ENHANCEMENT OF LONG-TERM CIDR-BASED ESTRUS
SYNCHRONIZATION PROTOCOLS TO OPTIMIZE TIMED
ARTIFICIAL INSEMINATION RESULTS FOR BEEF HEIFERS AND
COWS

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ENHANCEMENT OF LONG-TERM CIDR-BASED ESTRUS
SYNCHRONIZATION PROTOCOLS TO OPTIMIZE TIMED ARTIFICIAL
INSEMINATION RESULTS FOR BEEF HEIFERS AND COWS

presented by Jordan Matthew Thomas

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DEDICATION

When I applied to graduate school in the fall of 2011, I thought I knew what I was getting into. Now nearing the end of my graduate program and reflecting back, I can honestly say I did not have even the slightest idea then of what the next few years had in store. I expected there would be a lot of hard work, stress, caffeine, etc.---all of which came in spades, especially the caffeine. Knowing the faculty with whom I would be studying, I knew I could also expect a great education. But I never anticipated all the new places I would have the opportunity to visit and, more importantly, the new people I would have the good fortune to meet. This dissertation is dedicated to those people, particularly the farm and ranch families with whom I have had the privilege of working collaboratively these last few years. I have learned so much from you, not only because of the data generated through this research, but because of the opportunities to spend time on your operations and to get to know you personally. Time and time again, you welcomed me into your homes and treated me like part of the family. I am not often one to get sentimental, but I get a lump in my throat as I look back on those experiences and remember your hospitality. Professionally, the research presented here would not have been possible without your animals, facilities, and labor. And personally, the experiences that culminated in this research would not have been so rich if it were not for your friendship.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
BCS	Body condition score
CIDR	Controlled internal drug release insert
CL	Corpus luteum
cm	Centimeter(s)
d	Day(s)
E ₂	Estradiol-17 β
FDA	Food and Drug Administration
FSH	Follicle stimulating hormone
FTAI	Fixed-time artificial insemination
g	Gram(s)
GnRH	Gonadotropin-releasing hormone
h	Hour(s)
hd	Head
im	Intramuscular
kg	Kilogram(s)
LFD	Largest follicle diameter
LH	Luteinizing hormone
mg	Milligram(s)
MGA	Melengestrol acetate
ml	Milliliter(s)
mm	Millimeter

ng	Nanogram(s)
P ₄	Progesterone
PG	Prostaglandin F _{2α}
pg	Picogram(s)
RTS	Reproductive tract score
s	Second(s)
SAS	Statistical Analysis System
SE	Standard error
STAI	Split-time artificial insemination
μg	Microgram(s)
yr	Year(s)

**ENHANCEMENT OF LONG-TERM CIDR-BASED ESTRUS
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ABSTRACT

A series of experiments was conducted to evaluate alterations to long-term progestin-based estrus synchronization protocols that may result in enhanced pregnancy rates to timed artificial insemination (AI). In the first series of experiments, a new approach called the 9-d CIDR-PG protocol was developed and tested in mature beef cows (Chapters 2-4) and also evaluated in beef heifers (Chapter 5). In the second series of experiments (Chapters 6 and 7), a modified timed AI approach called split-time AI was evaluated in conjunction with use of sex-sorted semen.

In Chapter 2, a pilot experiment was designed to test the hypothesis that administration of prostaglandin $F_{2\alpha}$ prior to progestin treatment would allow for a reduced duration of progestin treatment in a long-term progestin-based estrus synchronization protocol. A modified pre-synchronization treatment was compared to a standard long-term controlled internal drug release (CIDR) treatment, and treatments were compared on the basis of ovarian follicular dynamics, estrous response rate, synchrony of estrus expression, and pregnancy rates resulting from timed AI in postpartum beef cows. Estrus was synchronized for 85 cows, with cows assigned to one

of two treatments based on age, days postpartum (DPP), and body condition score (BCS). Cows assigned to the 14-d CIDR-PG protocol received a CIDR insert (1.38 g progesterone) on Day 0, CIDR removal on Day 14, and administration of PG (25 mg im) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and administration of PG on Day 30. In both treatments, split-time AI was performed based on estrous response. At 72 h after PG (Day 33), cows having expressed estrus received timed AI; cows that failed to express estrus by 72 h received timed AI 24 h later (96 h after PG on Day 34), with gonadotropin-releasing hormone (GnRH; 100 µg im) administered to non-estrous cows. Estrus detection transmitters were used from CIDR removal until AI to determine onset time of estrus expression both after CIDR removal and after PG. Ovarian ultrasonography was performed at CIDR removal on Day 14, at PG on Day 30, and at AI on Day 33 or Day 34. At CIDR removal on Day 14, diameter of the largest follicle present on the ovary (LFD) was similar between treatments. The proportion of cows expressing estrus following CIDR removal tended to be higher ($P = 0.09$) among cows assigned to the 9-d CIDR-PG treatment (93%; 40/43) than among cows assigned to the 14-d CIDR-PG treatment (81%; 34/42). Following PG, a significantly higher proportion ($P = 0.02$) of cows expressed estrus following synchronization with the 9-d CIDR-PG treatment (91%; 39/43) than the 14-d CIDR-PG treatment (69%; 29/42). Consequently, pregnancy rate to TAI tended to be increased ($P = 0.09$) among the 9-d CIDR-PG treatment (76.7%; 33/43) compared to the 14-d CIDR-PG (59.5%; 25/42). In summary, a long-term CIDR-based estrous synchronization protocol for postpartum beef cows was enhanced through administration of PG at CIDR insertion

and at CIDR removal.

In Chapter 3, an experiment was designed to evaluate endocrine parameters, ovarian dynamics, and pregnancy rates to fixed-time artificial insemination (FTAI) following the 9-d CIDR-PG protocol in comparison to the 14-d CIDR-PG protocol. While both are long-term protocols using CIDR treatment for presynchronization, the 9-d CIDR-PG protocol differs from the 14-d CIDR-PG protocol in that prostaglandin $F_{2\alpha}$ (PG) is administered at CIDR insertion and removal to facilitate a decreased length of progestin treatment and potentially enhance response to the presynchronization treatment. Estrus was synchronized for 393 mature beef cows across five locations. Treatments were represented in each location, and cows within each location were randomly assigned to one of the two protocols based on age, DPP, and BCS. Cows assigned to the 14-d CIDR-PG treatment received a CIDR insert on Day 0 with removal of CIDR on Day 14, and 25 mg PG 16 d after CIDR removal on Day 30. Cows assigned the 9-d CIDR-PG treatment received 25 mg PG and a CIDR insert on Day 5; 25 mg PG and removal of CIDR on Day 14; and 25 mg PG 16 d after CIDR removal on Day 30. In both treatments, cows received FTAI on Day 33, 72 h after PG. All cows were administered 100 μ g GnRH concurrent with insemination. For a subset of animals in each treatment, ovarian ultrasound was performed and blood samples were collected for determination of serum estradiol concentrations at CIDR removal, PG administration, and FTAI. Protocols were compared on the basis of estrous response and pregnancy rate resulting from FTAI. Serum estradiol concentrations, follicle size, and estrous response did not differ based on treatment. However, cows assigned to the 9-d CIDR-PG protocol tended to achieve greater FTAI pregnancy rates than cows assigned to the 14-d CIDR-PG protocol (62% versus 52%; $P =$

0.07). Across treatments, greater pregnancy rates tended ($P = 0.10$) to be achieved by cows that expressed estrus prior to FTAI (69% for 9-d CIDR-PG, 58% for 14-d CIDR-PG) than by cows that failed to express estrus (55% for 9-d CIDR-PG, 47% for 14-d CIDR-PG). In summary, the 9-d CIDR-PG protocol is an effective protocol for synchronization of estrus among mature beef cows, and pregnancy rates to FTAI tended to be improved through use of the 9-d CIDR-PG compared to the 14-d CIDR-PG protocol.

In Chapter 4, response of mature beef cows following the 9-d CIDR-PG protocol was characterized in reference to a short-term protocol commonly used in commercial production. Mature beef cows ($n = 480$) across four locations were assigned based on age and days postpartum to either the 9-d CIDR-PG protocol [25 mg PG and insertion of a CIDR on Day -28; 25 mg PG and CIDR removal on Day -19; and 25 mg PG on Day -3] or the 7-d CO-Synch + CIDR protocol [100 μ g GnRH and CIDR insertion on Day -10; 25 mg PG and CIDR removal on Day -3]. On Day -3, estrus detection aids (Estrotect[®]) were applied, and a subset of cows ($n = 203$) were fitted with radiotelemetric, pressure-sensitive devices (HeatWatch[®]) to characterize timing of estrus onset. Split-time artificial insemination (STAI) was performed on Day 0 or 1 based on timing of estrus expression, with cows failing to express estrus by Day 1 administered 100 μ g GnRH at AI. In Locations 1, 2, and 3, blood samples were collected for radioimmunoassay on Day -38 and Day -28 to determine pretreatment estrous cyclicity status based on serum progesterone concentrations. Additional blood samples were collected at PG administration and at STAI for determination of serum estradiol concentrations. Transrectal ovarian ultrasound was performed at PG administration and AI for a subset of

cows (n = 154) to assess ovarian follicle size. Response following the 9-d CIDR-PG protocol differed ($P < 0.05$) from the 7-d CO-Synch + CIDR protocol with respect to mean interval from PG to estrus onset (71.2 ± 1.3 h versus 56.5 ± 1.6 h), variance in interval to estrus, ovulatory follicle size at AI (13.7 ± 0.2 mm versus 14.6 ± 0.2), and serum estradiol concentrations at AI (6.9 ± 0.3 pg/ml versus 6.0 ± 0.3 pg/ml). Similar pregnancy rates to AI were obtained among cows following the 7-d CO-Synch + CIDR (66%; 163/247) and 9-d CIDR-PG (70%; 163/233) treatments. In summary, when compared among mature beef cows to the 7-d CO-Synch + CIDR protocol, the 9-d CIDR-PG protocol resulted in later timing and enhanced synchrony of estrus expression, greater serum estradiol concentrations and smaller follicle size at AI, and similar pregnancy rates following STAI.

In Chapter 5, two long-term, CIDR-based estrus synchronization protocols were evaluated among *Bos indicus*-influenced and *Bos taurus* beef heifers. Treatments were evaluated on the basis of estrous response and pregnancy rate resulting from FTAI, and these outcomes were analyzed retrospectively relative to reproductive tract score (RTS; Scale 1-5) at treatment initiation. Estrus was synchronized for 1139 heifers in three locations, and heifers were assigned to one of two treatments within each location based on RTS. Heifers assigned to the 14-d CIDR-PG protocol received a CIDR insert on Day 0, CIDR removal on Day 14, administration of PG (25 mg im) on Day 30, and administration of GnRH (100 µg im) concurrent with FTAI on Day 33, 66 h after PG. Heifers assigned to the 9-d CIDR-PG protocol received administration of PG concurrent with CIDR insertion on Day 5, administration of PG concurrent with CIDR removal on Day 14, administration of PG on Day 30, and administration of GnRH concurrent with

FTAI on Day 33, 66 h after PG. Estrus detection aids were applied at CIDR removal on Day 14 and at PG on Day 30 to evaluate estrous response rate. Mean RTS differed ($P < 0.0001$) based on biological type due to higher rates of estrous cyclicity (RTS 4 and 5) among *Bos taurus* heifers (72%; 416/574) than among *Bos indicus*-influenced heifers (27%; 150/565). The proportion of heifers expressing estrus following CIDR removal was greater ($P = 0.01$) among heifers assigned to the 14-d CIDR-PG treatment (88%; 492/559) compared to the 9-d CIDR-PG treatment (83%; 480/580). Estrous response following CIDR removal was also higher ($P < 0.0001$) among *Bos taurus* (95%; 547/574) compared to *Bos indicus*-influenced (75%; 425/565) heifers. Rate of estrous response prior to FTAI did not differ significantly based on treatment but was higher ($P < 0.0001$) among *Bos taurus* heifers (60%; 344/574) than among *Bos indicus*-influenced heifers (45%; 253/565). However, the effect of biological type on estrous response was not significant when RTS was included in the model, as RTS significantly ($P < 0.0001$) affected the rate of estrous response both at CIDR removal and prior to FTAI. Across treatments and biological types, heifers that expressed estrus prior to AI achieved higher ($P < 0.0001$) AI pregnancy rates than heifers failing to express estrus. Pregnancy rates to FTAI did not differ significantly based on treatment in either biological type. Higher rates of estrous cyclicity among *Bos taurus* heifers resulted in higher FTAI pregnancy rates among *Bos taurus* (51%; 290/574) compared to *Bos indicus*-influenced heifers (39%; 218/565). However, pregnancy rates of respective RTS did not differ based on biological type. In summary, long-term CIDR-based protocols provide a simple, effective method of estrus synchronization in *Bos indicus*-influenced and *Bos taurus* beef heifers. Moreover, these results highlight the importance of management practices that result in high rates of

estrous cyclicity prior to protocol initiation, particularly among later maturing breeds and biological types.

In Chapter 6, an experiment was designed to evaluate the relative fertility of SexedULTRA™ sex-sorted semen compared to conventional, non-sex-sorted semen when used among beef heifers in conjunction with split-time AI following the 14-d CIDR-PG protocol. Units of conventional semen were generated with 25.0×10^6 live cells per 0.5 ml straw prior to freezing, and units of sex-sorted semen were generated using the SexedULTRA™ Genesis III sorting technology with 4.0×10^6 live cells per 0.25 ml straw prior to freezing. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%. Estrus was synchronized in 851 heifers at four locations using the 14-d CIDR-PG protocol: CIDR insert on Day 0, CIDR removal on Day 14, and administration of PG (25 mg im) on Day 30. Estrus detection aids were applied at PG on Day 30 to evaluate estrous response rate, and split-time AI was performed based on estrous response. At 66 h after PG (Day 33), heifers having expressed estrus received timed AI. Heifers failing to express estrus by 66 h received timed AI 24 h later (90 h after PG on Day 34). Heifers failing to express estrus by 90 h were administered GnRH (100 µg im) concurrent with AI. Heifers were preassigned to treatment (insemination with either conventional or SexedULTRA™ sex-sorted semen), and treatments were balanced within each location based on source, reproductive tract score, and weight. Heifers were exposed for natural service beginning 14 d after AI for the remainder of a 60 d breeding season. Pregnancy rates to AI across locations tended to be higher ($P = 0.09$) for heifers inseminated with conventional semen (60%; 257/429) compared to sex-sorted semen (52%; 218/422). Higher pregnancy rates to AI ($P <$

0.0001) were obtained among heifers that expressed estrus prior to AI than among heifers that failed to express estrus prior to AI at 90 h. Total pregnancy rates at the end of the 60 d breeding season did not differ between heifers that received sex-sorted semen at AI (89%; 376/422) and heifers that received conventional semen at AI (89%; 382/429). In summary, the pregnancy rates observed suggest that SexedULTRA™ sex-sorted semen can be used effectively for timed AI of beef heifers when split-time AI is performed following the 14-d CIDR-PG protocol.

In Chapter 7, fertility of SexedULTRA™ sex-sorted semen and conventional, non-sex-sorted semen was compared following either FTAI or STAI of mature suckled beef cows. Units of sex-sorted and conventional semen were produced using contemporaneous ejaculates from three commercially available AI sires. Units of conventional semen were generated with 25.0×10^6 live cells per 0.25 ml straw prior to freezing, and units of sex-sorted semen were generated using the SexedULTRA™ Genesis III sorting technology with 4.0×10^6 live cells per 0.25 ml straw prior to freezing. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%. Cows (n = 922) across seven locations were treated with the 7-d CO-Synch + CIDR protocol [administration of GnRH and insertion of a CIDR on Day -10, followed by administration of PG and removal of CIDR inserts on Day -3]. Cows were preassigned based on age, body condition score, and days postpartum to one of the following four treatments: FTAI with SexedULTRA™ sex-sorted semen, FTAI with conventional semen, STAI with SexedULTRA™ sex-sorted semen, or STAI with conventional semen. On Day -3, estrus detection aids (Estroject®) were applied. For cows in FTAI treatments, AI was performed on Day 0 at 66 h after PG administration and

CIDR removal, and 100 µg GnRH was administered concurrent with AI. For cows in STAI treatments, AI was performed on either Day 0 or 1, at 66 or 90 h after PG administration and CIDR removal, based on timing of estrus expression. On Day 1 at 90 h after PG administration and CIDR removal, 100 µg GnRH was administered concurrent with AI to any STAI-treated cows that had failed to express estrus. Total estrous response prior to AI was greater ($P < 0.001$) among cows receiving STAI (90%) compared to those receiving FTAI (80%) due to the additional 24 h for potential estrus expression when performing STAI. Greater pregnancy rates ($P < 0.0001$) were obtained among cows inseminated with conventional semen (68%; 315/464) compared to cows inseminated with SexedULTRATM sex-sorted semen (51%; 235/458). However, pregnancy rates did not differ between cows inseminated using a FTAI versus STAI approach, regardless of whether insemination was performed with conventional semen (69% versus 67%) or SexedULTRATM sex-sorted semen (50% versus 53%). Pregnancy rates to AI were affected ($P = 0.001$) by the interaction of bull and semen type. Greater pregnancy rates were obtained with conventional semen versus SexedULTRATM sex-sorted semen when using semen from Bull A (69% [111/161] versus 38% [59/157]; $P < 0.0001$) and Bull B (75% [121/161] versus 58% [92/157]; $P < 0.01$), whereas pregnancy rates to AI did not differ between conventional and SexedULTRATM sex-sorted when using semen from Bull C (58% [82/142] versus 58% [84/144]). In summary, greater pregnancy were obtained among cows receiving timed AI with conventional semen compared to SexedULTRATM sex-sorted semen, although the relative fertility of SexedULTRATM sex-sorted semen and conventional semen varied across bulls. Although likely resulting in lower average pregnancy rates than those obtained with conventional semen, use of SexedULTRATM

sex-sorted semen for timed AI of mature beef cows may result in pregnancy rates of a satisfactory level for some production settings.

CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

Commercial beef cattle production is seasonal in nature, with producers generally weaning all calves at a predetermined annual time point irrespective of variation in calf age. As a result, implementing a calving season of defined length is essential to ensure a calf crop of relatively uniform age and weight at weaning. Naturally, length of the calving season is a direct function of length of the breeding season; therefore, management for optimal breeding performance is imperative. The efficiencies associated with improved calf crop uniformity are myriad, and an exhaustive discussion of the impact of calving season length on beef cattle production efficiency is beyond the intended scope of this review. However, to provide broader context for this review, one key benefit of assisted reproductive technologies should be highlighted briefly: the potential to increase the proportion of females conceiving early in the breeding season and consequently calving early in the calving season.

Enhanced pregnancy success early in the breeding season skews the calving distribution earlier and therefore increases the average age and weight of calves at weaning (Dzuik and Bellows, 1983). For example, Schafer et al. (1990) reported that calves of females that became pregnant during a synchronized period were on average 13 d older and 9.5 kg heavier at weaning than calves of females in which no estrus synchronization was used. Enhancing the proportion of calves born early in the calving season also results in additional time postpartum for females to resume normal estrous cycles, thereby decreasing the likelihood that females will fail to become pregnant during

the subsequent breeding season. Since failure to become pregnant during the breeding season accounts for the most significant loss of reproductive efficiency among beef females (Bellows and Short, 1990), approaches that alleviate this reproductive failure result in cumulative enhancement of the reproductive performance of the herd over successive years (Lamb, 2015). Although assisted reproductive technologies must still be cost-effective in order to be profitable, these approaches represent tremendous opportunities for beef producers to improve production efficiency, particularly when considering the potential to use animals with high merit genetics for traits of production interest (Trenkle and Willham, 1977).

For the purposes of this review, discussion of assisted reproductive technologies will be largely restricted to estrus synchronization and artificial insemination, as these technologies are the focus of the research presented in this dissertation. Having previously reviewed (Thomas, 2013) the physiology of the estrous cycle, estrus synchronization, factors affecting fertility in artificial insemination programs, and unique considerations relative to use of sex-sorted semen, the present review will center around one overarching question: whether the body of literature suggests any opportunity to enhance timed AI pregnancy rates through improved estrous cycle control or through optimization of timing of insemination. Much attention will be given in particular to factors that may influence results when using sex-sorted semen. Therefore, this review is organized into the following specific areas of interest: (1) use of long-term, presynchronization-based protocols for estrous cycle control, (2) factors limiting successful use of sex-sorted semen in timed artificial insemination, and (3) research in the field of split-time artificial insemination.

USE OF LONG-TERM, PRESYNCHRONIZATION-BASED PROTOCOLS FOR ESTROUS CYCLE CONTROL

Several protocols and strategies for estrus synchronization have been previously published. Many of these are cost-effective, simple to follow, and result in reasonably high pregnancy rates. However, while pregnancy rates are generally satisfactory when insemination is performed based on detected estrus, few protocols generate the degree of synchrony required to achieve optimal pregnancy rates following an appointment breeding or fixed-time artificial insemination (FTAI). In addition to factors that improve general fertility and embryonic survival, continued improvement in pregnancy rates to FTAI is likely dependent upon enhancements in estrus synchronization that improve (1) total estrous response rate prior to AI and (2) synchrony of estrus and ovulation. Synchrony of estrus expression is largely dependent upon synchrony of ovarian follicular waves within the group of females undergoing estrus synchronization. Published protocols employ varying strategies to establish this follicular synchrony. For example, many protocols rely on administration of gonadotropin-releasing hormone (GnRH) early in the protocol, such as the commonly used 7-d CO-Synch + CIDR (Lamb et al., 2006) and 5-d CO-Synch + CIDR (Bridges et al., 2008) protocols. In these protocols, follicular synchrony is dependent upon successful response to GnRH. Numerous other protocols make use of exogenous estradiol esters to establish follicular synchrony; however, estradiol is not approved for legal use in estrus synchronization programs in many countries, including the United States.

Presynchronization as a Concept. The development of short-term protocols to synchronize estrus prior to timed AI for beef females has largely stemmed from the

development of estrus synchronization methods for dairy cows, with the Ovsynch protocol in particular having a significant impact (Macmillan and Thatcher, 1991; Pursley et al., 1995; Pursley et al., 1997). Research efforts in dairy cows quickly highlighted the fact that response of cows to GnRH administration varies greatly based on stage of the estrous cycle (Vasconcelos et al., 1999; Moreira et al., 2000). This variation in response to GnRH contributed to variability at later points in the protocol with respect to follicle size, circulating progesterone concentrations, timing of CL regression, and pregnancy rates to FTAI. Further research efforts to minimize this variability resulted in a new concept in the field of estrus synchronization: presynchronization programs. Rather than simply accepting that not every female will respond to GnRH administration on Day 0 of a protocol, presynchronization treatments in advance of GnRH administration are intended to decrease variation in stage of the estrous cycle and therefore improve response rates to GnRH.

