were inseminated with conventional semen. In all locations, cows were exposed to bulls for natural service beginning 14 days after FTAI.

2.4 Pregnancy Diagnosis

Pregnancy per artificial insemination (P/AI) was determined 70 to 75 days after artificial insemination by transrectal ultrasonography, using a SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer (SonoSite, Inc., Bothwell, WA) in all locations. Pregnancies resulting from AI were distinguished from pregnancies resulting from natural service based on fetal size.

2.5 Statistical Analysis

Statistical analyses were performed using SAS (SAS 9.4 Inst. Inc., Cary, NC). A general linear model (PROC GLM) was used to confirm balance across treatments with respect to DPP, BCS, and age of cows. A mixed model (PROC GLIMMIX) using the binary distribution link logit function was used to evaluate the proportion of cows expressing estrus prior to FTAI. Variables tested for inclusion were treatment (66 vs 72 h), age, BCS, and DPP. A mixed model (PROC GLIMMIX) using the binary distribution link logit function was used to evaluate the proportion of estrous cows that became pregnant to FTAI. Variables tested for inclusion were treatment (66 vs 72 h), age, BCS, and DPP. Location, technician and sex (e.g. chromosomal units of the sorted units) were included as random effects.

Results

3.1 Summary

Age, DPP, body condition score (BCS), and the proportion of cows expressing estrus prior to FTAI are summarized by treatment and location in Table 3.1. Age, DPP, and BCS did not differ between treatments within each location.

1.2 Estrus Expression

The proportion of cows expressing estrus prior to FTAI in each treatment is summarized in Table 1. Estrus detection patch score proportions are summarized in Table 3.2. The proportion of cows expressing estrus prior to FTAI did not differ ($P > 0.10$) between treatments (66 h: 71% [146/205]; 72 h: 76% [158/209]). Additionally, age and DPP did not have a significant effect on estrus expression. The proportion of cows that expressed estrus prior to FTAI was affected by BCS ($P < 0.01$) across treatments, with a greater proportion of cows expressing estrus in greater BCS compared to cows in lesser BCS.

1.3 Pregnancy Rate

P/AI of estrous cows is summarized in table 3.3 Analysis of P/AI was restricted to cows that expressed estrus prior to FTAI and received sex-sorted semen. Among estrous cows, P/AI did not differ between treatments (66 h: 45% [68/146]; 72 h: 40% [63/158]).

Additionally, age, BCS, DPP, and location did not affect ($P > 0.10$) P/AI.

Discussion

In multiple previous experiments, treatment of cows with the 7 & 7 Synch protocol resulted in a large proportion of cows expressing estrus following PG and CIDR removal (Andersen et al., 2020; Bonacker et al., 2020a). Cows treated with 7 & 7 Synch had a greater ($P < 0.005$) proportion of cows expressing estrus early in the distribution of the onset of estrus activity compared to the 7-Day CO-Synch + CIDR (Bonacker et al., 2020a). A protocol that increases the proportion of cows expressing estrus early in the distribution of the onset of estrus activity could optimize the number of cows that are being serviced closer to the timing of ovulation. Due to the synchrony and timing of estrus activity, we hypothesized that delaying FTAI with 7 & 7 Synch could increase P/AI of estrous cows receiving sexed semen for FTAI. However, later timing of FTAI did not improve P/AI in the present experiment. These data are similar to results observed in previous experiments with the 7-Day CO-Synch + CIDR and 5-Day CO-Synch + CIDR protocols (Hall et al., 2017b; Nash et al., 2012). When evaluated among cows treated with the 7-Day CO-Synch + CIDR protocol, prolonging the interval from CIDR removal to FTAI from 66 to 74 hours did not result in improved P/AI with sexed semen (Nash et al., 2012). When evaluated among postpartum cows treated with the 5-Day CO-Synch + CIDR protocol, prolonging the interval from CIDR removal to FTAI interval from 72 to 80 hours did not improve P/AI with sexed semen despite resulting in a greater proportion of cows expressing estrus (Hall et al., 2017).