Moreira et al. (2001) evaluated a presynchronization approach for use prior to Ovsynch, employing PG administration to manage stage of the estrous cycle. Administration of PG occurred twice, with a 14 d interval between PG administrations and the final administration of PG occurring twelve days before initiation of an Ovsynch protocol. Presynchronization using this approach, which became known as Presynch-12, enhanced pregnancy rates to FTAI among cyclic cows when compared to an Ovsynch protocol in which no presynchronization was used. Numerous further research efforts were undertaken to determine the optimal schedule for PG administration (Presynch-10, Presynch-11, Presynch-14, etc.), and several alternative presynchronization programs have been developed using both administration of PG and GnRH in the treatment

schedule (Double Ovsynch, G6G, etc.). For an extensive discussion of the development of Ovsynch and of the varying presynchronization protocols used in dairy cows prior to Ovsynch, see review by Wiltbank and Pursley (2014). Ultimately, the concept of presynchronization has become widely accepted by both researchers and practitioners in the field of dairy cattle reproduction.

In contrast, the concept of presynchronization prior to a short-term protocol has not been embraced widely by producers in the beef industry or by researchers in the field of beef cattle reproduction. This is likely due to the fact that implementing presynchronization programs similar to those used in dairy cows would involve additional cost and labor. While dairy producers already handle cattle regularly for milking, beef producers evaluate the feasibility of an estrus synchronization protocol largely based on the number of animal handling events, often referred to as “trips through the chute.” Estrus synchronization protocols that could offer some of the benefits of presynchronization while also minimizing animal handling are therefore of significant interest.

Long-term Progestin-based Protocols. Currently, the only widely-used protocols for beef cattle that involve presynchronization are long-term progestin-based protocols. In these protocols, presynchronization is accomplished through treatment with an exogenous progestin for an extended period of time (e.g. 14 d). There are currently two widely-used progestin products approved by the United States Food and Drug Administration (FDA) for synchronization of estrus: the feed additive progestin melengestrol acetate (MGA) and an intravaginal progesterone-releasing device called the Controlled Internal Drug Release (CIDR). Compared to endogenous progesterone, MGA

has an 11.1-fold higher binding affinity for the progesterone receptor (Perry et al., 2005). As a result, it is highly effective for suppression of estrus and ovulation when fed at a low concentration, such as the recommended 0.5 mg/hd/d (Imwalle et al., 2002; Patterson et al., 1990; Zimbelman and Smith, 1966a, 1966b). Although research has demonstrated effectiveness of MGA in both heifers and cows, the original FDA clearance trials sought label approval only for use in heifers. Therefore, use as a feed additive for mature cows is strictly prohibited by FDA at this time. The CIDR insert is a T-shaped, silicone-coated nylon device with either 1.38 g or 1.9 g of progesterone impregnated in the silicone coating. The CIDR device marketed in the United States is the 1.38 g progesterone product, while the 1.9 g progesterone CIDR device is marketed in other countries, such as Brazil. Progesterone is continuously released while the progesterone device is inserted, and serum concentrations of progesterone are maintained at > 2.0 ng/ml in the absence of a CL beginning 1 h after CIDR insertion (Chenault et al., 2003; Perry et al., 2004; Lamb et al., 2006). In comparison to MGA, progesterone from CIDR devices is cleared faster from the body (12 to 24 h), and synchrony of estrus is improved following CIDR treatment compared to MGA treatment (Kojima et al., 2004; Tauck et al., 2007). In addition, the CIDR insert has fairly broad FDA label approval for synchronization of estrus in lactating dairy cows, suckled beef cows, and replacement beef and dairy heifers.

Progestin treatment can effectively induce estrous cyclicity among a large proportion of peripubertal heifers and anestrus postpartum cows (Gonzalez-Padilla et al., 1975; Patterson et al., 1990). Treatment with a progestin increases the frequency of LH pulse release (Imwalle et al., 1998; Smith and Day, 1990) and down-regulates expression of estradiol receptor in the hypothalamus (Anderson and Day, 1996; Anderson

et al., 1996). Conceptually, treatment with a progestin mimics endogenous increases in progesterone that may occur prior to the initiation of normal estrous cycles. For peripubertal heifers, treatment with a progestin simulates an increase in progesterone that occurs prior to the first pubertal estrous cycle (Berardinelli et al., 1979). Likewise, for anestrus postpartum cows, progestin treatment simulates a short luteal phase that often occurs prior to the first normal estrous cycle (Donaldson et al., 1970; Schams et al., 1978; Odde et al., 1980; Peters and Riley, 1982).

Treatment with a progestin can also have effects in cyclic females, depending on stage of the estrous cycle. Since progestin treatment does not extend the lifespan of the corpus luteum, cycling females in the luteal phase may undergo luteolysis spontaneously during progestin treatment based on stage of cycle. In the absence of luteal progesterone, low concentrations of an exogenous progestin result in the formation of persistent follicles, characterized by an extended period of dominance and increased estradiol production (Zimbelman and Smith, 1966a,b; CRRP, 1996; Risley, 2008). This effect is attributed to an altered pulsatile pattern of gonadotropin release. Pulse frequency of LH release is increased, but progesterone concentrations remain sufficiently high to suppress an LH surge that would induce ovulation (Ireland and Roche, 1982; Kojima et al., 1992; Savio et al., 1993; Custer et al., 1994; Fortune, 1994; Kojima et al., 1995; Kinder et al., 1996). Since this effect occurs only when progesterone concentrations are at subluteal levels, the effectiveness of progestin for presynchronization of cyclic cows can be considered to be dependent on luteolysis. Given the length of the luteal phase, long-term progestin-based presynchronization protocols have generally involved treatment with a progestin for 14 d. This length of progestin treatment has been considered adequate to

ensure that a sufficiently high proportion of cyclic cows will have either undergone luteolysis during the course of progestin treatment or will be undergoing luteolysis shortly after progestin treatment ends.

Among females lacking luteal progesterone during treatment with an exogenous progestin, synchronous expression of estrus occurs shortly after progestin treatment ceases (e.g. withdrawal of MGA from the feed or removal of CIDR inserts). This initial estrus is subfertile due to the reduced fertility of persistent follicles, with reductions in fertility correlating with the length of time that follicular dominance was artificially extended through progestin treatment (Patterson et al., 1989). Therefore, insemination following this estrus is strongly discouraged. However, the synchrony of estrus expression that occurs following progestin removal has a presynchronizing effect, as recruitment of the next follicular wave occurs in a similarly synchronous manner. Thus, synchrony of ovarian follicular development is established for the subsequent estrous cycle.

Development of the MGA-PG Protocol. Temporally speaking, development of estrus synchronization methods using progestin treatment alone or in combination with other hormones predated development of methods involving use of gonadotropins to synchronize ovarian follicular waves. For example, the Syncro-Mate B system developed in the 1980s made use of a 6 mg ear implant of the progestin norgestomet for a period of 7 to 9 d, with 5 mg estradiol valerate and 3 mg norgestomet administered as an injection concurrent with the ear implant (Semler et al., 1984; Whittier et al., 1986). However, as the focus of estrus synchronization research shifted towards control of ovarian follicular waves, the opportunity to use progestins for presynchronization became apparent. The

MGA-PG protocol was developed as a long-term, presynchronization-based protocol in which MGA was fed for a period of 14 d, followed by administration of PG 17 to 19 d after the final day of MGA feeding (Brown et al., 1988; Deutscher, 2000; Lamb et al., 2000; Nix et al., 1998). This approach enhanced results compared to treatment with PG only (Patterson et al., 1995) and compared to Syncro-Mate B (Brown et al., 1988). The MGA-PG protocol remains a commonly used protocol for estrus synchronization of beef heifers and is suitable for use prior to either estrus detection based AI programs or FTAI performed at 72 h after PG administration.

Development of the 14-d CIDR-PG Protocol. The 14-d CIDR-PG protocol is a similar long-term progestin-based estrus synchronization protocol that is currently seeing widespread industry adoption for use among beef heifers. Estrous response rates and synchrony of estrus expression following this protocol are well characterized among heifers (Leitman et al., 2009a,b; Mallory et al., 2010) and contribute to successful application of FTAI. In the 14-d CIDR-PG protocol, a CIDR is inserted on Day 0 and removed on Day 14, PG is administered on Day 30, and AI is performed either following detected estrus or at a predetermined time point on Day 33. Unlike the CIDR-Select protocol that preceded the 14-d CIDR-PG protocol, GnRH is not administered 7 d prior to PG in the treatment schedule. Interestingly, when GnRH administration was included in the treatment schedule on Day 23, both synchrony of estrus expression and pregnancy rates following FTAI were decreased among beef heifers (Leitman et al., 2009a,b). Two separate reports by Nash et al. (2011, 2012a) also evaluated the effect of GnRH administration on Day 23 of the treatment schedule for mature cows, the first reporting similar pregnancy rates between treatments and the second reporting lower pregnancy

rates among cows receiving GnRH on Day 23. Together, these results supported removal of GnRH administration from the 14-d CIDR-PG treatment schedule. Therefore, synchrony of follicular development in the 14-d CIDR-PG protocol is established entirely through the physiology previously discussed relative to progestin treatment as a presynchronization method.

Successful results have also been obtained using the 14-d CIDR-PG protocol in dairy heifers, dairy cows, and in beef cows of varying age and parity. Escalante et al. (2013a) evaluated the 14-d CIDR-PG protocol among dairy heifers on the basis of ovarian follicular dynamics and luteal function, with results indicating successful synchronization response. Large field trials with dairy heifers further indicated that high pregnancy rates may be obtained when FTAI is performed following this protocol (Mallory et al., 2013; Escalante et al., 2013a; Claypool et al., 2015). Escalante et al. (2013b) also evaluated use of the 14-d CIDR-PG protocol among mature dairy cows in a grazing based dairy system, obtaining successful pregnancy rates to timed AI. In beef cows, pregnancy rates to FTAI following the 14-d CIDR-PG protocol were comparable to those obtained following the 7-d CO-Synch + CIDR protocol, in studies both in primiparous two-year-old beef cows (Abel et al., 2017) and in mature beef cows across various ages and parities (Nash et al., 2013).

Response of Mature Cows to the 14-d CIDR-PG Protocol. Although the 14-d CIDR-PG protocol is effective for synchronization of estrus across several classes of females, results from previous publications suggest the treatment schedule may not be optimized for mature cows. Nash et al. (2013) reported that mature cows exhibited a less synchronous initial estrus after CIDR removal and that the mean interval to estrus was

extended among cows compared to data published for heifers. This variation in timing of estrus expression suggests that a higher proportion of cows may fail to undergo luteolysis by the end of a 14 d CIDR treatment or may undergo luteolysis very late in CIDR treatment. As development of a persistent follicle during progesterone treatment is contingent upon spontaneous luteolysis, this could potentially result in more variation in physiological maturity of the dominant follicle at the time of CIDR removal. Estrous response after PG and prior to FTAI was also much lower among mature cows (23%) compared to heifers (78%) following the 14-d CIDR-PG protocol (Mallory et al., 2011; Nash et al., 2012a).

One aspect of the data regarding use of the 14-d CIDR-PG protocol in mature cows is counterintuitive and deserves further discussion: pregnancy rates to FTAI are quite high considering the low rates of estrous response observed. Nash et al. (2012a) observed a 23% estrous response prior to FTAI among cows treated with 14-d CIDR-PG protocol as compared to a 49% estrous response prior to FTAI among cows treated with the 7-d CO-Synch + CIDR protocol ($P < 0.0001$). However, pregnancy rates were comparable between treatments (54% versus 56%). Martin et al. (2014) also observed low estrous response (29.7%) among mature cows but acceptable pregnancy rates to FTAI (51.5%) following the 14-d CIDR-PG protocol. Abel et al. (2017) compared the 14-d CIDR-PG and 7-d CO-Synch + CIDR protocols specifically among primiparous two-year-old cows and observed similar results. Estrous response was lower ($P < 0.0001$) among 14-d CIDR-PG treated cows (43%) compared to 7-d CO-Synch + CIDR treated cows (74%), yet pregnancy rates were comparable between treatments (63% versus 64%).

Martin et al. (2013) demonstrated an improvement among mature cows in estrous response rate prior to FTAI when using a 19 d interval to PG following CIDR removal compared to a 16 d interval (47.4% vs 29.7%; $P < 0.01$). This extended interval to PG also tended to improve pregnancy rates following FTAI among mature cows. However, this modification adds 3 d in length to the protocol. Currently, the 14-d CIDR-PG protocol has seen little industry use among mature, suckled beef cows, due largely to the logistical challenge of using a protocol of longer duration in groups of cows with varying lengths of postpartum intervals. Therefore, modifications that decrease protocol length and enhance estrous response are of significant interest.

Mechanisms Potentially Involved in Fertility. To date, no publications have specifically described mechanisms that result in high fertility when using long-term progestin-based estrus synchronization protocols among mature cows. Due to the high pregnancy rates observed, it is plausible factors associated with high fertility are sufficiently enhanced despite the low rates of estrous response observed. There are some indications in the literature that estradiol production may be enhanced when using long-term progestin-based estrus synchronization protocols. Fralix et al. (1996) found that cows undergoing a presynchronization treatment with melengestrol acetate (MGA) for 14 days had increased estradiol concentrations during the second estrous cycle after MGA treatment. A somewhat similar effect may have been observed by Abel et al. (2017) in a comparison of long- and short-term CIDR-based protocols (the 14-d CIDR-PG and 7-d CO-Synch + CIDR protocols) among primiparous two-year-old beef cows. Among cows treated with the long-term protocol in that study, estradiol concentrations at FTAI were nearly double those observed among cows treated with the short-term 7-d CO-Synch +

CIDR protocol (8.0 ± 0.7 pg versus 4.2 ± 0.8 pg; $P < 0.001$). Mechanisms that may be involved in elevated estradiol production following long-term progestin-based protocols have not been precisely elucidated.

One hypothesis as to why long-term protocols offer potential for high pregnancy rates is that a relatively limited degree of intervention takes place during final development of the follicle, thus minimizing potential for negative effects on fertility. From this perspective, there are numerous opportunities by which follicular quality could potentially be compromised when using a short-term protocol like the 7-d CO-Synch + CIDR protocol. First, as previously discussed, a proportion of cows fail to respond to GnRH administration on Day 0 of the 7-d CO-Synch + CIDR protocol. If those females are also late in the luteal phase and undergo luteolysis during the course of CIDR treatment, the follicle present at PG administration will be sub-fertile due to the persistent follicle physiology previously discussed. Second, among those cows that do successfully respond to GnRH administration on Day 0 of the protocol, a proportion have a CL and subsequently form an accessory corpus luteum after the GnRH-induced ovulation. The developing follicle therefore develops under the influence of somewhat higher circulating concentrations of progesterone. Additionally, because a proportion of cows ovulate in response to GnRH administration and a proportion of cows do not, there are effectively two different subpopulations of cows with respect to stage of follicular wave at the time of CIDR removal and PG administration on Day 7. Lastly, among cows responding to GnRH on Day 0, recruitment of the next follicular wave follows ovulation of the previous dominant follicle in response to the large pharmacological dose of exogenous GnRH. This is a somewhat different set of physiological events compared to the typical transition

from first to second follicular wave, in which recruitment of the new follicular wave results from a transient rise in FSH following atresia of the previous dominant follicle (Adams et al., 1992a). The extent to which each of these factors impacts overall fertility (if at all) is an area of ongoing research. However, it is worth noting that these considerations related to follicular quality are avoided entirely when a long-term progestin based protocol is used, simply because administration of GnRH is not required to generate follicular synchrony.

FACTORS LIMITING SUCCESSFUL USE OF SEX-SORTED SEMEN IN TIMED ARTIFICIAL INSEMINATION

Pre-selection of progeny sex has been of interest to commercial animal agriculture for decades, as effective, low-cost methods to select the sex of offspring would improve efficiency (Seidel, 2003a,b). A flow cytometric method for cell sorting was developed in the 1980s, allowing for sorting of mammalian sperm cells based on differences in total DNA content between sperm cells bearing an X versus Y sex chromosome (Garner et al., 1983; Johnson et al., 1989). This method can be used to generate sorted populations of sperm cells bearing the desired sex chromosome at very high accuracies (Johnson, 2000; Seidel, 2014). Translational research efforts were quickly undertaken to evaluate use of sex-sorted bovine sperm in artificial insemination (Seidel et al., 1999; Schenk et al., 1999), and sex-sorted semen has been commercially available for several years for use in both beef and dairy cattle. Calves conceived using sex-sorted semen are phenotypically normal (Tubman et al., 2004), and it was estimated in 2009 that sex-sorted semen had been used for AI of approximately one million bovine females (Garner, 2009).

Economic Considerations. Generally, progeny of one sex resulting from a planned mating are of a higher value than the alternative sex, though the sex desired and the value difference between the sexes varies across animal agricultural industries and even from mating to mating. In bovine species in particular, the ability to preselect the sex of progeny allows for more efficient utilization of maternally or terminally favorable genetics. In the dairy industry, for example, purebred Holstein bull calves are of low value, as dairy-type calves perform suboptimally as a meat production animal. Sex selection in dairy systems allows for more focused production of progeny, either for production of replacement females or production of crossbred male calves that may offer more value in meat production. Likewise, in beef systems, predetermination of progeny sex could allow for more precise genetic emphasis. Maternally favorable genetics could be emphasized when producing replacement heifers, and more terminally favorable genetics relative to growth and carcass merit could be emphasized when producing male calves. Ultimately, successful use of sex-sorted semen in the beef and dairy industries would offer several economic advantages due to increased rates of genetic improvement and improved efficiency in resource use per unit of production.

Despite the potential value of this technology, use of sex-sorted semen in commercial production has been limited. In data summarized by the USDA Animal and Plant Health Inspection Service, artificial insemination rather than natural service accounts for 72.5% of all pregnancies in the dairy industry (APHIS, 2009). Among dairy animals receiving AI, sex-sorted semen is used to inseminate 11.4% of dairy heifers and 3.5% of dairy cows (APHIS, 2009). Use of sex-sorted semen is considerably more limited in the beef industry, as is use of AI in general. The most recent National Animal

Health Monitoring Survey (NAHMS, 2008) indicated only 7.6% of beef producers use AI. Moreover, use of AI in commercial beef production generally consists of a single AI service followed by natural service through the remainder of the breeding season. Data regarding use of sex-sorted semen in beef cattle are limited; however, it is likely sex-sorted semen is used in less than 1% of beef AI services. Although the direct cost of sex-sorted semen is fairly moderate (generally \$15 - \$30 more per unit than the cost of conventional semen), indirect costs have limited adoption of this technology, particularly in the beef industry (Seidel, 2003a,b). Indirect costs stem primarily from reduced pregnancy rates obtained when using sex-sorted compared to conventional semen (Hohenboken, 1999; Cooke et al., 2014).

Biological Considerations. Historically, lower pregnancy rates have been obtained with sex-sorted semen than with conventional semen collected from the same bull. Although variable, research efforts generally reported pregnancy rates with sex-sorted semen of an average factor of .75 relative to pregnancy rates obtained with conventional semen, provided AI was performed following detected estrus (Seidel, 2003a,b). For example, insemination with sex-sorted semen would result in a 45% first service conception rate rather than a 60% first service conception rate to conventional semen. The reduced fertility associated with use of sex-sorted semen is attributed to several factors but can be broadly described as resultant from (1) fewer numbers of sperm cells being placed in each unit of semen and (2) cellular stress to sperm cells following the sorting process and subsequent cryopreservation.

The quantity of sperm cells that can be economically placed in each unit of sex-sorted semen is limited by the efficiency of the sorting procedure and by the accuracy at

which the sorting equipment is set to operate. When operating to achieve a relatively high purity of the desired sex (e.g. > 90% accuracy for the desired sex, or a 90:10 sex ratio), the sex-sorting process is inherently inefficient. Lower-cost products which skew the sex ratio to a lesser degree (for example 70:30 rather than 90:10) have been developed and tested, but for whatever reason these products appear to gain little market traction. When operating to achieve the typical >90% accuracy for sex-sorting, about 60% of sperm cells cannot be sorted and are therefore discarded. Thus, 20% of the ejaculate is sex-sorted for X chromosome content and 20% sexed for Y chromosome content (Seidel and Garner, 2002; Seidel, 2007). Although not true in every case, sires whose collections are being sex-sorted were often selected for production of sex-sorted semen because of favorable characteristics for either male or female calves, not both. Hence, typically only 20% of the sperm cells of a given collection are then useful for producing desired units of sorted semen content (Seidel and Garner, 2002). In order to generate a sufficient number of saleable units from a collection, fewer sperm cells must be placed in an AI dose of sex-sorted semen. Historically, sex-sorted semen has been produced and marketed at 2×10^6 cells per dose as compared to 20×10^6 cells per dose in conventional semen. The lower sperm cell number per dose has been demonstrated to negatively impact fertility, though the degree to which low sperm cell numbers detrimentally affect fertility appears to be bull-dependent (Den Daas et al., 1998; DeJarnette et al., 2008).

Sex-sorted semen is also characterized by decreased sperm cell quality. Cellular damage to the sperm cell occurs as a result of the sex-sorting process, and this damage likely impacts fertility (Sartori et al., 2004). The staining of sperm with Hoeschst 33342 dye has not been demonstrated to negatively impact sperm motility or DNA integrity, but

the interaction of dye exposure and exposure to the laser may result in some damage (Garner, 2006). Laser intensity itself does not appear to impact DNA integrity of the resulting embryo in swine and likely does not in cattle (Guthrie, 2002). High pressure within the system (50psi) was also shown to decrease post-thaw sperm motility (Suh et al., 2005). Recent changes to the procedures used to sort semen have involved decreases in the internal pressure of the system and modifications to the laser approaches used, with some indication of decreased sperm cell damage and improved fertility (Schenk et al., 2009; Sharpe and Evans, 2009). Cellular debris produced during the sorting process can result in the production of reactive oxygen species, which is another mechanism by which sperm could be damaged (Ichikawa, 1999). However, effects of these reactive oxygen species have been mitigated by inclusion of antioxidant agents in the sorting fluid (Pena, 2003).

An additional fertility-limiting result of the sex-sorting process that has been well characterized is pre-capacitation of sperm cells after sex-sorting. Sex-sorting induces a higher proportion of pre-capacitated sperm cells (Hollinshead et al., 2003; Lu et al., 2004). The initiation of capacitation sets up a limit for the lifespan of a sperm cell, so pre-capacitation induced by the sorting process could negatively impact sperm longevity. In the ram, sex-sorted sperm cells are released sooner from the oviduct than non-sex-sorted sperm cells, suggesting another mechanism for impaired sperm longevity after sex-sorting (Hollinshead et al., 2003). These factors and others limit the fertile lifespan of sperm cells that have been sex-sorted, complicating efforts to use sex-sorted semen in industry settings.

Current Status in the Beef and Dairy Industries. While use of sex-sorted semen is much more common among dairy than among beef animals, use in the dairy industry is primarily in breeding programs for heifers rather than mature dairy cows (APHIS, 2009). Poor adoption of sex-sorted semen in breeding programs for mature dairy cows is due largely to decreased fertility of sex-sorted semen compared to conventional semen in lactating cows (Hall and Glaze, 2014). Certainly, however, there would be several opportunities for incorporation of sex-sorted semen into breeding programs for lactating dairy cows if fertility of sex-sorted semen were to become more comparable to that of conventional semen. These opportunities include (1) production of heifers from genetically elite lactating dairy cows through use of X-sorted semen; (2) reduction of the number of low-quality male progeny, which are suboptimal animals for feedlot performance; and (3) incorporation of targeted crossbreeding with terminal beef sires for generation of higher value male feedlot animals.

With the development of methods that result in higher fertility with sex-sorted semen, there would likely be an opportunity to dramatically expand use of sex-sorted semen among dairy heifers as well. From the perspective of genetic improvement, retention of heifer progeny generated from dairy heifers results in a decreased generation interval and therefore increased rate of genetic progress for traits of interest. As sex-sorted semen is used in less than 12% of dairy heifer breeding programs (APHIS, 2009), a significant portion of dairy heifer matings currently result in phenotypically low-value male progeny rather than production of genetically high-value female progeny. Improved breeding strategies for use of sex-sorted semen among dairy heifers are therefore of significant interest.

In the beef industry, indirect costs of using sex-sorted semen are the limiting factor for adoption of this technology among both heifers and mature cows. Considerations relative to early conception among heifers differ between beef and dairy due to the differing structures of the respective industries. The effect of low first service pregnancy rates following estrus synchronization (i.e. by using sex-sorted rather than conventional semen) would be primarily manifest as an increase in the average days-on-feed in dairy heifer development operations (Claypool et al., 2015). However, cost considerations are more complex for beef producers given the seasonal constraints of beef production systems. Well managed beef herds use breeding and calving seasons of limited length, and low first service conception rates shift females into later conception periods and therefore later calving periods. Later conception significantly decreases average age of calves at weaning and therefore pounds of calf weaned (Dziuk and Bellows 1983), and later age at conception also has implications for lifetime productivity of beef heifers (Lesmeister et al., 1973; Kill et al., 2012). Therefore, the indirect cost of low first service AI pregnancy rates are generally quite high in beef cow-calf operations.

In both beef and dairy, lower pregnancy rates to sex-sorted semen also limit the beneficial impact of sex-selection technology, as the degree to which progeny sex ratio can be effectively skewed is limited by conception rate. For example, inseminating 100 heifers with conventional semen at a 60% pregnancy rate would result in 30 pregnancies of the desired sex, while a 45% pregnancy rate to sex-sorted semen would result in 41 pregnancies of the desired sex (assuming a 50:50 and 92:8 sex ratio for conventional and sex-sorted semen respectively). This modest increase in the number of desired sex progeny has not offset the direct and indirect costs associated with sex-sorted semen in

many previous research efforts (Hohenboken, 1999; Seidel, 2003a,b; Meyer et al., 2012; Cooke et al., 2014).

Moreover, beef AI systems increasingly rely on protocols which facilitate fixed-time artificial insemination (FTAI), in which all females are inseminated at a predetermined time point following estrus synchronization. Time and labor considerations have been identified as key factors limiting adoption of advanced reproductive technologies in the beef industry (NAHMS, 2008); therefore, extensive research efforts by our lab and others have centered around development of timed AI protocols that do not require the labor and time commitment associated with estrus detection. Development of these protocols has had a substantial impact on use of AI in the beef industry, with sales of beef semen having doubled since the publishing of recommended timed AI protocols by the Beef Reproduction Task Force in 2005 (Patterson et al., 2016). However, while several protocols have been developed to facilitate FTAI with conventional semen, previous attempts to use sex-sorted semen in FTAI treatments have generally resulted in reduced pregnancy rates relative to conventional semen (Hall et al., 2010; Rhinehart et al., 2011; Sá Filho et al., 2012; Thomas et al., 2014; Cooke et al., 2014). To be economically viable for widespread adoption in the beef industry, therefore, sex-sorted semen must achieve pregnancy rates comparable to conventional semen and must be suitable for use in timed AI protocols.

Opportunities to Expand Use. Recently, fertility of sex-sorted semen has improved substantially following research efforts aimed at decreasing the degree of cellular damage occurring to sperm cells during the sex-sorting process (Sharpe and Evans, 2009; Evans, 2010; DeGraaf et al., 2014; Vishwanath, 2015). The technological

enhancements resulting from this research have led to the development of next-generation sex-sorting technology, from which sex-sorted semen is presently available for both dairy and beef breeds and marketed commercially under the trade name SexedULTRA™ (Sexing Technologies, Navasota, TX). In addition to offering improved sperm cell quality, the improved speed and efficiency of the sorting technology facilitates use of higher sperm cell numbers per unit (Vishwanath, 2015), above the typical 2.0×10^6 concentration that had become standard for units of sex-sorted semen (Seidel et al., 1999; Amann, 1999; DeJarnette et al., 2008). Although the specific factors enhanced in the technology used to produce SexedULTRA™ sex-sorted semen are proprietary, results from large field trials suggest fertility of the SexedULTRA™ product is substantially improved. In an experiment using semen from eight Holstein bulls for insemination of over 6,000 heifers in 41 commercial locations, pregnancy rates to AI following detected estrus were improved by 4.5 percentage points among dairy heifers inseminated with SexedULTRA™ sex-sorted semen (46.1%) compared to sex-sorted semen produced using the previous generation XY technology (41.6%) (Vishwanath, 2015). A separate trial using semen from five bulls for insemination of over 6,000 dairy animals based on detected estrus indicated no difference between pregnancy rates to SexedULTRA™ semen at a concentration of 4.0×10^6 cells per unit and pregnancy rates to conventional semen at a concentration of 15×10^6 cells per unit (Vishwanath, 2015).

While use of sex-sorted semen in AI is still limited relative to use of conventional semen, growing industry interest in use of sex-sorted semen is evidenced by an increase in the number of bulls for which sex-sorted semen is commercially available. This is particularly evident in the beef industry, in which the number of beef bulls for which sex-

sorted semen is available for purchase has historically been quite low. In 2008, for example, sex-sorted semen was not marketed for any beef bulls by major US AI studs. However, by 2011, the number of beef bulls for which sex-sorted semen could be purchased had already increased to 70, with several different beef breeds represented (Hall, 2011). More recently, the release of a beef bull lineup marketed directly by Sexing Technologies has further increased the availability of sex-sorted semen from beef bulls (Hall and Glaze, 2014).

RESEARCH IN THE FIELD OF SPLIT-TIME ARTIFICIAL INSEMINATION

The elimination of estrus detection from FTAI protocols greatly decreases the labor and inconvenience of carrying out an AI program. As a result, increasing numbers of beef cows are being inseminated by appointment. However, previous studies from our lab report varying pregnancy rates to FTAI based on estrus expression prior to FTAI, with females that expressed estrus performing significantly better than females that failed to express estrus prior to FTAI (Busch et al., 2007; Busch et al., 2008; Wilson et al., 2010; Mallory et al., 2011; Mallory et al., 2013). In a meta-analysis across published FTAI literature, Richardson et al. (2016) found that females that failed to express estrus prior to FTAI achieved 27% lower pregnancy rates than estrous females. Lack of estrous response when performing FTAI is particularly problematic when using sex-sorted semen, as conception rates to sex-sorted semen are currently maximized when cows are inseminated following an observed estrus (Seidel, 2007). Additionally, as previously discussed in this review, insemination closer to the time of ovulation is generally advantageous when using sex-sorted semen. Studies conducted by our lab using sex-

sorted semen suggested that fertility of non-estrous females in particular is reduced when sex-sorted semen is used in FTAI (Mallory et al., 2013; Nash et al., 2012b; Thomas et al., 2014a).

Initial Trials in Mature Cows. Thomas et al. (2014a) developed a strategy in which insemination was delayed until 20 h after GnRH administration for cows failing to express estrus prior to FTAI. This approach is occasionally referenced by the phrase “delayed insemination of non-estrous females” but has been more commonly referred to as “split-time AI,” in order to indicate that estrous and non-estrous females are split for insemination at two separate time points. In the first trial using split-time AI (Thomas et al., 2014a), estrus was synchronized for 656 suckled beef cows with the 7-day CO-Synch + CIDR protocol. Estrus detection aids (Estroject) were applied at PG and CIDR removal on d 7, and estrous status was recorded at GnRH on d 10. Cows were assigned to one of three treatments: (1) FTAI (concurrent with GnRH, 66 h after CIDR removal) with conventional semen regardless of estrous status; (2) FTAI with sex-sorted semen regardless of estrous status; or (3) FTAI with sex-sorted semen for cows having expressed estrus, and delayed AI 20 h after final GnRH for cows failing to express estrus. Higher pregnancy rates ($P < 0.0001$) were achieved with conventional semen (77%) than with sex-sorted semen (47%) among cows that expressed estrus. However, among cows that failed to express estrus, delayed insemination with sex-sorted semen yielded higher ($P < 0.0001$) pregnancy rates than with sex-sorted semen at the standard time (3% versus 36%, respectively). Furthermore, among cows that failed to express estrus, FTAI pregnancy rates using sex-sorted semen at the delayed time (36%) were comparable ($P = 0.9$) to those achieved using conventional semen at the standard time (37%). These results

indicated that delaying insemination of non-estrous cows by 20 h from the standard FTAI improved pregnancy rates substantially when using sex-sorted semen.

In a subsequent trial, Thomas et al. (2014b) also evaluated this approach when using conventional, non-sex-sorted semen. Estrus was synchronized for 951 mature, suckled cows across nine locations using the 7-d CO-Synch + CIDR protocol. Cows within each location were assigned to one of two treatments based on age, days postpartum (DPP), and body condition score (BCS): 1) FTAI (concurrent with GnRH, 66 h after PG) regardless of estrous expression or; 2) FTAI for cows expressing estrus, and delayed AI (20 h after GnRH) for cows failing to express estrus. In contrast to the results obtained using sex-sorted semen, the results from this field trial showed no significant effect of treatment on AI pregnancy rate when using conventional semen ($P = .76$).

It was initially hypothesized that delaying insemination of non-estrous females until 20 h after GnRH administration would better align the lifespan of viable, capacitated sperm with the timing of ovulation, potentially allowing for higher FTAI pregnancy rates. Insemination at the optimal time relative to estrus may be more or less critical based on individual bull fertility (Macmillan and Watson, 1975). Therefore, it is possible that enhancement of pregnancy rates by optimizing timing of insemination may occur only in certain situations, such as when using sex-sorted semen or when using conventional semen from sires with sub-optimal sperm cell longevity. However, the results from Thomas et al. (2014b) suggested that development of a favorable female environment may be the more critical factor involved in increasing the pregnancy rates of non-estrous females receiving delayed insemination. Estrus expression prior to AI is associated with improved fertility for a number of reasons. Initiation of estrus in cattle is preceded by a

rise in serum concentrations of estradiol (Allrich, 1994), and preovulatory estradiol coordinates several physiological processes that contribute to the establishment and maintenance of pregnancy, including effects on follicular cells, the oocyte, gamete transport, and preparation of the uterine environment (Pohler et al., 2012). Lower estradiol concentrations among non-estrous females may also affect pregnancy rates by impairing sperm transport in the female reproductive tract. Perry et al. (2008) suggested that preovulatory concentrations of estradiol during standing estrus may modulate uterine pH and thereby affect sperm transport. In the trial by Thomas et al. (2014b), delaying insemination of non-estrous cows allowed time for initiation of estrus among a large percentage of these cows. When receiving delayed insemination 20 h after GnRH administration, cows that expressed estrus during the 20 h delay period achieved significantly higher pregnancy rates than those cows that still had not expressed estrus by the time of delayed insemination (67% versus 40%). This increase in pregnancy rates may be attributed to development of more favorable conditions within the female reproductive tract due to the endocrine changes occurring around the time of onset of estrus.

Initial Trial in Heifers. Use of a split-time AI approach has been similarly evaluated following the 14-d CIDR-PG protocol for beef heifers. Thomas et al. (2014b) tested the same hypothesis that pregnancy rates in beef heifers after FTAI may be improved by delaying insemination of heifers that have not expressed estrus prior to the standard FTAI time. Higher pregnancy rates ($P = 0.02$) were obtained among non-estrous heifers that were inseminated at the delayed time (49%) compared to non-estrous heifers inseminated at the FTAI time point (34%). This resulted in an overall increase ($P = 0.01$)

in pregnancy rates following AI for the group of heifers in which STAI was performed (54%) as compared to those in which FTAI was performed (46%).

Optimal Timing of GnRH Administration for Mature Cows. Bishop et al. (2016) designed an experiment to evaluate timing of GnRH administration in beef cows based on estrous status when performing STAI. Treatment of non-estrous females with exogenous GnRH at the standard FTAI time was hypothesized to limit subsequent estrus activity during the 20-24 day period prior to insemination, as GnRH down-regulates the aromatase enzyme that is necessary for estradiol production (Voss and Fortune, 1992). Estrus was synchronized for 622 cows across six locations using the 7-d CO-Synch + CIDR protocol. Estrous detection aids (Estroject) were applied at CIDR removal and PG on d 7, with estrus recorded at 66 and 90 h after PG on d 10 and 11, respectively. Timing of insemination was based on expression of estrus 66 h after PG. Cows in each treatment that exhibited estrus by 66 h were inseminated; whereas AI was delayed 24 h until 90 h after PG for cows failing to exhibit estrus by 66 h. Cows in treatment 1 were administered GnRH 66 h after PG irrespective of estrus expression, whereas in treatment 2, cows were administered GnRH coincident with delayed insemination only if non-estrous at 66 h after PG. Treatment affected overall estrous response (1 = 85%; 2 = 90%; $P = 0.04$) but did not affect total AI pregnancy rate (1 = 58%; 2 = 57%; $P = 0.89$). There were no differences between treatments in estrous response at 66 h (1 = 73%; 2 = 75%; $P = 0.47$); and pregnancy rate resulting from AI for cows inseminated at 66 h was not influenced by GnRH (1 = 63%; 2 = 59%; $P = 0.51$). Estrous response during the 24 h delay period differed between treatments (1 = 45%; 2 = 61%; $P = 0.04$), although AI pregnancy rate for cows inseminated at 90 h did not differ (1 = 44%; 2 = 49%; $P = 0.51$).

This study indicated that when split-time AI is used in conjunction with the 7-d CO-Synch + CIDR protocol, administration of GnRH at AI to cows that exhibit estrus by 66 h after PG is not warranted. Additionally, the authors noted that, although pregnancy rates of non-estrous cows did not differ significantly based on the time point at which GnRH was administered, GnRH administration at the earlier time point appeared to have a suppressive effect on estrous response during the delay period.

In a similarly designed experiment, Hill et al. (2016b) also evaluated the effectiveness of GnRH administration for both estrous cows and non-estrous cows. In this experiment, the time points used for STAI were 65 h and 84 h after CIDR removal and PG administration. Pregnancy rates to AI did not differ ($P = 0.68$) between estrous cows receiving GnRH administration at AI (61.9%) and estrous cows not receiving GnRH administration (60.4%). For cows failing to express estrus prior to 65 h and therefore receiving delayed insemination at 84 h, the authors evaluated either administration of GnRH at 65 h or no administration of GnRH. Administration of GnRH at 65 h was found to increase ($P < 0.01$) pregnancy rates only among the proportion of non-estrous cows that still failed to express estrus by 84 h (GnRH = 33.4% [$n = 146$] vs. no GnRH = 15.0% [$n = 128$]). Unfortunately, the authors did not include in the experimental design a third treatment in which GnRH was administered to non-estrous cows concurrent with delayed insemination. Bishop et al. (2016) proposed that the optimal time point for administration of GnRH is at the later time point, concurrent with delayed insemination, due largely to the fact that administration of GnRH at the early time point was observed to have a suppressive effect on estrous response occurring during the delay period.

Bishop et al. (2017b) designed a subsequent experiment to compare pregnancy rates in postpartum beef cows following either FTAI or STAI in which GnRH administration was performed at the later time point concurrent with delayed insemination. Estrus was synchronized for 671 cows following administration of the 7-d CO-Synch + CIDR protocol. Cows assigned to the FTAI treatment ($n = 333$; FTAI) were inseminated at 66 h after PG and GnRH was administered concurrent with insemination regardless of estrus expression. For cows in the split-time AI treatment ($n = 338$; STAI), STAI was performed at 66 and 90 h after PG, and estrus was recorded at these times. Cows in the STAI treatment that exhibited estrus by 66 h were inseminated at that time and did not receive GnRH, whereas AI was delayed 24 h until 90 h after PG for cows that failed to exhibit estrus by 66 h. Gonadotropin-releasing hormone was administered concurrent with AI at 90 h only to cows failing to exhibit estrus. Estrus expression that occurred during the 24 delay period among cows assigned to the STAI treatment increased the total proportion of cows that expressed estrus prior to insemination (FTAI = 60%; STAI = 86%; $P < 0.001$). Pregnancy rates for cows inseminated at 66 h that exhibited estrus did not differ between treatments (FTAI = 58%; STAI = 58%; $P = 0.93$); however, pregnancy rates among cows that had failed to express estrus by 66 h was improved when STAI was performed and insemination and GnRH administration were postponed by 24 h (FTAI = 35%; STAI = 51%; $P = 0.01$). Consequently, total AI pregnancy rate tended to be higher for cows that received STAI (FTAI = 49%; STAI = 56%; $P = 0.06$).

Unclear Effectiveness of GnRH Administration for Heifers. Other experiments in heifers similarly evaluated the optimal timing and necessity of GnRH administration

when using STAI (Bishop et al., 2016, 2017a). Results from these studies clearly demonstrated that administration of GnRH was not required for heifers that exhibit estrus prior to AI, and that administration of GnRH to heifers that fail to exhibit estrus by 66 h could be delayed to 90 h, concurrent with insemination. This work supports the hypothesis that higher pregnancy rates resulting from STAI compared to FTAI are due primarily to higher estrous response rates prior to insemination, in contrast to the hypothesis that timing of insemination in STAI is more optimally aligned relative to the timing of GnRH-induced ovulations (Thomas et al., 2014a,b). Despite the high overall estrous response that is observed in heifers when split-time AI is practiced, a percentage of heifers fail to exhibit estrus prior to 90 h after PG (Bishop et al., 2016, 2017a). Pregnancy rates resulting from AI are generally reduced among these heifers compared with those that exhibit estrus (Richardson et al., 2016), despite the fact GnRH is routinely administered concurrent with AI. Bishop et al. (2017a) reported that, when split-time AI was used in conjunction with the 14-day CIDR-PG protocol, comparable pregnancy rates were achieved among non-estrous heifers at 90 hours after PG regardless of whether or not GnRH was administered concurrent with AI. These results continue to raise questions regarding the general efficacy of GnRH in beef heifers.

Response to GnRH administration differs between heifers and mature cows (Pursley et al., 1995; Macmillan and Thatcher, 1991; Moreira et al., 2000). Response of heifers to GnRH is highly dependent on day of the estrous cycle (Atkins et al., 2008). Exogenous GnRH successfully induces ovulation in a higher proportion of cows than heifers (66% versus 50%) when administered at a random stage of the estrous cycle, with this difference being largely attributed to differences in follicular maturity (Pursley et al.,

1995; Geary and Whittier, 1998). As a result, in applied research, use of GnRH in estrus synchronization protocols for heifers has been questioned by several publications (Lamb et al., 2006; Leitman et al., 2009a,b; Bishop et al., 2017a). Some of these data sets are counterintuitive from a physiological perspective. For example, Leitman et al. (2009a,b) reported that when using a 14 d CIDR presynchronization program, administration of GnRH to heifers 7 d prior to PG administration actually had a negative effect, reducing synchrony of estrus expression following PG. This publication ultimately resulted in development of the 14-d CIDR-PG protocol, a widely popular estrus synchronization protocol for beef heifers that does not rely on GnRH administration at any point in the protocol prior to AI.

Based on physiological differences between heifers and cows, administration of GnRH and the timing of GnRH administration may be more consequential for cows. For heifers, results observed by Bishop et al. (2017a) suggest GnRH may potentially be eliminated entirely from the protocol when using STAI following the 14-d CIDR-PG. By contrast, Hill et al. (2016b) evaluated removal of GnRH entirely from a split-time AI protocol for mature cows and observed that pregnancy rates were decreased for non-estrous cows if GnRH was not administered (42% with GnRH administration; 31% without administration of GnRH).

Despite potential physiological differences between mature cows and heifers in response to GnRH administration, the split-time AI approaches currently recommended (Thomas et al., 2016a) do not differ between beef cows following the 7-d CO-Synch + CIDR protocol and beef heifers following the 14-d CIDR-PG protocol. Females expressing estrus by the standard FTAI time point (e.g. 66 h) should receive AI at that

time, and females failing to express estrus should receive AI 20-24 hours later. Administration of GnRH may be restricted only to those females that fail to express estrus by the delayed insemination time point.

Timing of Split-Time Artificial Insemination. Several other authors have since evaluated approaches involving delay of insemination for females failing to express estrus by the time of FTAI. However, the designs of these experiments have varied widely and, unfortunately in several cases, preclude meaningful interpretation of results. In separate experiments, Markwood et al. (2014) and Hill et al. (2016a) evaluated delaying insemination of non-estrous mature cows following the 7-d CO-Synch PG protocol, with experimental designs that somewhat mirror the original STAI work by Thomas et al. (2014a,b). These authors reach similar conclusions in analysis of their results, though important differences exist with respect to experimental design. Markwood et al., (2014) established two insemination time points 18 h apart, at 58 and 76 h after PG administration. Cattle were randomly assigned to receive insemination at one of the two time points, with activation status of estrus detection aids (Estroject) recorded at AI. Pregnancy results were then analyzed retrospectively with respect to timing of AI and estrous status at AI. The authors observed a tendency ($P = 0.07$) for an interaction between timing of AI and estrous status. However, only numerically higher ($P = 0.13$) pregnancy rates were obtained among cows with non-activated estrus detection aids when insemination was performed at the later time point (54.4% versus 47.0%). Hill et al. (2016a) evaluated activation status of estrus detection aids (Estroject) for all cows at 60 h after PG administration, considered all estrous females as positive control in the analysis, and assigned the remaining non-estrous females to one of three treatments: (1) GnRH

administration concurrent with AI at 60 h (effectively a FTAI approach); (2) GnRH administration at 60 h and delayed AI at 75 h; or (3) GnRH administration concurrent with delayed AI at 75 h. Cows expressing estrus prior to 60 h achieved the highest pregnancy rates (64.2%; $P < 0.01$). Among non-estrous cows, cows receiving insemination at 60 h achieved lower pregnancy rates (41.7%; $P < 0.05$) than cows receiving AI at 75 h, regardless of whether GnRH was administered at 60 h (52.8%) or concurrent with delayed AI at 75 h (50.0%).

In these experiments and others, authors have made use of shorter (15 h or 18 h) delay periods rather than a 20 h delay period as published by Thomas et al. (2014a,b) or the 24 h delay period as published by Bishop et al. (2016, 2017a,b). It is likely the decisions to use shorter delay periods were based primarily on practical considerations relative to daylight hours and outdoor temperatures at certain times of the day. Similarly, some authors seem to find a 66 h interval from PG to FTAI inconvenient, which likely influenced the selection of the earlier 58 h time point by Markwood et al. (2014) and 60 h by Hill et al. (2016a). Later work by some of these authors have moved toward a longer delay period as well as toward a later set of AI time points. For example, a subsequent trial by Hill et al. (2016b) used a 19 h delay period with STAI performed at 65 or 84 h. Recent work from the same lab (Stevenson et al., 2017) also supports use of a longer 20 h delay period as well as a later set of time points for STAI. Pregnancy rates were evaluated following two alternative sets of time points for STAI, 55 and 75 h after PG versus 65 and 85 h after PG. Results support use of the later set of time points, 65 and 85 hours, which are similar to the time points originally used by Thomas et al. (2014a,b). When using the later set of time points (65 h and 85 h after PG), a higher proportion of cows

expressed estrus by the first 65 h time point (62.0% versus 41.9%), a higher total proportion of cows expressed estrus overall by the later 85 h time point (76.7% versus 66.7%), and overall pregnancy rates were increased (61.0% versus 51.3%).