Recent experiments among heifers have suggested delaying FTAI with sexed semen could potentially result in improvements to P/AI, but that these improvements have been protocol-dependent. When administering PG 7 days prior to the 7-Day CO-

Synch + CIDR protocol and delaying insemination with sexed semen from 54 h to 72 h, an improvement in P/AI with sexed semen was observed among heifers (Oosthuizen et al., 2021). A greater proportion of heifers that did not receive presynchronization had expressed estrus prior to FTAI when FTAI was performed at 72 h compared to 54 h. Similarly, results from an experiment evaluating later timepoints for split-time AI among heifers treated with the 14-Day CIDR-PG protocol showed a significant increase in the proportion of heifers expressing estrus by 72 h compared to 66 h (Ketchum et al., 2021). Although no significant improvement with P/AI with sexed semen was noted in this particular experiment, a trial with greater numbers may be warranted to further evaluate later timing of FTAI with sexed semen among heifers treated with the 14-Day CIDR-PG protocol. Increasing the proportion of heifers expressing estrus prior to FTAI by delaying insemination could provide a method to increase the number of animals being serviced with sexed semen.

Several investigators have suggested that sexed semen has increased sensitivity to the timing of insemination in relation to ovulation and onset of estrus (Bombardelli et al., 2016; Sales et al., 2011). It has been proposed that delaying insemination with sexed semen until closer to the timing of ovulation increases P/AI with sexed semen (Bombardelli et al., 2016; Sales et al., 2011). With conventional semen, P/AI is optimized when insemination occurs approximately 12-18 hours after the onset of estrus (Dalton et al., 2001; Dransfield et al., 1998; Hockey et al., 2010; Trimberger, 1948), as ovulation occurs approximately 24-32 hours after the onset of estrus (Brewster and Cole, 1941; Walker et al., 1996). Since the fertile lifespan of sexed semen can be reduced relative to that of conventional semen, the optimal timing of AI with sexed semen may differ from

the timing traditionally recommended as optimal for conventional semen. Therefore, it has been hypothesized that P/AI with sexed semen is optimized when insemination occurs 18-24 hours after the onset of estrus in order to shorten the interval from insemination to ovulation (Schenk et al., 2009; Seidel et al., 1999). Although several investigators have observed an increase in P/AI with sexed semen when insemination was delayed closer to the timing of ovulation (Bombardelli et al., 2016; Sales et al., 2011), in the present experiment, delaying FTAI from 66 to 72 hours did not improve P/AI with sexed semen. Modifying the timing of FTAI may result in AI being performed too late or too early for subsets of animals within the group. The lifespan of the oocyte following ovulation is shorter than the lifespan of sperm following insemination (Hawk, 1987, 1983). Depending on the estrus synchronization protocol and the distribution of the timing of estrus expression generated, delaying the timing of FTAI with sexed semen may result in placement of sperm occurring too late relative to the timing of ovulation for a subset of cows, even if the conception rate of another subset of cows is marginally improved due to more favorable timing. Timing of insemination clearly affects fertility in general, and insemination closer to ovulation could be particularly critical for some bulls (Dalton et al., 2001; Dransfield et al., 1998; MacMillan and Watson, 1975; Saacke et al., 2000; Trimberger, 1948). This could likewise be the case for sexed versus conventional semen from some bulls, as bulls appear to differ with respect to the resiliency of their sperm cells to the sex-sorting procedure and subsequent cryopreservation (Hall et al., 2017).