Prior to any research related to development of STAI, the optimal time point for FTAI following the 7-d CO-Synch had been a subject of research and debate. Although a comparison of a 60 to 66 h interval from PG administration to FTAI has not been rigorously evaluated, Busch et al. (2008) compared pregnancy rates obtained when FTAI was performed at either 54 or 66 h after PG following estrus synchronization using the 7-d CO-Synch + CIDR protocol. Pregnancy rates to FTAI were improved when FTAI was performed at 66 h rather than 54 h. In addition, because FTAI was performed later in the distribution of estrus onset times, a higher proportion of cows expressed estrus prior to FTAI when FTAI was performed at 66 h rather than 54 h (50% versus 26%). Moreover, cows that exhibited estrus prior to FTAI also obtained higher pregnancy rates when inseminated at the later time (81% at 66 h versus 65% at 54 h), potentially due to the fact that insemination occurred at a time point closer to ovulation on average. In part based on these results, performing FTAI at 66 h when using the 7-d CO-Synch + CIDR protocol is frequently recommended (Patterson et al., 2017a).

Thomas et al. (2013) proposed that optimal timing of AI may be altered slightly when STAI rather than FTAI is performed. The rationale proposed was that, because timed AI is essentially a compromise point that maximizes pregnancy outcome across a distribution of ovulatory times, the opportunity to use two AI time points to cover the same distribution would likely result in a different optimal time for the first AI time point. While this rationale may seem intuitive, use of a different (e.g. earlier) time point for the

first AI may not result in increased pregnancy rates if the distribution of estrus onset times and therefore ovulatory times is a non-normal distribution. Previously published distributions of estrus onsets for several protocols indicate that these distributions are generally skewed. Selection of earlier time points for STAI could then arguably result in decreased pregnancy rates, primarily for two reasons. First, artificial insemination of estrous females would occur at a time point further from ovulation on average, since the average interval from estrus onset to insemination would be reduced. Secondly and perhaps more critically, lower total rates of estrous response could be observed when using earlier STAI time points, since a smaller portion of the distribution of estrus onset times would be observed.

There may be some indications in the STAI literature of reduced success when using earlier time points. For example, total estrous response ranged from 65% to 78% in trials using earlier STAI time points, in which the delayed insemination occurred at ≤ 85 h after PG (Markwood et al., 2014; Hill et al., 2016a,b; Stevenson et al., 2017). By contrast, total estrous response ranged from 85% to 90% in trials using later STAI time points, in which the delayed insemination occurred between 86 and 90 h after PG (Thomas et al., 2014a,b; Bishop et al., 2016, 2017a,b). These trials differ with respect to the time points at which GnRH was administered, which could affect total estrous response (see discussions by Bishop et al., 2016, 2017a,b). However, the expectation that a later set of times for STAI would result in higher cumulative rates of estrous response is certainly consistent with the published distribution of estrus onset times for cows following the 7-d CO-Synch + CIDR protocol (Wilson et al., 2010).

Research Using Shorter Delay Times. Several other authors have since presented findings in which insemination was delayed by much shorter time intervals for cows or heifers failing to express estrus. Shaw et al. (2016) evaluated an 8 h delay of AI for non-estrous heifers following a 7-d CO-Synch + CIDR protocol. However, the time point used for FTAI was 48 h after PG, despite the fact that a later time point of 54 h after PG is recommended when using this protocol in heifers (Patterson et al., 2017b). As previously discussed, use of an earlier set of time points for STAI likely results in poor estrous response due to the fact that a smaller portion of the distribution of estrus onset times is observed. This may explain the particularly poor estrous response (38.7%) the authors observed in this experiment. The proportion of non-estrous heifers that went on to express estrus during the 8 h delay period was not characterized but could be speculated to be low due to the short duration of the delay period and the administration of GnRH at 48 h rather than concurrent with delayed insemination. Perhaps not surprisingly then, no significant difference ($P = 0.83$) was observed between the pregnancy rates of non-estrous heifers whether insemination occurred concurrent with GnRH at 48 h after PG (39.4%) or insemination was delayed 8 h until 56 h after PG (42.6%).

In a similar experiment evaluating a very short delay period, Dias et al. (2017) evaluated an 8 h delay period for mature cows following the 7-d CO-Synch + CIDR protocol. Again, no significant difference was observed between the pregnancy rates of non-estrous cows inseminated concurrent with GnRH at 66 h after PG (43.3%) and non-estrous cows inseminated at 74 h, 8 h after GnRH administration (50.9%). Demeterco et al. (2017) evaluated yet another variant in timing of STAI for cows following the 7-d CO-Synch + CIDR protocol, comparing FTAI at 72 h after PG administration to a STAI

approach in which GnRH administration and AI were delayed by 12 h for cows failing to express estrus. It should be noted that 72 h is 6 to 12 h later than the recommended time for FTAI as published by the Beef Reproduction Task Force. Unfortunately, only 149 animals were included in this trial, and it therefore lacks statistical power to make meaningful analyses relative to pregnancy rate. Overall, it is regrettable that each of the trials discussed in this section did not evaluate time points for STAI that had been previously shown to be successful (e.g. use of the industry standard FTAI time and a 20 to 24 h delay period).

Research Using Other Protocols. Research efforts related to use of STAI following other estrus synchronization protocols have been somewhat limited. In an experiment using sex-sorted semen, Hall et al. (2017) evaluated an 8 h delay of the FTAI time point for both estrous and non-estrous cows following the 5-d CO-Synch + CIDR protocol. Although only a single FTAI time point was used rather than a set of two time points as in a STAI approach, estrous status information at the FTAI time points provides similar information. Pregnancy rates were higher for cows that expressed estrus, and estrous response was higher among cows for which the insemination time was delayed. However, overall pregnancy rates to sex-sorted semen were not affected by timing of insemination or by the interaction of timing of insemination and estrous status.

Nielson et al. (2015) evaluated the effect of delaying insemination of non-estrous heifers in conjunction with the MGA-PG protocol. Heifers were detected for estrus and inseminated based on estrus expression until 72 h after PG administration, at which time heifers that had failed to express estrus were assigned to receive either AI concurrent with GnRH administration at 72 h or delayed AI 16 h after GnRH administration. Pregnancy

rates did not differ ($P = 0.56$) between non-estrous heifers inseminated at 72 h (56%) and those inseminated at 88 h (47%). Unfortunately, however, this trial lacked statistical power to either detect differences or demonstrate equivalence relative to pregnancy rate, as the two treatments were comprised of only 70 and 64 non-estrous heifers respectively.

Abel et al. (2017) compared FTAI and STAI following the 14-d CIDR-PG specifically among two-year-old, primiparous beef cows. Cows undergoing STAI as opposed to FTAI obtained a higher total estrous response rate, due to the additional estrous response occurring during the 24 h delay period (64% versus 42%; $P < 0.0001$). However, pregnancy rates resulting from FTAI were similar ($P = 0.60$) between STAI (55%) and FTAI (56%) treatments. Unfortunately, in this trial, GnRH was administered at the beginning of the delay period rather than concurrent with AI at the delayed time point. It is still unclear to what extent early GnRH administration may have influenced these results.

It remains possible that the relative advantage of STAI over FTAI is somewhat protocol dependent. Overall, more research efforts are needed to evaluate use of STAI following other estrus synchronization protocols. However, it is critical that this research be done with some level of consistency relative to timing of STAI, timing of GnRH administration, and length of the delay period.

SUMMARY AND DISCUSSION

In recent years, the focus in ruminant reproductive research has largely shifted away from applied work in the field of estrus synchronization and toward more basic research relative to embryonic loss. Certainly, high rates of early embryonic loss occur in

cattle and represent an area of much-needed research. In informal conversations, some researchers suggest that further research efforts in the field of estrus synchronization are unlikely to yield improvements in pregnancy rates to AI, arguing that conception rates are likely already maximized based on our understanding of fertilization rates and subsequent rates of early embryonic loss. The rates of fertilization and embryonic loss typically referenced come from an often-cited review in which fertilization rates among heifers and cows were found to be 88% and 90% respectively, but calving rate to a single service was found to be 55% (Sreenan and Diskin, 1986). Since pregnancy rates to AI are often in this range, the perception of many seems to be that there is little room left for improvement. Although perhaps understandable, this thinking makes several questionable assumptions.

First, with respect to the review itself, it is important to note that (1) the pregnancy rates reported are based upon breeding following detected estrus rather than following an estrus synchronization and timed AI approach; (2) the data set consists entirely of females that express estrus prior to breeding, whereas timed AI results reflect pregnancy rates for non-estrous females as well; and (3) the results do not provide insight into timing of insemination relative to ovulation when performing AI with semen of compromised quality, such as sex-sorted semen. With these considerations in mind, it is likely incorrect to assume fertilization rates following FTAI are as high as those reported. Sreenan and Diskin (1986) even note in the original review that “the fertilisation [sic] rates recorded here probably over-estimate those achieved on farms.”

When performing AI at a fixed-time irrespective of estrous status or timing of estrous onset, endocrine parameters that would affect gamete transport, fertilization rate, and establishment of pregnancy vary widely between females (see review by Pohler et

al., 2012). Variation in timing of AI could result in poor fertilization rate if insemination occurs too early relative to ovulation. Conditions within the reproductive tract of females that have not expressed estrus could be less favorable for support and transport of sperm, further decreasing the average pregnancy rate of the group receiving FTAI. Even ignoring direct effects on fertilization rate, estrus synchronization and FTAI introduce several opportunities to directly impact embryo quality and potentially affect rates of embryonic loss. Estrus synchronization itself could have large impacts on follicle quality, steroidogenic activity surrounding insemination/ovulation, and oocyte competence. Induction of ovulation among non-estrous females could result in poor embryo quality and higher rates of embryonic loss. Variation in timing of AI relative to ovulation could result in an “aged oocyte” and decreased embryo quality (Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001). The field of estrus synchronization is relatively new in human history and the field of timed AI even newer; it is unlikely that current results are fully optimized.

Based on the research efforts previously reviewed, it is the author’s opinion that numerous opportunities exist to enhance estrous cycle control and improve pregnancy rates to timed AI. Several opportunities appear particularly promising and worthy of further research. For example, endocrine parameters and pregnancy rates to FTAI following long-term progestin-based protocols are promising among mature cows, despite the fact that the treatment schedule is likely not yet optimized specifically for mature cows. Likewise, while opportunities to use sex-sorted semen for AI of beef cattle are expanding, results obtained using sex-sorted semen are likely not yet optimal. Lastly, alternative timed AI approaches that may increase pregnancy rates above those typically

obtained with FTAI are of significant interest. It is with these considerations in mind that the following experiments were designed.

CHAPTER 2

THE 9-D CIDR-PG PROTOCOL: INCORPORATION OF PROSTAGLANDIN F_{2α} PRETREATMENT INTO A LONG-TERM PROGESTIN-BASED ESTRUS SYNCHRONIZATION PROTOCOL FOR POSTPARTUM BEEF COWS

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ABSTRACT

A pilot experiment was designed to test the hypothesis that administration of prostaglandin F_{2α} prior to progestin treatment would allow for a reduced duration of progestin treatment in a long-term progestin-based estrus synchronization protocol. A modified pre-synchronization treatment was compared to a standard long-term CIDR treatment, and treatments were compared on the basis of ovarian follicular dynamics, estrous response rate, synchrony of estrus expression, and pregnancy rates resulting from timed artificial insemination (AI) in postpartum beef cows. Estrous was synchronized for 85 cows, with cows assigned to one of two treatments based on age, days postpartum (DPP), and body condition score (BCS). Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert (1.38 g progesterone) on Day 0, CIDR removal on Day 14, and administration of PG (25 mg im) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and administration of PG on Day 30. In both treatments, split-time AI was performed based on estrous response. At 72 h after PG (Day 33), cows having expressed estrus received timed AI; cows that failed to express estrus by 72 h received timed AI 24 h later (96 h after PG on Day 34), with

gonadotropin-releasing hormone (GnRH; 100 µg im) administered to non-estrous cows. Estrus detection transmitters were used from CIDR removal until AI to determine onset time of estrus expression both after CIDR removal and after PG. Ovarian ultrasonography was performed at CIDR removal on Day 14, at PG on Day 30, and at AI on Day 33 or Day 34. At CIDR removal on Day 14, diameter of the largest follicle present on the ovary (LFD) was similar between treatments. The proportion of cows expressing estrus following CIDR removal tended to be higher ($P = 0.09$) among cows assigned to the 9-d CIDR-PG treatment (93%; 40/43) than among cows assigned to the 14-d CIDR-PG treatment (81%; 34/42). Following PG, a significantly higher proportion ($P = 0.02$) of cows expressed estrus following synchronization with the 9-d CIDR-PG treatment (91%; 39/43) than the 14-d CIDR-PG treatment (69%; 29/42). Consequently, pregnancy rate to TAI tended to be increased ($P = 0.09$) among the 9-d CIDR-PG treatment (76.7%; 33/43) compared to the 14-d CIDR-PG (59.5%; 25/42). In summary, a long-term CIDR-based estrous synchronization protocol for postpartum beef cows was enhanced through administration of PG at CIDR insertion and at CIDR removal.

INTRODUCTION

The 14-d CIDR-PG protocol is a long-term progestin-based estrus synchronization protocol currently seeing widespread industry adoption. Estrous response rate and synchrony of estrus expression following this protocol were characterized among heifers (Leitman et al., 2009a,b; Mallory et al., 2010) and contribute to successful application of fixed-time artificial insemination (FTAI). Recent work has evaluated the 14-d CIDR-PG protocol in mature beef cows and noted several differences among cows

compared to heifers. Nash et al. (2013) reported that mature cows exhibit a less synchronous initial estrus after CIDR removal and an extended mean interval to estrus compared to the data previously published in heifers. Total estrous response after PG and prior to FTAI was also lower among cows (23%) compared to heifers (78%) following the 14-d CIDR-PG protocol (Nash et al., 2012a; Mallory et al., 2011). Furthermore, in comparison to the 7-d CO-Synch + CIDR protocol, estrous response prior to FTAI was reduced in the 14-d CIDR-PG protocol (23% vs 49%), although pregnancy rates to FTAI were comparable. Martin et al. (2013) demonstrated an improvement among mature cows in estrous response rate prior to FTAI when using a 19 d interval to PG following CIDR removal compared to a 16 d interval (47.4% vs 29.7%; $P < 0.01$). This extended interval to PG also tended to improve timed AI pregnancy rates among mature cows. However, this modification adds 3 d in length to the protocol. Currently, the 14-d CIDR-PG protocol has seen little industry use among mature, suckled beef cows, due largely to the logistical challenge of using a protocol of longer duration in groups of cows with varying lengths of postpartum intervals. Therefore, modifications that decrease protocol length and enhance estrous response are of significant interest. In the absence of luteal progesterone, low concentrations of an exogenous progestin result in the formation of persistent follicles, characterized by an extended period of dominance and increased estradiol production (Zimbelman and Smith, 1966a; Stock and Fortune, 1993; CRRP, 1996; Risley, 2008). This effect is attributed to an altered pulsatile pattern of gonadotropin release due to the decrease in endogenous progesterone production following PG-induced luteolysis (Ireland and Roche, 1982; Kojima et al., 1992; Savio et al., 1993; Custer et al., 1994; Fortune, 1994; Kojima et al., 1995; Kinder et al., 1996). We

hypothesized that a large dominant follicle would develop earlier during CIDR treatment following administration of PG at CIDR insertion, and that by Day 9 of CIDR treatment, follicle size would be similar in comparison to Day 14 of CIDR treatment alone. Therefore, the present study was conducted as a pilot effort to evaluate the effect of administering PG in conjunction with a long-term CIDR-based protocol. The study was designed to reduce length of the treatment schedule and to compare and evaluate a modified long-term protocol on the basis of follicular dynamics and related reproductive endpoints.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Animals. Estrus was synchronized for crossbred lactating beef cows ($n = 85$). Cows were assigned to one of two treatments (Figure 2.1) based on age, days postpartum (DPP), and BCS (Richards et al., 1986) using a 1 to 9 scale (1 = emaciated and 9 = obese). Cows assigned to the 14-d CIDR-PG protocol received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, CIDR removal on Day 14, and PG (25 mg im; Lutalyse, Zoetis, Madison, NJ) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and PG on Day 30. In both treatments, split-time AI was performed based on estrous response (Thomas et al., 2014a,b; Bishop et al., 2016, 2017b). At 72 h after PG (Day 33), cows that had expressed estrus received timed AI. Cows that failed to express estrus by 72 h received timed AI 24 h later (96 h after PG on

Day 34), with GnRH (100 µg im; Cystorelin, Merial, Athens, GA) administered to non-estrous cows concurrent with AI. Times of PG administration, GnRH administration, and AI were recorded for each cow. Insemination was performed using conventional frozen semen sourced from one collection of a single AI sire. Two technicians were pre-assigned and balanced across treatments based on cow age, DPP, and BCS. Fourteen d after AI, cows were exposed to fertile bulls for the remainder of the 45 d breeding season.

Estrus detection. Cows were fitted with HeatWatch estrus detection transmitters (DDx Inc., Denver, CO) from the time of CIDR removal until FTAI for continuous estrus detection. Estrus was defined as cows receiving ≥ 3 mounts of ≥ 2 s in duration within a 4-h period, with the onset of estrus determined as the first mount within that time period (Busch et al., 2007). Transmitters were removed from cows expressing estrus by 72 h after PG but were not removed from the remaining cows until 96 h to characterize estrus expression between 72 and 96 h.

Ovarian ultrasonography. Transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) was performed to assess ovarian follicular dynamics and presence of corpora lutea. Dominant follicles were measured, and measurements reported are the largest follicle diameter (LFD). Ultrasonography was performed at CIDR removal on Day 14, at PG administration on Day 30, and at AI on Day 33 and 34.

Pregnancy diagnosis. Pregnancy rate to AI was determined by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) 74 d after AI (Day 107).

Statistical analysis. Treatment differences for age, BCS, DPP, LFD

measurements, and timing of estrus expression were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to analyze treatment differences in the proportion of cows with CL present at CIDR removal, proportion of cows with CL present at PG on Day 30, and pregnancy rates based on estrous expression. Estrous response rate after CIDR removal, estrous response rate after PG administration, pregnancy rate to AI, and final pregnancy rate were analyzed with the GENMOD procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function.

RESULTS

Cow age, BCS, DPP at AI, and estrous response rates are shown for each treatment in Table 2.1. Cows did not differ based on treatment for age ($P = 0.35$), BCS ($P = 0.98$), or DPP (0.98). Estrous response rate and timing of estrous expression after CIDR removal on Day 14 and after PG administration on Day 30 are shown in Table 2.2. Ultrasound data collected at CIDR removal on Day 14, at PG on Day 30, and at AI on Day 33 and 34 are presented in Table 2.3.

At CIDR removal on Day 14, diameter of the largest follicle present on the ovary (LFD) was similar ($P = 0.66$) among the 14-d CIDR-PG (14.3 ± 0.4 mm) and the 9-d CIDR-PG (14.3 ± 0.4 mm) treated cows. Estrous expression following CIDR removal is graphed for each treatment in Figure 2.2. The mean interval from CIDR removal to estrus was similar ($P = 0.15$) among cows assigned to the 14-d CIDR-PG treatment (48.3 ± 3.3 h) and cows assigned to the 9-d CIDR-PG treatment (48.2 ± 2.4 h). However, the

proportion of cows expressing estrus following CIDR removal tended to be higher ($P = 0.097$) among cows assigned to the 9-d CIDR-PG treatment (93%; 40/43) than among cows assigned to the 14-d CIDR-PG treatment (81%; 34/42). Among cows in the 9-d CIDR-PG treatment, 16% (7/43) of the cows had CL present at CIDR removal on Day 14, and of these cows, 100% (7/7) expressed estrus following CIDR removal. The mean interval from CIDR removal to the onset of estrus for cows in the 9-d CIDR-PG treatment was numerically extended among cows with CL at CIDR removal (67.6 ± 3.3 h) compared to cows without CL (48.2 ± 2.2 h), although differences in these intervals did not differ ($P = 0.35$). Among cows in the 14-d CIDR-PG treatment, 10% (4/42) of the cows had CL present at CIDR removal, and 75% (3/4) of these cows failed to express estrus following CIDR removal.

At PG on Day 30, LFD tended ($P = 0.09$) to be larger among cows in the 9-d CIDR-PG treatment (11.6 ± 0.3 mm) than the 14-d CIDR-PG treatment (11.3 ± 0.2 mm). The proportion of cows with corpora lutea at PG on Day 30 was similar ($P = 0.24$) in both the 14-d CIDR-PG treatment (95%; 40/42) and the 9-d CIDR-PG treatment (100%; 43/43). Timing of estrous expression following PG is graphed for cows in each treatment in Figure 2.3. Following PG, a higher proportion ($P = 0.02$) of cows expressed estrus following synchronization with the 9-d CIDR-PG treatment (91%; 39/43) than the 14-d CIDR-PG treatment (69%; 29/42). The mean interval from PG to estrus was numerically shortened among cows following the 9-d CIDR-PG treatment (70.7 ± 1.4 h) compared to the 14-d CIDR-PG treatment (78.0 ± 1.9 h); however this differences was not significant ($P = 0.30$).

At AI on Day 33 and 34, LFD did not differ ($P = 0.11$) between the 14-d CIDR-

PG (13.3 ± 0.3 mm) and 9-d CIDR-PG (13.7 ± 0.2 mm) treated cows. Cows that expressed estrus prior to AI had greater LFD than cows that failed to express estrus in both the 14-d CIDR-PG (13.9 ± 0.3 mm versus 11.9 ± 0.7 mm) and 9-d CIDR-PG (13.9 ± 0.3 mm versus 11.9 ± 0.4 mm) treated groups. However, no differences in LFD based on treatment were apparent within estrous cows or within non-estrous cows respectively.

Timed AI pregnancy rates based on treatment and estrous response are shown in Table 2.4. Across treatments, cows that expressed estrus prior to AI tended ($P = 0.08$) to achieve higher AI pregnancy rates than cows failing to express estrus. Cows that expressed estrus prior to AI achieved similar ($P = 0.50$) pregnancy rates (14-d CIDR-PG [72%; 21/29]; 9-d CIDR-PG [79%; 31/39]). Similarly, no difference between treatments ($P = 0.48$) was detected when pregnancy rates of cows that failed to express estrus prior to AI were compared (14-d CIDR-PG [31%; 4/13]; 9-d CIDR-PG [50%; 2/4]). However, overall timed AI pregnancy rate tended to be increased ($P = 0.09$) in the 9-d CIDR-PG treatment (76.7%; 33/43) than in the 14-d CIDR-PG treatment (59.5%; 25/42) due to the increased proportion of cows that expressed estrus prior to AI following the 9-d CIDR-PG treatment. Timed AI pregnancy rate did not differ ($P = 0.44$) based on technician. Final pregnancy rate at the end of a 45 d breeding period did not differ ($P = 0.32$) between cows assigned to the 14-d CIDR-PG (93%; 39/42) and 9-d CIDR-PG (88%; 38/43) treatments.

DISCUSSION

Several protocols and strategies for estrus synchronization have been previously published. Many of these are cost-effective, simple to follow, and achieve reasonably high pregnancy rates. However, while pregnancy rates are generally satisfactory when

insemination is performed based on detected estrus, few protocols generate the degree of synchrony required to achieve optimal pregnancy rates to FTAI. In addition to factors that improve general conception rates, continued improvement in timed AI pregnancy rate is likely dependent upon enhancements in estrus synchronization that improve (1) total estrous response rate prior to AI and (2) synchrony of estrus and ovulation.

Synchrony of estrus expression is largely dependent upon synchrony of ovarian follicular waves among a group of females undergoing estrus synchronization. Published protocols employ varying strategies to establish follicular synchrony. Many protocols rely on administration of gonadotropin-releasing hormone (GnRH) early in the protocol, such as the commonly used 7-d CO-Synch + CIDR (Lamb et al., 2001) and 5-d CO-Synch + CIDR (Bridges et al., 2008) protocols. In these protocols, follicular synchrony is dependent upon successful response to GnRH. Other protocols make use of exogenous estradiol to establish follicular synchrony; however, estradiol cannot be used legally for estrus synchronization in many countries, including the United States. Alternatively, the 14-d CIDR-PG (Leitman et al., 2009a,b) protocol attempts to establish follicular synchrony through a synchronous initial estrus event occurring after CIDR removal. In this case, establishment of subsequent follicular synchrony to facilitate timed AI is dependent upon the initial estrus event at CIDR removal. To establish optimal synchrony, this estrus event must (1) occur among the maximum proportion of females and (2) occur over a narrow time period so as to minimize subsequent variation.

Achieving a greater overall estrous response and a highly synchronous initial estrus following CIDR removal is dependent upon uniformity in the development of dominant follicles during the period of CIDR treatment and a subsequent rapid decline in

progesterone. A 14 d period of CIDR insertion or feeding of melengestrol acetate (MGA) can achieve a reasonable degree of follicular uniformity and progesterone decline simply from the length of progestin treatment. Exogenous progesterone does not extend luteal lifespan. Hence, as luteolysis occurs and endogenous progesterone concentrations decline during progestin exposure, dominant follicles will persist for an extended time before ovulation or atresia. Therefore, based upon length of the luteal phase of the estrous cycle, the majority of females should not have CL and should have a large dominant follicle after 14 d of progestin exposure.

In contrast, the 9-d CIDR-PG protocol utilizes PG at CIDR insertion to induce luteolysis among females with responsive CL (≥ 5 d post-estrus). Females responding to PG at CIDR insertion subsequently lack endogenous luteal progesterone during the 9 d of progestin (CIDR) treatment, creating an endocrine environment similar to that described by others (Zimbelman and Smith, 1966a; CRRP, 1996; Risley, 2008) for development of persistent follicles. Of course, a proportion of these females (generally < 5 d post-estrus) will possess corpora lutea incapable of responding to PG at CIDR insertion and can be expected to retain CL through CIDR treatment. Therefore, PG is administered at CIDR removal to maximize the proportion of females expressing an initial estrus following CIDR removal. Cows retaining CL through CIDR treatment were anticipated to have more variation in LFD at CIDR removal and to differ somewhat in subsequent estrous response rate and timing of estrus. The mean interval from CIDR removal in the 9-d CIDR-PG treatment to estrus onset was numerically extended among cows with CL at CIDR removal (67.6 ± 3.3 h) compared to cows with no CL (48.2 ± 2.2 h), although this difference in intervals was not significant ($P = 0.35$). Additionally, 100% (7/7) of these

cows exhibited estrus following CIDR removal, indicating a successful response rate to PG at CIDR removal.

This experiment is not the first reported attempt to reduce endogenous progesterone production as a means of influencing follicular development. Inclusion of PG at early time points within protocols has been a topic of interest for use in both beef and dairy females and in both *Bos taurus* and *Bos indicus* (Grant et al., 2011; Stevenson and Pulley, 2012; Perry et al., 2012; Hill et al., 2014; Williams et al., 2015). However, these efforts to date have focused on protocols that achieve follicular synchrony using GnRH rather than a synchronous initial estrus event. In the present study, use of PG at CIDR insertion and at CIDR removal facilitated shortening of a long-term progestin treatment from 14 d to 9 d while establishing similarly sized dominant follicles at CIDR removal. While this results in only a 5 d reduction in protocol length, it is important to note that decreased protocol length is of significant practical importance when long-term protocols are used in groups of suckled beef cows that range in postpartum interval, as the manufacturer's label on CIDR inserts recommends that beef cows be ≥ 20 d postpartum at the time of CIDR insertion.

In the design of this experiment, AI was conducted using a split-time AI approach to allow for a longer period of estrus expression, determined using HeatWatch. However, future studies should evaluate the optimal timing of fixed-time AI (FTAI) as well as compare the success of FTAI to split-time AI when using the 9-d CIDR-PG protocol. Furthermore, future work should evaluate the 9-d CIDR-PG protocol in comparison to short-term protocols more commonly used in mature beef cows, as the estrous response rate and synchrony of estrus observed following the 9-d CIDR-PG protocol is

noteworthy. Following PG administration on Day 30, 91% of cows expressed estrus over a 32 h time period, from 59 to 91 h following PG administration (Figure 2.2). Previous work from our laboratory comparing the 7-d CO-Synch + CIDR and the 5-d CO-Synch + CIDR protocols found total estrous response following these protocols to be 85% and 79% respectively, with estrus expression occurring over a > 84 h time period (Wilson et al., 2010). Likewise, future work should evaluate the effectiveness of the 9-d CIDR-PG approach compared to the 14-d CIDR-PG in heifers. Previously published data from our laboratory (Mallory et al., 2010) found that 71% of heifers express estrus following CIDR removal in the 14-d CIDR-PG protocol, in contrast to the 93% estrous response we observed among cows following CIDR removal in the 9-d CIDR-PG treatment. As this initial estrus establishes synchrony, improvement in estrous response rate at CIDR removal may improve final estrous response and timed AI pregnancy rates in heifers as well. Although we acknowledge the limited number of cows involved in this pilot study, the high estrous response and the synchrony of estrus observed following administration of this protocol may offer an opportunity for future use of this protocol not only in timed AI but in other reproductive applications such as embryo transfer.

In summary, we demonstrate that administration of PG at CIDR insertion and removal allowed for a shortened length of progestin treatment, reducing the overall length of the 14-d CIDR-PG protocol by 5 d. The shorter 9-d CIDR-PG protocol resulted in similar follicular dynamics at time points of interest and offered potential improvements in estrous response rate, synchrony of estrus, and timed AI pregnancy rates when compared to the longer 14-d CIDR-PG protocol.

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Table 2.1. Cow age, body condition score (BCS), days postpartum (DPP), and estrous response within treatments.

Treatment ¹	Age, y	BCS ²	DPP, d	Estrous by 72 h ³		Estrous 72 - 96 h ⁴		Non-estrous by 96 h ⁵	
				Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	7.7 ± .4	5.6 ± .1	74.5 ± 1.5	11/42	26	18/42	43	13/42	31
9-d CIDR-PG	7.5 ± .4	5.6 ± .1	75.4 ± 1.5	24/43	56	15/43	35	4/43	9
Combined	7.6 ± .3	5.6 ± .1	75.0 ± 1.1	35/85	41	33/85	39	17/85	20

¹Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0, CIDR removal on Day 14, and administration of PG (25 mg im) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and administration of PG on Day 30. In both treatments, timed AI was performed using a split-time AI approach based on estrous response. At 72 h after PG (Day 33), cows having expressed estrus received timed AI; cows having not expressed estrus by 72 h received timed AI 24 h later (96 h after PG on Day 34), with gonadotropin-releasing hormone (GnRH; 100 µg im) administered to non-estrous cows concurrent with AI.

²Body condition score (mean ± SE) of cows at treatment assignment on Day 0 (1 to 9 scale, where 1 = emaciated and 9 = obese).

³Proportion of cows expressing estrus by 72 h after PG administration on (Day 30) subsequently inseminated at 72 h (Day 33).

⁴Proportion of cows expressing estrus between 72 h and 96 h after PG administration (Day 30) and subsequently inseminated at 96 h (Day 34).

⁵Proportion of cows failing to express estrus by 96 h after PG administration (Day 30) and subsequently administered GnRH and inseminated at 96 h (Day 34).

Table 2.2. Estrous response¹ rate and timing of estrus following CIDR removal (Day 14) and following PG (Day 30) within treatments.

Treatment ²	Estrous following CIDR removal		Interval to estrus from CIDR removal	Estrous following PG		Interval to estrus from PG
	Proportion	%	h	Proportion	%	h
14-d CIDR-PG	34/42	81	48.3 ± 3.3	29/42	69	78.0 ± 1.9
9-d CIDR-PG	40/43	93	48.2 ± 2.4	39/43	91	70.7 ± 1.4

¹Rate and timing of estrus expression determined using HeatWatch transmitters. Estrus defined as cows receiving ≥3 mounts of ≥2 s in duration within a 4-h period, with the onset of estrus determined as the first mount within that time period.

²See Table 2.1 for description of treatment protocols.

Table 2.3. Ultrasound data¹ collected at CIDR removal, at PG administration, and at AI.

Treatment ²	LFD CIDR removal	CL present CIDR removal		LFD PG	CL present PG		LFD AI
	mm	Proportion	%	mm	Proportion	%	mm
14-d CIDR-PG	14.3 ± 0.4	4/42	10	11.3 ± 0.2	40/42	95	13.3 ± 0.3
9-d CIDR- PG	14.3 ± 0.4	7/43	16	11.6 ± 0.3	43/43	100	13.7 ± 0.2

¹Transrectal ultrasonography was performed to assess ovarian follicular dynamics and presence of corpora lutea. Dominant follicles were measured, and measurements reported are the largest follicle diameter (LFD). Ultrasonography was performed at CIDR removal on Day 14, at PG administration on Day 30, and at AI on Day 33 and 34.

²See Table 2.1 for description of treatment protocols.

Table 2.4. Timed AI pregnancy rates¹ based on estrous response and treatment.

Treatment ²	Overall		Estrous by 72 h ³		Estrous 72 - 96 h ⁴		Non-estrous by 96 h ⁵	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	25/42	60	6/11	55	15/18	83	4/13	31
9-d CIDR-PG	33/43	77	16/24	67	15/15	100	2/4	50

¹Pregnancy rate to AI determined by transrectal ultrasonography 74 d after AI (Day 107).

²See Table 2.1 for description of treatment protocols.

³Pregnancy rate of cows expressing estrus by 72 h after PG administration on (Day 30) subsequently inseminated at 72 h (Day 33).

⁴Pregnancy rate of cows expressing estrus between 72 h and 96 h after PG administration (Day 30) and subsequently inseminated at 96 h (Day 34).

⁵Pregnancy rate of cows failing to express estrus by 96 h after PG administration (Day 30) and subsequently administered GnRH and inseminated at 96 h (Day 34).

Figure 2.1

Figure 2.1. Treatment schedule for the 14-d CIDR-PG and 9-d CIDR-PG protocols. Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0, CIDR removal on Day 14, and administration of PG (25 mg im) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and administration of PG on Day 30. In both treatments, timed AI was performed using a split-time AI approach based on estrous response. At 72 h after PG (Day 33), cows having expressed estrus received timed AI; cows having not expressed estrus by 72 h received timed AI 24 h later (96 h after PG on Day 34), with gonadotropin-releasing hormone (GnRH; 100 µg im) administered to non-estrous cows concurrent with AI.

Figure 2.2

Figure 2.2. Percentage of cows in 14-d CIDR-PG (gray bar) and 9-d CIDR-PG (black bar) treatments expressing estrus after CIDR removal. No response indicates no expression of estrus between CIDR removal (Day 14) and PG administration (Day 30). See Figure 2.1 for description of treatment protocols.

Figure 2.3

Figure 2.3. Percentage of cows in 14-d CIDR-PG (gray bar) and 9-d CIDR-PG (black bar) treatments expressing estrus after PG administration (Day 33). No response indicates no expression of estrus by 96 h after PG administration. See Figure 2.1 for description of treatment protocols.

CHAPTER 3

THE 9-D CIDR-PG PROTOCOL II: CHARACTERIZATION OF ENDOCRINE PARAMETERS, OVARIAN DYNAMICS, AND PREGNANCY RATES TO FIXED- TIME AI FOLLOWING USE OF LONG-TERM CIDR-BASED ESTRUS SYNCHRONIZATION AMONG MATURE BEEF COWS

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ABSTRACT

An experiment was designed to evaluate endocrine parameters, ovarian dynamics, and pregnancy rates to fixed-time artificial insemination (FTAI) following the 9-d CIDR-PG protocol in comparison to the 14-d CIDR-PG protocol. While both are long-term protocols using CIDR treatment for presynchronization, the 9-d CIDR-PG protocol differs from the 14-d CIDR-PG protocol in that prostaglandin $F_{2\alpha}$ (PG) is administered at CIDR insertion and removal to facilitate a decreased length of progestin treatment and potentially enhance response to the presynchronization treatment. Estrus was synchronized for 393 mature beef cows across five locations. Treatments were represented in each location, and cows within each location were randomly assigned to one of the two protocols based on age, days postpartum (DPP), and body condition score (BCS). Cows assigned to the 14-d CIDR-PG treatment received a CIDR insert (1.38 g progesterone) on Day 0 with removal of CIDR on Day 14, and 25 mg PG 16 d after CIDR removal on Day 30. Cows assigned the 9-d CIDR-PG treatment received 25 mg PG and a CIDR insert (1.38 g progesterone) on Day 5; 25 mg PG and removal of CIDR

on Day 14; and 25 mg PG 16 d after CIDR removal on Day 30. In both treatments, cows received FTAI on Day 33, 72 h after PG. All cows were administered 100 µg gonadotropin-releasing hormone (GnRH) concurrent with insemination. For a subset of animals in each treatment, ovarian ultrasound was performed and blood samples were collected for determination of serum estradiol concentrations at CIDR removal, PG administration, and FTAI. Protocols were compared on the basis of estrous response and pregnancy rate resulting from FTAI. Serum estradiol concentrations, follicle size, and estrous response did not differ based on treatment. However, cows assigned to the 9-d CIDR-PG protocol tended to achieve greater FTAI pregnancy rates than cows assigned to the 14-d CIDR-PG protocol (62% versus 52%; $P = 0.07$). Across treatments, greater pregnancy rates tended ($P = 0.10$) to be achieved by cows that expressed estrus prior to FTAI (69% for 9-d CIDR-PG, 58% for 14-d CIDR-PG) than by cows that failed to express estrus (55% for 9-d CIDR-PG, 47% for 14-d CIDR-PG). In summary, the 9-d CIDR-PG protocol is an effective protocol for synchronization of estrus among mature beef cows, and pregnancy rates to FTAI tended to be improved through use of the 9-d CIDR-PG compared to the 14-d CIDR-PG protocol.

INTRODUCTION

The 14-d CIDR-PG protocol is a simple, highly effective protocol for synchronization of estrus among beef replacement heifers. Due to the high rate of estrous response and synchrony of estrus observed following the 14-d CIDR-PG protocol, acceptable pregnancy rates can be obtained using fixed-time artificial insemination

(FTAI) among beef heifers following this protocol (Leitman et al., 2009a,b; Mallory et al., 2010). In contrast, when the 14-d CIDR-PG protocol is used among mature beef cows, lower rates of estrous response are observed after PG administration prior to FTAI (Nash et al., 2012a; Mallory et al., 2011). While pregnancy rates to FTAI have been acceptable among cows even in the absence of high estrous response, differences in response between cows and heifers suggest that this protocol may not be optimized for all age classes of beef females.

In the 14-d CIDR-PG estrus synchronization protocol, a 14 d period of CIDR treatment is used for presynchronization prior to prostaglandin $F_{2\alpha}$ (PG) administration 16 d after CIDR removal on Day 30 of the protocol, at which point the majority of females should have a large, second-wave dominant follicle and a mature corpus luteum (CL). The length of the CIDR treatment is intended to establish large preovulatory follicles among the majority of females, so as to generate a synchronous initial estrus event following CIDR removal. However, comparing data previously published among cows to those published among heifers, estrus expression after CIDR removal is less synchronous among mature cows, and mean interval to estrus after CIDR removal is extended (Nash et al., 2013). This variation in timing of estrus expression may indicate a higher proportion of cows fail to undergo luteolysis by the end of a 14 d CIDR treatment or undergo luteolysis very late in CIDR treatment. As development of a persistent follicle during progesterone treatment is contingent upon spontaneous luteolysis, this could potentially result in more variation in physiological maturity of the dominant follicle at the time of CIDR removal. We hypothesize that these differences in timing of estrus expression after

CIDR removal largely explain the less synchronous estrus expression observed among mature cows following PG, as the initial estrus expression and subsequent ovulation after CIDR removal serves to presynchronize follicular waves prior to administration of PG on Day 30 of the protocol.

In a previous pilot study, we found that administration of PG at CIDR insertion and removal facilitated a decrease in the length of CIDR treatment from 14 to 9 d (Thomas et al., 2016b). This was proposed as being advantageous for producers attempting to use long-term protocols among cows with varying postpartum intervals, given the manufacturer's recommendation that a CIDR device not be utilized among females <20 d postpartum. Moreover, in addition to achieving follicular dynamics similar to those obtained when using the 14-d CIDR-PG protocol, the shortened 9-d CIDR-PG protocol resulted in enhanced estrous response rate after PG. Pregnancy rates also tended to be increased among 9-d CIDR-PG treated cows compared to 14-d CIDR-PG treated cows. However, that experiment, which had been designed primarily to characterize follicular dynamics and timing of estrous response, lacked sufficient power to make meaningful interpretations of potential differences in pregnancy rate based on treatment. Similarly, insemination was performed using split-time AI (STAI) rather than FTAI so as to maximize estrous response (Thomas et al., 2014a,b; Bishop et al., 2016, 2017b). Therefore, the present experiment was designed as a larger scale field trial to compare the 9-d CIDR-PG and 14-d CIDR-PG protocols based on endocrine parameters, ovarian dynamics, and pregnancy rates to FTAI.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Animals. Estrus was synchronized for suckled beef cows ($n = 393$) of varying age and parity across five locations. Location 1 consisted of two-year-old primiparous suckled cows; Locations 2-4 consisted of cows of varying age and parity; and Location 5 consisted of mature, non-lactating multiparous cows. Within each location, cows were assigned to one of two treatments (Figure 3.1) based on age and days postpartum (DPP). Cows assigned to the 14-d CIDR-PG protocol received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, CIDR removal on Day 14, and PG (25 mg im; Lutalyse, Zoetis, Madison, NJ) on Day 30. Cows assigned to the 9-d CIDR-PG protocol received PG concurrent with CIDR insertion on Day 5, PG concurrent with CIDR removal on Day 14, and PG on Day 30. In both treatments, FTAI was performed on Day 33, 72 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 μ g im; Cystorelin, Merial, Athens, GA) was administered to all cows concurrent with FTAI. Body condition scores (BCS) were assigned to each animal on Day 0 using a 1 to 9 scale (1 = emaciated and 9 = obese) (Richards et al., 1986). Times of PG administration and FTAI were recorded for each cow. Units of conventional semen (approximately 20×10^6 live cells per 0.5 ml straw prior to freezing) from a single AI sire were used for FTAI within each location. Two technicians performed inseminations in Location 1, with technicians pre-assigned and balanced across treatments based on cow age, DPP, and BCS. Within remaining locations, a single technician performed all inseminations.

Fourteen d after AI, cows were exposed to fertile bulls for the remainder of a 60 d breeding season.

Estrus detection. Estrus detection aids (Estroject, Rockway Inc, Spring Valley, WI) were applied at PG administration on Day 30 and evaluated at FTAI on Day 33. Estrus expression was defined as removal of >50% of the rub-off coating on the estrus detection aid.

Blood collection and RIA. Among a subset of cows at Location 4 (n = 39), blood samples were collected via jugular venipuncture on Day -10 and 0 to determine pretreatment estrous cyclicity status by progesterone concentrations and on Days 14, 30, and 33 to determine serum estradiol (E₂) concentrations at CIDR removal, PG, and FTAI respectively. Blood samples were allowed to clot and stored at 4 °C for 24 h, with serum collected by centrifugation for 20 min at 1500 g and stored at -20 °C. Serum concentrations of progesterone were determined via a validated radioimmunoassay (RIA) (Pohler et al., 2016). The intra-assay CV was less than 5%. Cows were considered to be estrous cycling when P₄ concentrations were ≥ 0.5 ng/mL at one or both of the pretreatment blood sampling times. Blood samples collected at PG and FTAI were assayed in triplicate for estradiol, using the liquid-phase double-antibody RIA procedures described by Rozell and Keisler (1990) with modifications as follows. All assay buffers consisted of 0.1% gelatin, 0.01 M EDTA, 0.9% NaCl, 0.01 M phosphate, 0.01% sodium azide, 0.05% Tween-20, pH = 7.1 (PABET). As a standard measure of assay quality control and performance, pooled bovine sera was assayed in triplicate at 25, 40, 60, 100, 175, 200, and 300 μ L volumes, which were performed in parallel to the standard curves.

The estradiol assay utilized 3-¹²⁵Iodo-Estradiol-17 β (MP Biomedicals # 07138226) and rabbit anti-estradiol antisera (MP Biomedicals #07138216). A pre-precipitated sheep anti-rabbit second antibody was used for precipitation. The intra-assay CV was less than 15%.

Ovarian ultrasonography. Transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) was performed to assess ovarian follicle size and presence of corpora lutea. Follicles were measured, and measurements reported are the largest follicle diameter (LFD). Ultrasonography was performed at CIDR removal on Day 14, at PG administration on Day 30, and at FTAI on Day 33.

Pregnancy diagnosis. Pregnancy rate to FTAI was determined by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) 81 to 98 d after FTAI.

Statistical analysis. Treatment differences for age, DPP, BCS, and timing of insemination relative to PG were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Results are reported as mean $\pm$ standard error of the mean unless otherwise indicated. Significance was defined as $P \leq 0.05$, and a probability of $0.05 > P \geq 0.10$ indicated that significance was approached. Serum concentrations of estradiol-17 β and differences in mean LFD were analyzed as a two by two factorial for treatment and cyclicity status using PROC MIXED (SAS Inst. Inc., Cary, NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to analyze treatment differences in estrous response rates prior to FTAI, final pregnancy rate at the

breeding season, as well as FTAI pregnancy rates by technician within Location 1. Pregnancy rate to FTAI was analyzed using a generalized linear model via the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. The error term was specified as the treatment x location interaction. Variables tested for inclusion in the model were treatment, location, technician, age, DPP, BCS, estrous response prior to FTAI, and the following interactions: treatment x age, treatment x DPP, treatment x BCS, treatment x technician, and treatment x estrous response. Variables that did not significantly ($P > 0.10$) affect pregnancy rate to STAI were removed from the model. The final model for pregnancy rate to FTAI consisted of treatment, DPP, technician, and estrous response prior to FTAI. Least squares means were generated using this model and compared using Fisher's least significant difference.