Variation in timing of estrus expression is dependent upon an estrus synchronization protocol's efficacy in controlling the estrous cycle. For example, the 7-

Day CO-Synch + CIDR and the 5-Day CO-Synch + CIDR protocols begin with an initial administration of GnRH to synchronize the follicular wave. In order to ovulate in response to administration of exogenous GnRH, a follicle has to be of sufficient physiological maturity (Bo et al., 1995). However, cows begin the protocol in varying stages of the estrous cycle, and therefore not every cow will have a physiologically mature, LH-responsive follicle present on the ovary. Specifically, literature has observed that approximately 65% of cows fail to ovulate in response to GnRH administered at the start of an estrus synchronization protocol (Geary et al., 2000). To address the variation in response to GnRH and subsequent variation in timing of estrus expression, the 7 & 7 Synch protocol incorporates a presynchronization treatment to increase the proportion of cows with a physiologically mature follicle at the time of GnRH administration. By doing so, variation among cows in stage of cycle among cows is reduced in comparison to variation observed among cows treated with the 7-Day CO-Synch + CIDR protocol (Bonacker et al., 2020a). Consequently, synchrony of estrus expression is enhanced, with a greater proportion of cows expressing estrus in the early portion of the distribution of onset of estrus activity. Minimizing the variance in timing of estrus onset and, therefore, timing ovulation within the group of animals may explain the much of the improvement observed among cows in pregnancy rates to FTAI following 7 & 7 Synch.

At this power of test, P/AI of estrous cows did not differ when FTAI was performed at 66 or 72 h (66 h: 45% [68/146]; vs 72 h: 40%; [63/158]). The numerical reduction in P/AI of estrous cows when FTAI was performed at 72 h is of a magnitude that would be economically significant, however, and failure to detect a difference should not be misinterpreted as demonstration of equivalence. A larger sample size would be

required to determine whether 72 h is an acceptable timepoint at which to perform FTAI with sexed semen after treatment with 7 & 7 Synch.

Cows that fail to express estrus prior to FTAI have particularly decreased P/AI when sexed semen is used, perhaps due to the reduced sperm lifespan and timing of ovulation not being optimally aligned (de Sá Filho and Vasconcelos, 2011; Perry et al., 2020; Thomas et al., 2014, 2017, 2019). In order to optimize P/AI with sexed semen, one recommendation is to restrict use of sexed semen to cows that have expressed estrus prior to AI while using conventional semen on cows that failed to express estrus (Thomas and Andersen, 2020). Estrus synchronization protocols that result in a greater proportion of cows expressing estrus prior to FTAI may increase the adoption of sexed semen in the industry. In the present experiment, only a slight numerical increase in the proportion of cows expressing estrus prior to FTAI was observed when FTAI was performed at 72 h rather than 66 h. This was perhaps due to a large proportion of cows that express estrus prior to 66 h following 7 & 7 Synch. Previous experiments using other protocols have observed a significant increase in the proportion of animals expressing estrus prior to FTAI when FTAI is delayed until a later time (Hall et al., 2017; Ketchum et al., 2021). Increasing the proportion of cows expressing estrus prior to FTAI by delaying insemination would result in a greater number of cows being serviced with sexed semen. However, delaying the timing of insemination with sexed semen would only be a reliable method if the altered timing of FTAI did not compromise the conception rate to sexed semen. A trial with a larger sample size might provide sufficient power to observe differences in P/AI when delaying the timing of insemination.

In conclusion, extending the PG-AI interval of the 7 & 7 Synch protocol from 66 h to 72 h did not improve P/AI of estrous cows receiving sex-sorted semen. This is similar to results when later timing of FTAI with sexed semen was evaluated following other protocols for postpartum beef cows (Nash et al., 2021; Hall et al., 2017b). Improvement in P/AI with sexed semen is critical for widespread adoption of this reproductive technology in the beef industry. Currently, restricting use of sexed semen to cows that express estrus remains the most effective strategy to mitigate the degree to which P/AI is reduced when using sexed semen. Although this experiment did not observe increased P/AI with sexed semen when delaying timing of FTAI, use of the 7 & 7 Synch does result in a large proportion of cows expressing estrus prior to the time of FTAI. This provides the opportunity to generate a large proportion of females eligible to be serviced with sexed semen at the standard recommended time of 66 h, and there is no clear evidence from this experiment that recommended timing of FTAI should be altered.