RESULTS

Cow age, BCS, DPP, and estrous response rates are shown for each treatment and location in Table 3.1. Age, BCS, and DPP did not differ between treatments. Timing of FTAI did not differ between the 9-d CIDR-PG and 14-d CIDR-PG treatments (72.7 ± 0.1 h and 72.8 ± 0.1 h after PG administration). Within Location 1, pregnancy rates differed between technicians; however, there was no interaction of treatment x technician, as technicians were preassigned within treatments based on age, DPP, and BCS. Therefore, the effect of technician was included in the GLIMMIX model for pregnancy rate to FTAI. Pregnancy rate to FTAI tended to be affected by estrous response prior to FTAI ($P = 0.10$) and was significantly affected by DPP ($P = 0.018$); however, there was no

interaction of either estrous response or DPP with treatment.

Ovarian and endocrine data collected at CIDR removal, PG administration, and FTAI are presented in Tables 3-5. Treatments did not differ with respect to LFD at CIDR removal, PG administration, or FTAI. However, among cows assigned to the 14-d CIDR-PG protocol, LFD was larger ($P < 0.05$) at CIDR removal among anestrus cows (15.5 ± 0.8 mm) than among estrus cycling cows (13.0 ± 0.8 mm). Neither treatment nor pretreatment cyclicity status affected LFD at PG administration or FTAI, and all cows had corpora lutea at PG administration. Serum concentrations of estradiol-17 β did not differ based on treatment or pretreatment cyclicity status at CIDR removal, PG administration, or FTAI.

Pregnancy rates to FTAI based on treatment and estrous response are shown in Table 3.2. Cows assigned to the 9-d CIDR-PG protocol tended to achieve greater FTAI pregnancy rates than cows assigned to the 14-d CIDR-PG protocol (62% versus 52%; $P = 0.07$). Estrus response prior to FTAI did not differ between treatments (9-d CIDR-PG = 50%; 14-d CIDR-PG = 47%). Across treatments, greater pregnancy rates tended ($P = 0.10$) to be achieved by cows that expressed estrus prior to FTAI (69% for 9-d CIDR-PG, 58% for 14-d CIDR-PG) than among cows that failed to express estrus (55% for 9-d CIDR-PG, 47% for 14-d CIDR-PG). Final pregnancy rate at the end of a 60 d breeding period did not differ between cows assigned to the 14-d CIDR-PG (93%; 186/199) and 9-d CIDR-PG (95%; 184/194) treatments.

DISCUSSION

In long-term progestin-based estrus synchronization protocols, presynchronization is achieved through a synchronous, yet sub-fertile estrus that occurs following progestin withdrawal. The 9-d CIDR-PG protocol makes use of a unique approach to presynchronization, employing PG administration at progesterone device insertion and removal. In addition to reducing the length of the protocol by 5 d, the use of PG for induction of luteolysis may improve presynchronization response among mature cows compared to the standard 14-d CIDR-PG protocol, which relies on spontaneous luteolysis (Thomas et al., 2016b). The results presented here support those previously published, as pregnancy rate to FTAI tended to be increased following the 9-d CIDR-PG protocol compared to the 14-d CIDR-PG protocol.

Although the 14-d CIDR-PG protocol is highly effective for synchronization of estrus among beef heifers, results from previous publications suggest the treatment schedule is not optimized for mature cows. For example, when using the 14-d CIDR-PG protocol among mature cows ≥ 4 years of age, use of a 19 d rather than 16 d interval to PG significantly improved estrous response prior to FTAI (47.4% vs 29.7%; $P < 0.01$) and tended to improve pregnancy rates to FTAI (Martin et al., 2013). However, this modification adds 3 d in length to the protocol and did not improve results in young cows. In contrast, the 9-d CIDR-PG protocol reduces the length of the treatment schedule by 5 d, and the tendency for improved pregnancy rates to FTAI was observed uniformly across varying age classes of cows.

Regarding estrous response prior to FTAI, a previous pilot study comparing the 9-d CIDR-PG and 14-d CIDR-PG protocols evaluated a larger time period of potential estrus expression (until 96 h after PG) and found that total estrous response rate after PG was improved following the 9-d CIDR-PG protocol compared to the 14-d CIDR-PG (Thomas et al., 2016b). This work also noted exceptional synchrony in the timing of estrus onset, with 91% of cows detected in estrus over a 32 h time period (59 to 91 h after PG) following the 9-d CIDR-PG protocol. The improvement in estrous response and synchrony was attributed to the presynchronization approach used in the 9-d CIDR-PG protocol (Thomas et al., 2016b). In the present study, estrous response rate up to the point of FTAI did not differ based upon treatment. However, it should be noted that estrous response was characterized using Estroject estrus detection aids rather than HeatWatch transmitters, so any differences in timing of estrus expression cannot be evaluated in the present study. More importantly, the full distribution of estrus expression was not observed, as FTAI was conducted at 72 h after PG. Therefore, improved synchronization success would likely not be manifest as increased estrous response rate prior to FTAI in the present study. This interpretation may be supported by the greater pregnancy rate achieved among cows irrespective of estrous status following the 9-d CIDR-PG protocol compared to the 14-d CIDR-PG protocol (Table 3.2).

Given the tendency for improved AI pregnancy rate observed in the absence of demonstrable improvements in estrous response rate, it remains possible that improved fertility following the 9-d CIDR-PG protocol results from other factors rather than

enhanced estrous response. The 9-d CIDR-PG protocol makes use of PG administration during the presynchronization treatment schedule, and other authors have proposed that PG administration in and of itself may have favorable impacts on fertility among anestrous cows through mechanisms independent of luteolysis (Lopes et al., 2015). However, while cyclicity status was not robustly characterized for all animals in this dataset, there was no interaction of treatment with factors known to be associated with anestrous, such as age, DPP, or BCS.

It is also possible that protocols involving presynchronization may have differing effects in the subsequent estrous cycle occurring after presynchronization. For example, a 14 d feeding of melengestrol acetate (MGA) resulted in increased circulating estradiol concentrations among cows during the second estrous cycle after MGA withdrawal (Fralix et al., 1996). Similarly, in recent work comparing the 14-d CIDR-PG and 7-d CO-Synch + CIDR protocols specifically in two-year-old postpartum beef cows, circulating estradiol concentrations at PG administration and FTAI were markedly higher among cows following the long term 14-CIDR-PG compared to the short term 7-d CO-Synch + CIDR protocol (Abel et al., 2016). Moreover, pregnancy rates were similar between protocols despite markedly lower estrous response rates observed among 14-d CIDR-PG treated cows (Abel et al., 2016). These results raise questions relative to the potential effect of presynchronization treatments on circulating estradiol concentrations and fertility in the subsequent estrous cycle. However, it should be noted that no differences in circulating estradiol concentrations or ovarian follicle size were detected between the 14-d CIDR-PG and 9-d CIDR-PG treatments in the present experiment.

Given these results with long-term CIDR-based protocols, further work is needed to evaluate fertility in comparison to more commonly used short-term protocols, such as the 5-d and 7-d CO-Synch + CIDR protocols. Although these protocols generate acceptable pregnancy rates, the timing of progesterone treatment may be less than optimal for several reasons, including the potential for aged ovarian follicles among a proportion of cows not responding to GnRH administration on Day 0 of the protocol (Bridges et al., 2008, 2009). Moreover, in contrast to the timing of progesterone administration in long-term protocols involving progestin-based presynchronization, administration of progesterone in short-term CIDR-based protocols occurs during development of the ovulatory follicle. Elevated circulating progesterone concentrations during follicular development were demonstrated to affect ovulatory follicle size and may impair fertility through decreased steroidogenic capacity of the follicle and subsequent CL (Adams et al., 1992b; Carvalho et al., 2008; Dias et al., 2009; Mantovani et al., 2010; Sá Filho et al., 2010; Stevenson and Lamb, 2016). Previous publications have evaluated the long-term 14-d CIDR-PG protocol in comparison to the short-term 7-d CO-Synch + CIDR protocol, with results published specifically for primiparous two-year-old beef cows (Abel et al., 2016) and for mature beef cows of varying age and parity (Nash et al., 2012a). In both publications, estrous response prior to FTAI was lower following the 14-d CIDR-PG protocol but pregnancy rates to FTAI were comparable between protocols. Given the pregnancy rates observed in the present experiment, further research efforts are warranted to directly compare the 9-d CIDR-PG protocol to more commonly used short-

term protocols.

Lastly, although acceptable pregnancy rates were obtained when FTAI was conducted at 72 h after PG in this experiment, the optimal time point at which FTAI should be performed following the 9-d CIDR-PG protocol is unknown. Timing of AI relative to estrus onset is consequential, as inseminating too early relative to ovulation results in decreased fertilization rate, while inseminating too late results in lower embryo quality (Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001). Elsewhere we have proposed that the optimal time point for FTAI is the point at which estrous response rate is maximized but those animals expressing estrus earliest in the distribution are not inseminated too late relative to ovulation (Thomas and Patterson, 2014). Although the optimal time point must be determined through experimental efforts comparing different FTAI times, we speculate that optimal timing of FTAI following the 9-d CIDR-PG protocol may be later than 72 h after PG based on the previously characterized distribution of estrus onset times (Thomas et al., 2016b). In the absence of research efforts to determine the optimal time point for FTAI, producers electing to use the 9-d CIDR-PG protocol in conjunction with timed AI may be advised to conduct inseminations using a STAI rather than FTAI approach to achieve maximum pregnancy rates (Thomas et al., 2014a,b, 2016; Bishop et al., 2016, 2017a,b).

In summary, the 9-d CIDR-PG protocol is an effective long-term CIDR-based protocol for synchronization of estrus among mature beef cows. While requiring two additional doses of PG per cow, the 9-d CIDR-PG protocol offered a 5 d reduction in protocol length and tended to result in increased pregnancy rates to FTAI when compared

to the 14-d CIDR-PG protocol.

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Figure 3.1

14-d CIDR-PG

9-d CIDR-PG

Figure 3.1. Treatment schedule for the 14-d CIDR-PG and 9-d CIDR-PG protocols. Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Cows assigned to the 9-d CIDR-PG protocol received prostaglandin $F_{2\alpha}$ (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 72 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 μ g im) was administered to all cows concurrent with FTAI.

Table 3.1. Cow age, days postpartum (DPP), body condition score (BCS), and estrous response based on treatment and location.

Treatment ¹	Location ²	N	Age	DPP	BCS ³	Estrous Response Prior to FTAI ⁴	
						Proportion	%
14-d CIDR-PG		199	3.3 ± 0.1	86.2 ± 2.2	5.3 ± 0.1	93/199	47
	Location 1	101	2.2 ± 0.1	90.8 ± 1.2	5.1 ± 0.1	54/101	53
	Location 2	32	4.6 ± 0.5	77.0 ± 1.9	5.8 ± 0.1	14/32	44
	Location 3	13	5.5 ± 0.9	87.0 ± 3.2	5.3 ± 0.1	6/13	46
	Location 4	36	4.1 ± 0.3	81.2 ± 2.3	5.4 ± 0.1	11/36	31
	Location 5	17	3.5 ± 0.1		5.3 ± 0.1	8/17	47
9-d CIDR-PG		194	3.4 ± 0.1	86.4 ± 2.2	5.3 ± 0.1	97/194	50
	Location 1	98	2.2 ± 0.1	91.5 ± 1.2	5.1 ± 0.1	55/98	56
	Location 2	25	5.0 ± 0.5	79.0 ± 2.1	6.0 ± 0.1	11/25	44
	Location 3	17	5.2 ± 0.7	83.7 ± 3.0	5.3 ± 0.1	5/17	29
	Location 4	36	4.5 ± 0.4	79.1 ± 2.4	5.4 ± 0.1	14/36	39
	Location 5	18	3.4 ± 0.1		5.3 ± 0.1	12/18	67

¹Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Cows assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and fixed-time artificial insemination (FTAI) was performed on Day 33, 72 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all cows concurrent with FTAI.

²Location 1 consisted of exclusively two-year-old primiparous suckled cows; Locations 2 and 3 consisted of suckled cows of varying age and parity; and Location 4 consisted of mature, non-lactating multiparous cows.

³BCS evaluated at CIDR insertion on Day 0.

⁴Estrous response prior to FTAI, as determined by activation of an estrus detection aid (Estrotest) by 72 h after PG administration. Estrus expression was defined as removal of >50% of the rub-off coating on the estrus detection aid.

Table 3.2. Pregnancy rates¹ to fixed-time artificial insemination (FTAI) based on treatment² and estrous response³ prior to FTAI.

Treatment	Overall		Estrous		Non-Estrous	
	Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	104/199	52 ^a	54/93	58	50/106	47
Location 1	50/101	50	30/54	56	20/47	43
Location 2	19/32	59	7/14	50	12/18	67
Location 3	4/13	31	4/6	67	0/7	0
Location 4	18/36	50	7/11	64	11/25	44
Location 5	13/17	76	6/8	75	7/9	78
9-d CIDR-PG	120/194	62 ^b	67/97	69	53/97	55
Location 1	58/98	59	37/55	67	21/43	49
Location 2	17/25	68	6/11	55	11/14	79
Location 3	10/17	59	3/5	60	7/12	58
Location 4	20/36	56	10/14	71	10/22	45
Location 5	15/18	83	11/12	92	4/6	67

¹Pregnancy rate to AI determined by transrectal ultrasonography 81 to 92 d after FTAI.

²Cows assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Cows assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and fixed-time artificial insemination (FTAI) was performed on Day 33, 72 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all cows concurrent with FTAI.

³Estrous response prior to FTAI, as determined by activation of an estrus detection aid (Estrotect).

^{ab}Across locations, overall pregnancy rate to FTAI tended to be greater ($P = 0.07$) among 9-d CIDR-PG treated cows.

Table 3.3. Ultrasound data¹ collected at CIDR removal and estrous response following CIDR removal based on treatment and pre-treatment cyclicity status.

Treatment ²	LFD at CIDR removal	CL present at CIDR removal		Serum E ₂ at CIDR removal	Estrous response following CIDR removal	
	mm	Proportion	%	pg/ml	Proportion	%
14-d CIDR-PG	13.9 ± 0.7	3/20	15	5.6 ± 0.4	17/20	85
Anestrous	15.5 ± 0.8 ^a	0/7	0	5.8 ± 0.8	4/7	57
Estrous cycling	13.0 ± 0.8 ^b	3/13	23	5.5 ± 0.6	13/13	100
9-d CIDR-PG	13.9 ± 0.6	4/19	21	5.3 ± 0.4	18/19	95
Anestrous	13.9 ± 0.5	0/6	0	5.6 ± 1.1	6/6	100
Estrous cycling	14.0 ± 0.9	4/13	31	5.2 ± 0.4	12/13	92

¹Transrectal ultrasonography was performed to assess ovarian follicular dynamics and presence of corpora lutea. Dominant follicles were measured, and measurements reported are the largest follicle diameter (LFD).

²See Table 3.1 for description of treatment protocols.

^{ab}Diameter of the largest ovarian follicle present at CIDR removal was greater ($P = 0.05$) for anestrous compared to estrous cycling cows following the 14-d CIDR presynchronization.

Table 3.4. Ultrasound data¹ collected and serum estradiol concentrations at PG administration based on treatment and pre-treatment cyclicity status.

Treatment ²	LFD	CL present		Serum E ₂ concentrations
	mm	Proportion	%	pg/ml
14-d CIDR-PG	11.6 ± 0.5	20/20	100	5.2 ± 0.5
Anestrous	11.7 ± 0.6	7/7	100	5.8 ± 1.1
Estrous cycling	11.5 ± 0.6	13/13	100	4.9 ± 0.5
9-d CIDR-PG	10.9 ± 0.5	19/19	100	4.8 ± 0.3
Anestrous	9.8 ± 0.5	6/6	100	5.2 ± 0.9
Estrous cycling	11.3 ± 0.7	13/13	100	4.7 ± 0.3

¹Transrectal ultrasonography was performed to assess ovarian follicular dynamics and presence of corpora lutea. Dominant follicles were measured, and measurements reported are the largest follicle diameter (LFD).

²See Table 3.1 for description of treatment protocols.

Table 3.5. Ultrasound data¹ collected, serum estradiol concentrations, estrous response, and pregnancy rate to FTAI based on treatment and pre-treatment cyclicity status.

Treatment ²	LFD	Serum E ₂ concentrations	Estrous response		Pregnancy rate to FTAI	
	mm	pg/ml	Proportion	%	Proportion	%
14-d CIDR-PG	12.7 ± 0.6	6.9 ± 0.8	5/20	25	9/20	45
Anestrous	12.3 ± 0.6	7.9 ± 2.1	1/7	14	2/7	29
Estrous cycling	12.9 ± 0.8	6.4 ± 0.8	4/13	31	7/13	54
9-d CIDR-PG	13.4 ± 0.6	5.9 ± 0.5	5/19	26	11/19	58
Anestrous	13.0 ± 0.9	5.3 ± 0.5	2/6	33	5/7	71
Estrous cycling	13.5 ± 0.8	6.2 ± 0.7	3/12	25	6/12	50

¹Transrectal ultrasonography was performed to assess ovarian follicular dynamics and presence of corpora lutea. Dominant follicles were measured, and measurements reported are the largest follicle diameter (LFD).

²See Table 3.1 for description of treatment protocols.

CHAPTER 4

**THE 9-D CIDR-PG PROTOCOL III: EVALUATION OF SYNCHRONY OF
ESTRUS, ENDOCRINE PARAMETERS, OVARIAN DYNAMICS, AND
PREGNANCY RATES TO AI IN COMPARISON TO THE 7-D CO-SYNCH +
CIDR PROTOCOL**

ABSTRACT

Response of mature beef cows following a recently developed long-term estrus synchronization protocol was characterized in reference to a short-term protocol commonly used in commercial production. Mature beef cows (n = 480) across four locations were assigned based on age and days postpartum to either the 9-d CIDR-PG protocol [25 mg prostaglandin F_{2α} (PG) and insertion of a 1.38 g progesterone insert (CIDR) on Day -28; 25 mg PG and CIDR removal on Day -19; and 25 mg PG on Day -3] or the 7-d CO-Synch + CIDR protocol [100 µg gonadotropin-releasing hormone (GnRH) and CIDR insertion on Day -10; 25 mg PG and CIDR removal on Day -3]. On Day -3, estrus detection aids (Estroject[®]) were applied, and a subset of cows (n = 203) were fitted with radiotelemetric, pressure-sensitive devices (HeatWatch[®]) to characterize timing of estrus onset. Split-time artificial insemination (AI) was performed on Day 0 or 1 based on timing of estrus expression, with cows failing to express estrus by Day 1 administered 100 µg GnRH at AI. In Locations 1, 2, and 3, blood samples were collected for radioimmunoassay on Day -38 and Day -28 to determine pretreatment estrous cyclicity status based on serum progesterone concentrations. Additional blood samples were

collected at PG administration and at AI for determination of serum estradiol concentrations. Transrectal ovarian ultrasound was performed at PG administration and AI for a subset of cows ($n = 154$) to assess ovarian follicle size. Response following the 9-d CIDR-PG protocol differed ($P < 0.05$) from the 7-d CO-Synch + CIDR protocol with respect to mean interval from PG to estrus onset (71.2 ± 1.3 h versus 56.5 ± 1.6 h), variance in interval to estrus, ovulatory follicle size at AI (13.7 ± 0.2 mm versus 14.6 ± 0.2), and serum estradiol concentrations at AI (6.9 ± 0.3 pg/ml versus 6.0 ± 0.3 pg/ml). Similar pregnancy rates to AI were obtained among cows following the 7-d CO-Synch + CIDR (66%; 163/247) and 9-d CIDR-PG (70%; 163/233) treatments. In summary, when compared among mature beef cows to the 7-d CO-Synch + CIDR protocol, the 9-d CIDR-PG protocol resulted in later timing and enhanced synchrony of estrus expression, greater serum estradiol concentrations and smaller follicle size at AI, and similar pregnancy rates following STAI.

INTRODUCTION

Protocols commonly used for synchronization of estrus in the beef and dairy industries vary widely with respect to the approach used to generate synchrony of ovarian follicular development prior to timed artificial insemination (AI). In many protocols commonly used in the United States, gonadotropin-releasing hormone (GnRH) is administered at the start of the protocol in order to reduce variation in stage of ovarian follicular development. However, response to GnRH administered at any random point of the estrous cycle is variable based on the size and physiological maturity of the ovarian

follicle present (Vasconcelos et al., 1999; Moreira et al., 2000; Geary et al., 2000; Atkins et al., 2008). As a result, response rates of beef and dairy cows to GnRH in short-term protocols are generally reported to be in the range of 65% (Vasconcelos et al., 1999; Geary et al., 2000). However, response to GnRH is markedly higher (80-90%) among cows that are between days 5 and 9 of the estrous cycle (Vasconcelos et al., 1999; Moreira et al., 2000), with results likely optimal on day 6 (Bello et al., 2006). Therefore, numerous research efforts have focused on presynchronization programs to manage stage of the estrous cycle prior to administration of GnRH or other products.

In the dairy industry, the development of long-term, presynchronization based approaches for estrus synchronization has significantly enhanced reproductive performance of lactating dairy cows, with fertility to timed AI now exceeding results obtained with estrus detection alone (Wiltbank and Pursley, 2014). Protocols such as Pre-Synch OvSynch, G6G, or Double OvSynch are all effective options that can be implemented feasibly given the frequency with which animals are routinely handled in the dairy industry. By comparison, efforts to develop long-term, presynchronization-based protocols for beef cows have been limited. Short-term protocols such as the 7-d CO-Synch + CIDR (Lamb et al., 2006) or the 5-d CO-Synch + CIDR (Bridges et al., 2008) have been evaluated much more extensively in beef cattle, in part because short-term protocols are presumed to be more practical in commercial beef production settings.

One long-term, presynchronization-based protocol that has been evaluated somewhat extensively in beef cattle is the 14-d CIDR-PG protocol. This protocol is highly effectively for synchronization of estrus across a broad class of beef and dairy

females (Leitman et al., 2009a,b; Mallory et al., 2010; Nash et al., 2011, 2012a, 2013; Mallory et al., 2013; Escalante et al., 2013a,b; Claypool et al., 2015; Abel et al., 2017). However, although commonly used among beef heifers, few producers make use of this protocol among mature beef cows, primarily for two reasons. First, the 33 d length of the protocol presents a logistical challenge when synchronizing estrus among cows of varying postpartum intervals, due to the recommendation that use of CIDR inserts should be restricted to only those cows that are > 20 d postpartum. Second, rates of estrous response prior to FTAI following the 14-d CIDR-PG protocol are much lower among mature beef cows (23%; Nash et al., 2012a) than those observed among beef heifers. Although reasonably high pregnancy rates are obtained among cows despite this low estrous response (Nash et al., 2012a; Abel et al., 2017), lack of visible estrous response is unsettling to producers and likely limits adoption of this protocol for use in cows.

Low rates of estrous response prior to FTAI suggest the treatment schedule of the 14-d CIDR-PG protocol is not optimized specifically for mature cows. Martin et al. (2013) found estrous response rates could be enhanced among mature cows (>4 years of age) by using a 19 d rather than 16 d interval from CIDR removal to PG administration (47.4% vs 29.7%; $P < 0.01$). Use of this extended interval also tended to improve pregnancy rates following FTAI among mature cows. However, this modification adds 3 d in length to a protocol that is likely already too long for some commercial applications, and this modification did not improve results among young cows (2-3 years of age). In experimental efforts to enhance response of mature cows to long-term CIDR-based estrus synchronization, Thomas et al. (2016a) evaluated an alternative presynchronization

approach, termed the 9-d CIDR-PG protocol, in which administration of PG was included in the presynchronization treatment schedule. Rather than relying on occurrence of spontaneous luteolysis during progestin presynchronization, PG was administered to induce luteolysis at CIDR insertion, thereby generating a low progesterone milieu for development of persistent follicles among the majority of cyclic females. Since a proportion of cows may have unresponsive CL < 5 d of age at the time of the first PG administration, administration of PG was also included in the treatment schedule at CIDR removal. This approach facilitated a 5 d reduction in the length of CIDR treatment from 14 to 9 d, likewise decreasing total length of the protocol by 5 d. In addition, the shortened 9-d CIDR-PG protocol resulted in enhanced rates of estrous response after PG and tended to increase pregnancy rates to AI in comparison to the 14-d CIDR-PG treated cows. Moreover, although a preliminary data set with limited numbers, the synchrony of estrus expression observed in this study was noteworthy and resulted in interest in further evaluation of the 9-d CIDR-PG protocol. Subsequent research efforts comparing effectiveness of the 9-d CIDR-PG and 14-d CIDR-PG protocols found no difference between treatments in pregnancy rates to FTAI among heifers (Thomas et al., 2017a) but again indicated a tendency for improved pregnancy rates among mature cows (Thomas et al., 2017b).

Based on indications that synchrony of estrus expression and pregnancy rates to AI may be particularly high following the 9-d CIDR-PG protocol, the present trial was designed to provide complete characterization of this protocol in reference to an industry standard protocol. Therefore, the 9-d CIDR-PG and 7-d CO-Synch + CIDR protocols

were compared among mature beef cows, and response of cows was characterized with respect to pregnancy rates following STAI, timing and synchrony of estrus expression, serum estradiol concentrations, and ovarian follicle size and CL presence at time points of interest.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Animals and treatments. Estrus was synchronized for 480 suckled beef cows of varying age and parity across four locations. Within each location, cows were assigned to one of two treatments (Figure 4.1) based on age, days postpartum (DPP), and body condition score (BCS). Cows assigned to the 9-d CIDR-PG protocol received an Eazi-Breed CIDR[®] insert (1.38 g progesterone; Zoetis, Madison, NJ) and were administered PG (25 mg im dinoprost tromethamine; Lutalyse[®], Zoetis, Madison, NJ) on Day -28. On Day -19, CIDR inserts were removed and PG was administered. Cows were administered PG a final time on Day -3. Cows assigned to the 7-d CO-Synch + CIDR protocol received a CIDR insert on Day -10, and were administered GnRH (100 µg im gonadorelin diacetate tetrahydrate; Cystorelin[®]; Merial, Athens, GA) concurrent with CIDR insertion. On Day -3, CIDR inserts were removed and PG was administered.

Estrus detection. On Day -3, estrus detection aids (Estrotect[®], Rockway Inc, Spring Valley, WI) were applied, and a subset of cows (n = 203) were fitted with radiotelemetric, pressure-sensitive devices (HeatWatch[®], DDx Inc., Denver, CO) to

characterize timing of estrus onset. Estrus expression was defined as removal of >50% of the rub-off coating on the estrus detection aid. For cows fitted with transmitters, the time of estrus onset was defined as the first mount in a series of ≥ 3 mounts of ≥ 2 s in duration within a 4-h period (Busch et al., 2007).

Artificial insemination. Split-time artificial insemination (STAI) was performed on Day 0 or 1 based on timing of estrus expression (Thomas et al., 2014a,b; Bishop et al., 2016, 2017a,b). Due to differences between protocols in timing of estrus expression among cows, the optimal time points recommended (see review by Patterson et al., 2017a) for timed AI differ between the 7-d CO-Synch + CIDR protocol (66 h after CIDR removal and PG administration) and long-term protocols such as the 14-d CIDR-PG or the 9-d CIDR-PG protocols (72 h after PG administration). Therefore, estrous cows on Day 0 were scheduled to receive AI at 66 h after PG administration and CIDR removal in the 7-d CO-Synch + CIDR treatment and at 72 h after PG administration in the 9-d CIDR-PG treatment. In each treatment, insemination was delayed by 24 h (until 90 h or 96 h for the 7-d CO-Synch + CIDR or 9-d CIDR-PG treatments respectively) for cows failing to express estrus by the initial time point. Cows still failing to express estrus by the delayed time point on Day 1 were administered 100 μ g GnRH at AI. Times of PG administration and AI were recorded for each cow. Cows assigned to the 7-d CO-Synch + CIDR treatment received STAI at 66.6 ± 0.1 h or 90.0 ± 0.1 h after CIDR removal and PG administration. Cows assigned to the 9-d CIDR-PG treatment received STAI at 72.7 ± 0.1 h and 96.0 ± 0.1 h after PG administration. A single technician performed AI within each location. Units of conventional semen (approximately 20×10^6 live cells per 0.5 ml

straw prior to freezing) from commercial AI sires were used for AI. In locations using multiple AI sires, sires were used proportionally across treatments and were preassigned to cows within treatment based on age, DPP, and BCS. A single sire was used for AI in Locations 2 and 3, two sires were used in Location 1, and five sires were used in Location 4. Fourteen d after AI, cows were exposed to fertile bulls for the remainder of a 45 d breeding season.

Blood sampling and RIA. In Locations 1, 2, and 3, blood samples were collected for radioimmunoassay (RIA) on Day -38 and Day -28 to determine pretreatment estrous cyclicity status based on serum progesterone concentrations. Additional blood samples were collected at PG administration and at AI for determination of serum estradiol concentrations. Blood samples were allowed to clot and stored at 4 °C for 24 h, with serum collected by centrifugation and stored at -20 °C. Serum concentrations of progesterone were determined via a validated RIA (Pohler et al., 2016). The intra-assay CV was less than 5%. Cows were considered to be estrous cycling when P₄ concentrations were ≥ 0.5 ng/mL at one or both of the pretreatment blood sampling times. Blood samples collected at PG and FTAI were assayed in triplicate for estradiol, using the liquid-phase double-antibody RIA procedures described by Rozell and Keisler (1990) with modifications as follows. All assay buffers consisted of 0.1% gelatin, 0.01 M EDTA, 0.9% NaCl, 0.01 M phosphate, 0.01% sodium azide, 0.05% Tween-20, pH = 7.1 (PABET). As a standard measure of assay quality control and performance, pooled bovine sera were assayed in triplicate at 25, 40, 60, 100, 175, 200, and 300 μ L volumes, which were performed in parallel to the standard curves. The estradiol assay utilized 3-

¹²⁵Iodo-Estradiol-17 β (MP Biomedicals # 07138226) and rabbit anti-estradiol antisera (MP Biomedicals #07138216). A pre-precipitated sheep anti-rabbit second antibody was used for precipitation. The intra-assay CV was less than 15%.

Ovarian Ultrasonography. Transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) was performed for a subset of cows (n = 154) to assess ovarian follicle size and presence of corpora lutea. Follicles were measured, and measurements reported are the largest follicle diameter (LFD). Ultrasonography was performed at PG administration on Day -3 and at STAI on Day 0 or 1.

Pregnancy diagnosis. Pregnancy rate to AI was determined by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA), with pregnancies resulting from AI distinguished from those resulting from natural service based on fetal size (Curran et al., 1986), as heifers were not exposed to natural service bulls until 14 d after AI. Ultrasonography was performed on Day 84, Day 94, Day 67, and Day 76 in Locations 1-4 respectively.

Statistical analysis. Potential treatment differences for age, DPP, BCS, interval from PG administration to onset of estrus, and interval from onset of estrus to AI were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Variances associated with the interval to estrus were compared by F-test (greater variance divided by the smaller variance; Snedecor and Cochran, 1989). Serum concentrations of estradiol-17 β and mean LFD at PG administration and AI were analyzed as a two by two factorial for treatment and cyclicity status using PROC MIXED (SAS Inst. Inc., Cary,

NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to analyze treatment differences in pretreatment estrous cyclicity status, estrous response rates on Day 0 and Day 1, final pregnancy rate at the breeding season, as well as AI pregnancy rates by sire within Locations 1 and 4. Pregnancy rates to STAI were analyzed using a generalized linear model via the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. The error term was specified as the treatment x location interaction. Variables tested for inclusion in the model were treatment, location, bull, age, DPP, BCS, estrous response on Day 0, estrous response on Day 1, follicle size at PG administration, follicle size at AI, interval from PG administration to onset of estrus, interval from estrus onset to AI, and the following interactions: treatment x age, treatment x DPP, treatment x BCS, treatment x estrous response on Day 0, treatment x estrous response on Day 1, treatment x follicle size at PG, treatment x follicle size at AI, treatment x interval from PG administration to onset of estrus, and treatment x interval from onset of estrus to AI. Variables that did not significantly ($P > 0.10$) affect pregnancy rate to STAI were removed from the model. The final model for pregnancy rate to STAI consisted only of treatment. Least squares means were generated using this model and compared using Fisher's least significant difference.

RESULTS

Cow age, DPP, BCS, and estrous cyclicity status pretreatment are presented for each treatment and location in Table 4.1. Treatments did not differ with respect to mean cow age, DPP, and BCS. The proportion of cows determined to be estrous cycling prior to

treatment initiation did not differ between the 7-d CO-Synch + CIDR (74%; 103/140) and the 9-d CIDR-PG (72%; 97/134) treatments.

Several differences between treatments were observed relative to timing and synchrony of estrus expression. Rates of estrous response are presented for each treatment and location in Table 4.2, and Figure 4.2 presents timing of estrus onset among cows fitted with Heatwatch[®] transmitters. Mean interval to estrus onset differed ($P < 0.0001$) between cows treated with the 7-d CO-Synch + CIDR protocol (56.5 ± 1.6 h) and cows treated with the 9-d CIDR-PG protocol (71.2 ± 1.3 h). As a result, treatments differed with respect to the proportion of cows expressing estrus at the alternative time points for STAI. A greater proportion ($P < 0.0001$) of cows expressed estrus prior to the first time point for potential AI in the 7-d CO-Synch + CIDR treatment (58%; 143/247) compared to the 9-d CIDR-PG treatment (38%; 88/233). Likewise, mean interval from onset of estrus to AI on Day 0 was shorter ($P < 0.01$) among 9-d CIDR treated cows (11.1 ± 1.0 h) compared to 7-d CO-Synch + CIDR treated cows (16.4 ± 1.5 h). Conversely, a greater proportion ($P < 0.001$) of cows expressed estrus during the 24 h period prior to delayed insemination on Day 1 in the 9-d CIDR-PG treatment (43%; 101/233) compared to the 7-d CO-Synch + CIDR treatment (27%; 67/247). For cows that expressed estrus during the 24 h delay period, mean interval from onset of estrus to AI on Day 1 was similar between 9-d CIDR treated cows (16.2 ± 1.0 h) compared to 7-d CO-Synch + CIDR treated cows (15.7 ± 1.5 h). The cumulative proportion of cows expressing estrus prior to AI did not differ between treatments (85% and 81% for the 7-d CO-Synch + CIDR and 9-d CIDR-PG treatments respectively). However, timing of estrus expression

was more synchronous among 9-d CIDR-PG treated cows, with variance in interval to estrus being reduced ($P < 0.02$) among 9-d CIDR-PG treated cows compared to 7-d CO-Synch + CIDR treated cows.

Ovarian data and circulating estradiol concentrations at the time of PG administration on Day -3 are presented in Table 4.4 for estrous cycling and anestrous cows within each treatment. Size of the largest follicle present at PG administration did not differ based on treatment or cyclicity status, nor did the proportion of cows having CL at this time. Serum concentrations of estradiol at PG administration were similar between treatments. However, within each treatment, serum estradiol concentrations at PG administration were higher ($P = 0.02$) among cows determined to be estrous cycling prior to treatment initiation compared to cows determined to be anestrous.

Ovarian follicular data and circulating estradiol concentrations at AI are presented in Table 4.5. Size of the largest follicle present at AI differed between treatments, with cows treated with the 9-d CIDR-PG protocol having smaller ($P < 0.01$) mean diameter follicles (13.7 ± 0.2 mm) compared to cows treated with the 7-d CO-Synch + CIDR protocol (14.6 ± 0.2 mm); however, this difference was dependent on pretreatment cyclicity status. Follicle size at AI was similar between treatments for cows determined to be anestrous prior to treatment initiation. However, estrous cycling cows treated with the 9-d CIDR-PG protocol had smaller ($P < 0.01$) mean diameter follicles (13.6 ± 0.3 mm) at AI compared to cows treated with the 7-d CO-Synch + CIDR protocol, whether estrous cycling (14.6 ± 0.3 mm) or anestrous (14.5 ± 0.6). Conversely, serum estradiol concentrations at AI were higher ($P = 0.02$) among cows treated with the 9-d CIDR-PG

protocol (6.9 ± 0.3 pg/ml) compared to 7-d CO-Synch + CIDR treated cows (6.0 ± 0.3 pg/ml), regardless of whether cows were anestrous (6.7 ± 0.5 versus 5.3 ± 0.6 pg/ml; $P = 0.07$) or estrous cycling (7.0 ± 0.4 versus 6.2 ± 0.3 pg/ml; $P = 0.10$) prior to treatment initiation.

Similar pregnancy rates to AI were obtained among cows following the 7-d CO-Synch + CIDR (66%; 163/247) and 9-d CIDR-PG (70%; 163/233) treatments. There was no significant effect of the interaction of treatment and estrous response on pregnancy rates to AI. However, across treatments, higher pregnancy rates ($P < 0.001$) were obtained among cows that expressed estrus prior to AI (71%; 285/400) than among cows that failed to express estrus (51%; 41/80). Final pregnancy rates at the end of a 45 d breeding period did not differ between cows assigned to the 7-d CO-Synch + CIDR treatment (94%; 233/247) and cows assigned to the 9-d CIDR-PG treatment (93%; 216/233).

DISCUSSION

The 9-d CIDR-PG protocol is a highly effective long-term CIDR-based protocol for synchronization of estrus among mature beef cows. The high pregnancy rates obtained following STAI in this trial (70%; 163/233) support results previously published from a pilot study (Thomas et al., 2016) evaluating pregnancy rates to STAI following the 9-d CIDR-PG protocol (77%; 33/43). Although similar pregnancy rates were observed in this experiment among cows treated with the 9-d CIDR-PG and 7-d CO-Synch + CIDR protocols, treatments differed with respect to timing and synchrony of estrus expression as well as circulating concentrations of estradiol at the time of AI.

In a strict sense, the term proestrus refers to the period from luteolysis until *onset of estrus*, although several publications use the term “proestrus length” loosely when evaluating the effect of timing of luteolysis in relation to GnRH administration for ovulatory induction. In a series of publications, Bridges et al. (2008, 2009) reported that several factors associated with fertility were enhanced among cows having a longer period of time from luteolysis to induction of ovulation: circulating concentrations of estradiol at AI; circulating concentrations of progesterone in the subsequent luteal phase; normality of subsequent luteal phase length; and uterine expression of estradiol receptor in the endometrium and nuclear progesterone receptor in the deep glandular epithelium on Day 15.5 after GnRH administration. These studies highlight the fact that several important physiological processes occur during the span of time from luteolysis until the gonadotropin surge. However, as these trials likely evaluate effects of physiological maturity of the ovulatory follicle rather than “proestrus length,” it is unclear whether a longer period of time from PG administration to onset of spontaneous estrus is itself associated with enhanced fertility. In the present data, the average interval from PG administration to onset of estrus was substantially increased among 9-d CIDR-PG treated (71.2 ± 1.3 h) compared to 7-d CO-Synch + CIDR treated (56.5 ± 1.6 h) cows (see Figure 4.2). This longer proestrus suggests that preovulatory follicles among 9-d CIDR-PG treated cows were less physiologically mature when luteolysis was induced, despite similar follicle size and serum estradiol concentrations at the time of PG administration on Day -3. The consequences of the longer proestrus observed among 9-d CIDR-PG treated cows are unclear but merit further investigation based on the fertility observed.

Timing of insemination in relation to timing of ovulation also critically affects fertility, with insemination too late relative to ovulation resulting in poor embryo quality and insemination too early resulting in poor fertilization success (Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001). Timing of ovulation is related to timing of estrus onset, with ovulation occurring approximately 28-30 h after onset of estrus (Shams et al., 1977; Walker et al., 1996; White et al., 2002). Thus, synchrony of timing of estrus onset is of significant interest when evaluating estrus synchronization protocols for use with timed AI. Variance in interval to estrus following PG administration was reduced among 9-d CIDR-PG treated cows compared to 7-d CO-Synch + CIDR treated cows. Although treatments did not differ with respect to pregnancy rates to STAI in the present data, further research using the 9-d CIDR-PG protocol is necessary to evaluate potential effects of enhanced synchrony of estrus expression. For example, synchrony of estrus expression may be particularly consequential when performing timed AI using sex-sorted semen or semen from sires with suboptimal fertility in timed AI programs.

The high fertility observed in this trial is likely due in part to use of a STAI rather than FTAI approach, given the high rates of total estrous response observed (83% across treatments). Animals that fail to express estrus prior to FTAI achieve markedly lower pregnancy rates than those that express estrus (Richardson et al., 2016). Use of a STAI rather than FTAI approach affords an additional 24 h for potential expression of estrus among the synchronized group, thus minimizing the proportion of cow that fail to express estrus prior to AI (Thomas et al., 2014a,b; Bishop et al., 2016, 2017b). However, low rates of estrous response prior to AI are likely to be observed when FTAI is performed at

72 h following PG administration using the 9-d CIDR-PG protocol. In the present study, 38% (88/233) of cows treated with the 9-d CIDR-PG expressed estrus by 72 h after PG administration, similar to previously published (Thomas et al., 2016; 2017b) low estrous response rates of 50% (97/194) and 56% (24/43) by this time. This may indicate that use of STAI rather than FTAI should be encouraged following the 9-d CIDR-PG protocol, or that optimal timing for FTAI should be later than 72 h. Although reasonably high pregnancy rates have been reported (Thomas et al., 2017b) when FTAI was performed following the 9-d CIDR-PG protocol (62%; 120/194), the relative fertility of the 9-d CIDR-PG and 7-d CO-Synch + CIDR protocols when FTAI is performed has not been investigated.

Circulating concentrations of estradiol at AI also differed between treatments, with higher serum estradiol concentrations among cows treated with the 9-d CIDR-PG protocol. The biological significance of this difference is unclear in the context of the present data set, as pregnancy rates to AI did not differ significantly between treatments. However, preovulatory estradiol plays a critical role in several physiological processes that contribute to the establishment and maintenance of pregnancy, with effects of estradiol upon follicular cells, the oocyte, gamete transport, and the uterus (see review by Pohler et al., 2012). Some literature evaluating use of progestin presynchronization suggests serum estradiol concentrations may be elevated among cows treated with long-term progestin based protocols. Fralix et al. (1996) found that cows treated with melengestrol acetate (MGA) for 14 days had increased serum estradiol concentrations during the second estrous cycle after MGA treatment. A similar effect was observed by

Abel et al. (2017) in a comparison of the 14-d CIDR-PG and 7-d CO-Synch + CIDR protocols specifically among primiparous two-year-old beef cows. Among cows treated with the long-term protocol, serum estradiol concentrations at FTAI were nearly double those observed among cows treated with the short-term 7-d CO-Synch + CIDR protocol (8.0 ± 0.7 versus 4.2 ± 0.8 pg/ml; $P < 0.001$). Moreover, pregnancy rates were similar between long- and short-term treatments despite significantly lower rates of estrous response observed among 14-d CIDR-PG treated cows. In the present data, estradiol concentrations at AI were higher both among 9-d CIDR-PG treated cows that had been classified as anestrous prior to treatment initiation and among 9-d CIDR-PG treated cows classified as estrous cycling.

The largest magnitude difference between treatments in serum estradiol concentrations at AI was observed among anestrous cows (6.7 ± 0.5 versus 5.3 ± 0.6 pg/ml). Due to the design of the 9-d CIDR-PG and similar long-term progestin-based protocols, anestrous cows responding to the presynchronization treatment are induced to initiate normal estrous cycles following progestin treatment. Effectively, therefore, AI of these cows takes place on the second postpartum estrus. Earlier induction of cyclicity among anestrous cows could potentially be advantageous, as greater pregnancy rates are obtained among cows that have expressed estrus one or more times prior to insemination than among cows inseminated at their first postpartum estrus (Thatcher and Wilcox, 1973; Macmillan and Clayton, 1980).

Consideration of variation within treatment groups may shed light on potential differences between treatments. By design, long-term protocols make use of

presynchronization programs to minimize variation in stage of the estrous cycle. In contrast, short-term protocols like the 7-d CO-Synch + CIDR tolerate significant variability among the synchronized group. Previous research suggests that approximately 65% cows have an ovulation in response to GnRH administration on Day 0 of a short-term estrus synchronization protocol when no presynchronization is used (Vasconcelos et al., 1999; Moreira et al., 2000; Geary et al., 2000). Since a proportion of cows ovulate and a proportion of cows do not, there are effectively two different subpopulations of cows with respect to stage of the subsequent follicular wave. If those cows are also late in the luteal phase and undergo luteolysis during the course of CIDR treatment, the follicle present at PG administration may also be sub-fertile due to development of a persistent follicle (Stock and Fortune, 1993). Additionally, among those cows that do successfully respond to GnRH administration on Day 0 of the protocol, a proportion have a CL and subsequently form an accessory corpus luteum after the GnRH-induced ovulation. The developing follicle therefore develops under the influence of somewhat higher circulating concentrations of progesterone, since progesterone is elevated among cows with two corpora lutea (Price and Webb, 1989; Rajamahendran et al., 1992; Fricke et al., 1993). Elevated circulating progesterone concentrations during follicular development affect ovulatory follicle size and may impair fertility due to the decreased steroidogenic capacity of the follicle and subsequent CL (Adams et al., 1992b; Carvalho et al., 2008; Dias et al., 2009; Mantovani et al., 2010; Sá Filho et al., 2010; Stevenson and Lamb, 2016). The extent to which these factors impact overall fertility in the context of a 7-d CO-Synch + CIDR protocol is an area of ongoing research. However, it is worth noting

that these considerations are avoided when a long-term progestin based protocol is used, simply because follicular synchrony is established earlier in the protocol through progestin-based presynchronization.

In summary, the 9-d CIDR-PG protocol and 7-d CO-Synch + CIDR protocol resulted in similarly high fertility when STAI was performed. However, cows treated with the 9-d CIDR-PG protocol exhibited a longer proestrus, more synchronous expression of estrus, smaller mean diameter of the ovulatory follicle at AI, and greater serum estradiol concentrations at the time of AI.

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Table 4.1. Cow age, days postpartum (DPP), and body condition score (BCS) by treatment and location.