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Table 3.1. Cow age, days postpartum (DPP), body condition score (BCS), and proportion expressing estrus prior to fixed-time artificial insemination (FTAI) by treatment and location.

Treatment ¹		Estrus expression prior to FTAI ⁴				
Location	N	Age	DPP ²	BCS ³	Proportion	%
66 h	205	4.6 ± 2.2	76.6 ± 14.6	5.4 ± 0.5	146/205	71
Location 1	42	7.8 ± 2.5	76.3 ± 11.6	5.5 ± 0.4	31/42	74
Location 2	52	2.9 ± 1.0	81.8 ± 16.4	5.2 ± 0.4	26/52	50
Location 3	67	5.0 ± 0.0	72.1 ± 14.9	5.7 ± 0.3	52/67	78
Location 4	44	3.0 ± 0.0	77.4 ± 16.4	5.3 ± 0.4	37/44	84
72 h	209	4.7 ± 2.2	75.5 ± 14.2	5.5 ± 0.5	158/209	76
Location 1	46	7.7 ± 2.4	74.8 ± 12.2	5.8 ± 0.45	37/46	80
Location 2	53	3.1 ± 1.2	80.4 ± 15.0	5.2 ± 0.5	28/53	53
Location 3	65	5.0 ± 0.0	69.7 ± 15.6	5.7 ± 0.3	52/65	80
Location 4	44	3.0 ± 0.0	78.7 ± 12.7	5.3 ± 0.5	41/45	91

Data presented as mean ± SD

¹See Figure 3.1 for treatment descriptions.

²DPP calculated from calving date to FTAI date

³BCS of cows recorded on Day -17 using a scale of 1-9 (1 = emaciated and 9 = obese)

⁴Proportion of cows expressing estrus prior to FTAI, based on ≥ 50% of the patch coating rubbed off or a missing patch

Table 3.2. Estrus detection patch scores¹ by treatment.²

Patch Score	Proportion	%
FTAI at 66 h		
0	13/205	6
1	48/205	23
2	11/205	5
3	9/205	4
4	122/205	60
FTAI at 72 h		
0	20/209	10
1	43/209	21
2	7/209	3
3	9/209	4
4	130/209	62

¹ Estrus detection patches were scored on a system of 0 to 4: 0 = missing patch; 1 = 0-25% activated; 2 = 25-50% activated; 3 = 50-75% activated; and 4 = 75-100% activated (Pohler et al., 2016).

²Fixed-time artificial insemination occurred at 66 h or 72 h after PG administration on Day 0

Table 3.3. Pregnancy rates (P/AI)¹ to fixed-time artificial insemination (FTAI) among estrous cows receiving sex-sorted semen.²

Treatment ³	P/AI	
	Proportion	%
FTAI at 66 h	68/146	45
Location 1	18/31	58
Location 2	10/26	35
Location 3	16/52	31
Location 4	24/37	65
FTAI at 72 h	63/158	40
Location 1	12/37	32
Location 2	16/28	57
Location 3	18/52	35
Location 4	17/41	41

¹P/AI was determined via transrectal ultrasonography 70-75 days after AI

²Fixed-time artificial insemination was performed either at 66 or 72 hours after administration of PG

³FTAI was performed at 66 h or 72 h after PG administration on Day 0

Figure 3.1. Treatment schedule. Estrus was synchronized using the 7 & 7 Synch protocol. Cows received administration of PG and insertion of CIDR on Day 0, administration of GnRH on Day 7, and administration of PG coincident with CIDR removal on Day 14. Estroject™ estrus detection aids were applied to all cows on Day -3 and patch activation score were recorded at the time of FTAI on Day 0. Cows were blocked based on age, DPP, and BCS and randomly assigned to received FTAI at 66 h (n=205) or 72 h (n=209). Cows that expressed estrus at FTAI received sexed semen whereas cows that failed to express estrus received conventional semen.

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