Treatment ¹	N	Age	DPP	BCS	Estrous Cyclicity Proportion	%
7-d CO-Synch + CIDR	247	4.2 ± 0.2	89.5 ± 1.3	5.8 ± 0.1	103/140	74
Location 1	57	3.1 ± 0.2	84.3 ± 2.3	5.9 ± 0.1	39/57	68
Location 2	45	7.6 ± 0.4	72.7 ± 1.9	6.2 ± 0.1	29/45	64
Location 3	38	4.1 ± 0.4	86.5 ± 2.2	5.8 ± 0.1	35/38	92
Location 4	107	3.4 ± 0.3	101.8 ± 1.9	5.5 ± 0.1		
9-d CIDR-PG	233	4.2 ± 0.2	90.3 ± 1.3	5.8 ± 0.1	97/134	72
Location 1	55	3.0 ± 0.2	84.0 ± 2.3	6.0 ± 0.1	36/55	65
Location 2	43	7.5 ± 0.3	75.6 ± 1.7	6.2 ± 0.1	28/43	65
Location 3	36	4.6 ± 0.5	84.0 ± 2.5	5.8 ± 0.1	33/36	92
Location 4	99	3.3 ± 0.3	102.7 ± 2.0	5.4 ± 0.1		

¹See Figure 4.1 for treatment descriptions.

Table 4.2. Estrous response based on treatment and location.

Treatment ¹ Location	Estrous Response Prior to AI on Day 0 ²		Estrous Response During 24 h Delay ³		Total Estrous Response with STAI ⁴	
	Proportion	%	Proportion	%	Proportion	%
7-d CO-Synch + CIDR	143/247	58 ^a	67/247	27 ^x	210/247	85
Location 1	40/57	70	6/57	11	46/57	81
Location 2	23/45	51	16/45	36	39/45	87
Location 3	24/38	63	9/38	24	33/38	87
Location 4	56/107	52	36/107	34	92/107	86
9-d CIDR-PG	88/233	38 ^b	101/233	43 ^y	189/233	81
Location 1	25/55	45	21/55	38	46/55	84
Location 2	15/43	35	22/43	51	37/43	86
Location 3	4/36	11	26/36	72	30/36	83
Location 4	44/99	44	32/99	32	76/99	77

¹See Figure 4.1 for treatment descriptions.

²Estrous response prior to the recommended time point for FTAI for each protocol: 66 h after CIDR removal and PG administration for the 7-d CO-Synch + CIDR protocol and 72 h after PG administration for the 9-d CIDR-PG protocol.

³Estrous response occurring during the 24 h delay period for cows failing to express estrus by the standard FTAI time point: 66-90 h after CIDR removal and PG administration for the 7-d CO-Synch + CIDR protocol and 72-96 h after PG administration for the 9-d CIDR-PG protocol.

⁴Total cumulative estrous response occurring during STAI.

^{ab,xy}Percentages within column with different superscripts differ (P < 0.001).

Table 4.3. Pregnancy rates¹ to split-time artificial insemination (STAI) based on treatment² and estrous response³.

Treatment	Overall		Estrous by Initial AI Time		Estrous by Delayed AI Time		Non-Estrous	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%
7-d CO-Synch + CIDR	163/247	66	97/144	67 ^a	47/67	70 ^a	19/36	53 ^b
Location 1	40/57	70	26/41	63	6/6	100	8/10	80
Location 2	35/45	79	19/23	83	13/16	81	3/6	50
Location 3	24/38	63	17/24	71	5/9	56	2/5	40
Location 4	64/107	60	35/56	63	23/36	64	6/15	40
9-d CIDR-PG	163/233	70	66/88	75 ^a	75/101	74 ^a	22/44	50 ^b
Location 1	35/55	64	19/25	76	10/21	48	6/9	67
Location 2	35/43	81	11/15	73	20/22	91	4/6	67
Location 3	25/36	69	3/4	75	19/26	73	3/6	50
Location 4	68/99	69	33/44	75	26/32	81	9/23	39

¹Pregnancy rate to AI determined by transrectal ultrasonography 67 - 94 d after STAI.

²See Figure 4.1 for treatment descriptions.

³Estrous response as determined by activation of an estrus detection aid (Estrotest).

^{ab}Percentages with different superscripts differ (P < 0.001).

Table 4.4. Ovarian¹ follicle size, presence of CL, serum estradiol concentrations at PG administration based on treatment and pre-treatment estrous cyclicity status.

Treatment ²	LFD at PG administration	CL present at PG administration		Serum concentrations of estradiol-17 β at PG administration
	mm	Proportion	%	pg/ml
7-d CO-Synch + CIDR	12.6 $\pm$ 0.3	69/78	88	5.0 $\pm$ 0.2
Anestrous	13.1 $\pm$ 0.6	17/19	89	4.1 $\pm$ 0.4 ^x
Estrous cycling	12.4 $\pm$ 0.3	52/59	88	5.4 $\pm$ 0.3 ^y
9-d CIDR-PG	12.5 $\pm$ 0.3	69/76	91	4.8 $\pm$ 0.3
Anestrous	12.3 $\pm$ 0.6	18/19	95	3.9 $\pm$ 0.4 ^x
Estrous cycling	12.6 $\pm$ 0.3	51/57	89	5.2 $\pm$ 0.3 ^y

¹Transrectal ultrasonography was performed to measure ovarian follicle size and assess presence of corpora lutea. Follicles were measured, and measurements reported are the largest follicle diameter (LFD).

²See Table 4.1 for description of treatment protocols.

^{xy}Percentages with different superscripts differ (P = 0.02).

Table 4.5. Ovarian follicle size¹ and serum estradiol concentrations at AI based on treatment and pre-treatment estrous cyclicity status.

Treatment ²	LFD at AI	Serum concentrations of estradiol-17 β at AI
	mm	pg/ml
7-d CO-Synch + CIDR	14.6 $\pm$ 0.2 ^a	6.0 $\pm$ 0.3 ^c
Anestrous	14.5 $\pm$ 0.6 ^x	5.3 $\pm$ 0.6
Estrous cycling	14.6 $\pm$ 0.3 ^x	6.2 $\pm$ 0.3
9-d CIDR-PG	13.7 $\pm$ 0.2 ^b	6.9 $\pm$ 0.3 ^d
Anestrous	14.1 $\pm$ 0.5 ^{xy}	6.7 $\pm$ 0.5
Estrous cycling	13.6 $\pm$ 0.3 ^y	7.0 $\pm$ 0.4

¹Transrectal ultrasonography was performed to measure ovarian follicle size. Measurements reported are the largest follicle diameter (LFD).

²See Table 4.1 for description of treatment protocols.

^{ab}Percentages with different superscripts differ (P < 0.01).

^{xy}Percentages with different superscripts differ (P < 0.01).

^{cd}Percentages with different superscripts differ (P = 0.02).

Figure 4.1

Figure 4.1. Treatment schedule for the 7-d CO-Synch + CIDR and 9-d CIDR-PG protocols. Cows assigned to the 7-d CO-Synch + CIDR protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] and GnRH administration (100 µg im) on Day 18. On Day 25, CIDR inserts were removed, PG (25 mg im) was administered, and estrus detection aids (Estroject) were applied. On Day 28, 66 h after PG administration, cows with activated estrus detection aids received AI. On Day 29, 90 h after PG administration, all remaining cows received AI, and GnRH was administered to cows with non-activated estrus detection aids. Cows assigned to the 9-d CIDR-PG protocol received a CIDR insert and PG administration on Day 0. On Day 9, CIDR inserts were removed and PG was administered. On Day 25, PG was administered and estrus detection aids were applied. On Day 28, 72 h after PG administration, cows with activated estrus detection aids received AI. On Day 29, 96 h after PG administration, all remaining cows received AI, and GnRH was administered to cows with non-activated estrus detection aids.

Figure 4.2

Figure 4.2. Percentage of cows in 7-d CO-Synch + CIDR (gray bars) and 9-d CIDR-PG (black bars) treatments expressing estrus after PG administration on Day -3. No response indicates no expression of estrus prior to split-time AI. See Fig. 1 for description of treatment protocols.

CHAPTER 5

EVALUATION OF THE 14-D CIDR-PG AND 9-D CIDR-PG PROTOCOLS FOR SYNCHRONIZATION OF ESTRUS IN *BOS INDICUS*-INFLUENCED AND *BOS* *TAURUS* BEEF HEIFERS

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ABSTRACT

Two long-term, CIDR-based estrus synchronization protocols were evaluated among *Bos indicus*-influenced and *Bos taurus* beef heifers. Treatments were evaluated on the basis of estrous response and pregnancy rate resulting from fixed-time artificial insemination (FTAI), and these outcomes were analyzed retrospectively relative to reproductive tract score (RTS; Scale 1-5) at treatment initiation. Estrus was synchronized for 1139 heifers in three locations, and heifers were assigned to one of two treatments within each location based on RTS. Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert (1.38 g progesterone) on Day 0, CIDR removal on Day 14, administration of prostaglandin F_{2α} (PG; 25 mg im) on Day 30, and administration of gonadotropin-releasing hormone (GnRH; 100 µg im) concurrent with FTAI on Day 33, 66 h after PG. Heifers assigned to the 9-d CIDR-PG protocol received administration of PG concurrent with CIDR insertion on Day 5, administration of PG concurrent with CIDR removal on Day 14, administration of PG on Day 30, and administration of GnRH concurrent with FTAI on Day 33, 66 h after PG. Estrus detection aids were applied at CIDR removal on Day 14 and at PG on Day 30 to

evaluate estrous response rate. Mean RTS differed ($P < 0.0001$) based on biological type due to higher rates of estrous cyclicity (RTS 4 and 5) among *Bos taurus* heifers (72%; 416/574) than among *Bos indicus*-influenced heifers (27%; 150/565). The proportion of heifers expressing estrus following CIDR removal was greater ($P = 0.01$) among heifers assigned to the 14-d CIDR-PG treatment (88%; 492/559) compared to the 9-d CIDR-PG treatment (83%; 480/580). Estrous response following CIDR removal was also higher ($P < 0.0001$) among *Bos taurus* (95%; 547/574) compared to *Bos indicus*-influenced (75%; 425/565) heifers. Rate of estrous response prior to FTAI did not differ significantly based on treatment but was higher ($P < 0.0001$) among *Bos taurus* heifers (60%; 344/574) than among *Bos indicus*-influenced heifers (45%; 253/565). However, the effect of biological type on estrous response was not significant when RTS was included in the model, as RTS significantly ($P < 0.0001$) affected the rate of estrous response both at CIDR removal and prior to FTAI. Across treatments and biological types, heifers that expressed estrus prior to AI achieved higher ($P < 0.0001$) AI pregnancy rates than heifers failing to express estrus. Pregnancy rates to FTAI did not differ significantly based on treatment in either biological type. Higher rates of estrous cyclicity among *Bos taurus* heifers resulted in higher FTAI pregnancy rates among *Bos taurus* (51%; 290/574) compared to *Bos indicus*-influenced heifers (39%; 218/565). However, pregnancy rates of respective RTS did not differ based on biological type. In summary, long-term CIDR-based protocols provide a simple, effective method of estrus synchronization in *Bos indicus*-influenced and *Bos taurus* beef heifers. Moreover, these results highlight the importance of management practices that result in high rates of estrous cyclicity prior to protocol

initiation, particularly among later maturing breeds and biological types.

INTRODUCTION

Development of protocols to synchronize estrus and ovulation prior to fixed-time artificial insemination in crossbred beef heifers of *Bos indicus* influence has been limited in comparison to *Bos taurus* beef heifers. *Bos indicus*-influenced cattle are generally regarded to exhibit increased susceptibility to stress associated events and are unique from *Bos taurus* females based on their distinct reproductive physiology (Saldarriaga et al., 2007; Sartori et al., 2010). *Bos indicus*-influenced heifers generally reach puberty at later ages than *Bos taurus* females and differ from *Bos taurus* females in preovulatory follicular diameter, timing of follicular wave emergence, and sensitivity to progesterone effects on LH pulsatility (Chenoweth, 1994; Bó et al., 2003; Carvalho et al., 2008). These differences between biological types complicate the development of systems to effectively control the estrous cycles of *Bos indicus*-influenced cattle and have therefore limited the expanded use of AI in regions of the United States where these cattle are better adapted to tropical and subtropical environmental conditions (Saldarriaga et al., 2007; Sartori et al., 2010). Although several protocols have been developed in South America for synchronization of estrus in *Bos indicus* cattle (reviewed by Baruselli et al., 2012), these protocols generally use estradiol products not currently approved by the United States Food and Drug Administration for use in estrus synchronization.

The 14-d CIDR-PG protocol is a long-term CIDR-based estrus synchronization protocol that has been widely adopted for use among *Bos taurus* beef heifers in many

parts of the United States. Estrous response rate following this protocol has been well characterized among *Bos taurus* beef heifers (Leitman et al., 2009a,b; Mallory et al., 2010), and the synchrony and rate of estrous response provides a platform for effective use of fixed-time artificial insemination (FTAI). However, despite extensive use of the 14-d CIDR-PG protocol among *Bos taurus* replacement beef heifers, there are no published studies to date involving use of the protocol in *Bos indicus*-influenced heifers.

The 9-d CIDR-PG protocol is a similar long-term CIDR-based estrus synchronization protocol that was recently developed for use in mature beef cows. Preliminary evaluation suggested that the incorporation of prostaglandin $F_{2\alpha}$ (PG) treatment at CIDR insertion and removal facilitated a decreased length of progestin treatment and enhanced estrous response (Thomas et al., 2016), and subsequent work found that pregnancy rate to FTAI was increased among 9-d CIDR-PG treated compared to 14-d CIDR-PG treated cows (Thomas et al., 2017b). However, the 9-d CIDR-PG protocol has not yet been evaluated among either *Bos indicus*-influenced or *Bos taurus* beef heifers.

Treatment of prepubertal heifers with exogenous progesterone hastens attainment of puberty and enhances conception rates among both *Bos taurus* (Hall et al., 1997; Grings et al., 1998; Imwalle et al., 1998; Rasby et al., 1998) and *Bos indicus* (Claro et al., 2010) heifers. However, high levels of circulating progesterone decrease LH pulsatility and therefore have a suppressive effect relative to ovarian follicular development (Ireland and Roche, 1982; Roberson et al., 1989; Sirois and Fortune, 1990; Savio et al., 1993). With the hypothesis that this effect is particularly consequential among *Bos indicus*-

influenced females, previous studies evaluated strategies to reduce endogenous progesterone production or limit exogenous progesterone administration during treatment with a short-term progestin-based estrus synchronization protocol as a means of improving success among *Bos indicus*-influenced females (Carvalho et al., 2008; Peres et al., 2009; Mantovani et al., 2010; Cipriano et al., 2011; Williams et al., 2012).

This experiment was designed to characterize the relative effectiveness of long-term CIDR-based protocols among *Bos indicus*-influenced and *Bos taurus* heifers in their respective production systems in the midwestern and southeastern United States. We hypothesized that the 9-d CIDR-PG protocol would increase estrous response and FTAI pregnancy rates among *Bos taurus* and/or *Bos indicus*-influenced heifers in comparison to the 14-d CIDR-PG protocol.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Animals. On Day 0 prior to protocol initiation, individual animal weights and reproductive tract scores (RTS; Scale 1-5) (Anderson et al., 1991; Holm et al., 2009; Rosenkrans and Hardin, 2003) were obtained for beef replacement heifers (14 to 15 months of age) at three locations. In each location, heifers identified as having an abnormal or infantile (RTS 1) reproductive tract were culled and not exposed for estrus synchronization and AI (Location 1: n = 18; Location 2: n = 3; Location 3: n = 104). The remaining 1,139 heifers were assigned within location to treatments (Figure 5.1) based on

source and RTS. Location 1 (n = 533) and Location 2 (n = 41) consisted of exclusively *Bos taurus* commercial Angus heifers, and Location 3 (n = 565) consisted of exclusively crossbred heifers of *Bos indicus* influence, ranging from 1/8th to 3/8th Brahman. Heifers assigned to the 14-d CIDR-PG protocol received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol were administered prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) concurrent with CIDR insertion on Day 5 and concurrent with CIDR removal on Day 14. Heifers in both treatments were administered PG on Day 30, and fixed-time artificial insemination (FTAI) was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) was administered to heifers in both treatments concurrent with FTAI. Time of PG administration on Day 30 and time of FTAI on Day 33 was recorded for each heifer. Within each location, insemination was performed using conventional frozen-thawed semen sourced from a single AI sire. In Location 1 and Location 3, two technicians were pre-assigned and balanced across treatments based on heifer source, RTS, and weight. In Location 2, a single technician performed all inseminations. Fourteen d after AI, heifers were exposed to fertile bulls for the remainder of the breeding season.

Estrus detection. Estrus detection aids (Estroject, Rockway Inc, Spring Valley, WI) were applied at CIDR removal on Day 14 and evaluated at PG administration on Day 30 to determine rate of estrous response following CIDR removal. A new estrus detection aid was applied at PG administration on Day 30 and evaluated at FTAI on Day

33 to determine rate of estrous response following PG administration. Estrus was defined as removal of >50% of the scratch-off coating on the Estroject.

Pregnancy diagnosis. Pregnancy rate to FTAI was determined by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) 62 d to 90 d after FTAI. Pregnancies resulting from AI were distinguished from those resulting from natural service based on fetal size (Curran et al., 1986), as heifers were not exposed to natural service bulls until 14 d after AI.

Statistical analysis. Treatment differences for RTS, weight, and timing of insemination were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to initially assess potential differences in Locations 1 and 2 relative to estrous response and AI pregnancy rate. Locations 1 and 2, each containing *Bos taurus* heifers under similar management conditions, did not differ significantly; therefore, treatments were compared relative to biological type rather than location. Estrous response rate after CIDR removal, estrous response rate after PG administration, and pregnancy rate to FTAI were analyzed using a generalized linear model with the GENMOD procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. Each model included treatment, biological type, and RTS in the class statement, and the model included RTS, weight, treatment, biological type, and the following interactions: treatment x biological type, treatment x RTS, and treatment x RTS x biological type.

RESULTS

Heifer weight, mean RTS, timing of insemination, and estrous response rates are shown for each treatment and within each biological type in Table 5.1. Heifers did not differ based on treatment for RTS or weight. However, mean weight differed based ($P < 0.0001$) based on biological type, with higher weights observed at protocol initiation among *Bos taurus* heifers. Likewise, mean RTS differed ($P < 0.0001$) based on biological type, with higher rates of estrous cyclicity (RTS 4 and 5) observed at protocol initiation among *Bos taurus* heifers (72%; 416/574) than among *Bos indicus*-influenced heifers (25%; 141/565). The interval from PG to FTAI did not differ based on treatment or biological type. Within locations with two technicians, timed AI pregnancy rates did not differ based on technician.

Estrous response rates following CIDR removal based on treatment, biological type, and RTS are presented in Table 5.2. Using a generalized linear model, estrous response rate following CIDR removal was significantly influenced by weight ($P < .001$), RTS ($P < 0.0001$), biological type ($P < 0.0001$), and the interaction of treatment x RTS x biological type ($P = 0.02$). In this analysis, estrous response rate following CIDR removal did not differ significantly based on treatment for *Bos taurus* heifers ($P = 0.38$). However, among *Bos indicus*-influenced heifers, estrous response rate following CIDR removal tended ($P = 0.08$) to be higher among heifers assigned to the 14-d CIDR-PG treatment. For each treatment, estrous response rate after CIDR removal was higher ($P < 0.0001$) among *Bos taurus* compared to *Bos indicus*-influenced heifers.

While numerically higher rates of estrous response prior to FTAI were observed

among *Bos taurus* (60%; 344/574) compared to *Bos indicus*-influenced heifers (45%; 253/565), rate of estrous response prior to FTAI was not affected by biological type, treatment, or any interaction in the generalized linear model. Only RTS significantly ($P < 0.0001$) affected rate of estrous response prior to FTAI, and the effect of weight was not significant when RTS was included in the model.

Pregnancy rates to FTAI based on treatment, biological type, and estrous response are shown in Table 5.3. In each treatment, heifers that expressed estrus prior to AI achieved higher ($P < 0.0001$) pregnancy rates than heifers failing to express estrus. While numerically higher pregnancy rates to FTAI were observed among *Bos taurus* (51%; 290/574) compared to *Bos indicus*-influenced heifers (39%; 218/565), the effect of biological type was not significant ($P = 0.79$) when the effect of RTS ($P < 0.001$) was included in a generalized linear model. Similarly, pregnancy rate to FTAI was not significantly affected by weight, treatment, or any interaction when RTS was included in the model. Pregnancy rates to FTAI based on RTS, treatment, and biological type are presented in Table 5.4. Although lower rates of estrous cyclicity among *Bos indicus*-influenced heifers resulted in lower overall pregnancy rates to FTAI in comparison to *Bos taurus* females, FTAI pregnancy rates did not differ within respective RTS based on treatment or biological type.

DISCUSSION

When comparing the 14-d CIDR-PG and 9-d CIDR-PG treatments in heifers of either biological type, no improvement in FTAI pregnancy rate was noted using the 9-d

CIDR-PG protocol. It is the opinion of the authors that use of the 9-d CIDR-PG protocol in heifers should be discouraged in favor of the 14-d CIDR-PG protocol, considering that the 14-d CIDR-PG treatment schedule requires two fewer doses of PG per animal. Moreover, protocol compliance problems may be less likely with the simpler 14-d CIDR-PG protocol, in that CIDRs are removed on the same day of the week two weeks after the day of insertion. Nevertheless, the shorter 9-d CIDR-PG protocol may have some limited applications in heifers, such as in situations when a producer has missed the necessary date of protocol initiation for the 14-d CIDR-PG protocol but still intends to keep the target breeding date.

The similar pregnancy outcomes obtained among heifers across treatments are in contrast to those obtained when comparing the 14-d CIDR-PG and 9-d CIDR-PG protocols among mature *Bos taurus* cows. Previous work from our lab found that pregnancy rate to FTAI tended to be improved among *Bos taurus* cows treated with the 9-d CIDR-PG protocol compared to the 14-d CIDR-PG protocol (Thomas et al., 2017b). Improvements in FTAI pregnancy rate were attributed to previously reported (Thomas et al., 2016b) indications of improved synchronization success among mature cows using the 9-d CIDR-PG protocol, namely an improvement in estrous response after CIDR removal and after PG. Although timing of estrus expression was not characterized in the present study, estrous response rates were not improved among 9-d CIDR-PG-treated heifers at either time point.

We hypothesize that differences in the relative effectiveness of the 9-d CIDR-PG and 14-d CIDR-PG protocols can be explained largely by general differences between

mature cows and heifers in length of ovarian follicular waves, overall length of the estrous cycle, and rate of estrous cyclicity at protocol initiation. Cyclic cows in the early luteal phase likely benefit from induced luteolysis in the 9-d CIDR-PG presynchronization schedule, therefore exhibiting estrus earlier following CIDR removal. We speculate that, in contrast to mature cows, spontaneous luteolysis occurs sufficiently early during the 14-d CIDR-PG treatment among even cyclic heifers in the early luteal phase. Consequently, few cyclic, early luteal phase heifers may benefit from the induced luteolysis in the 9-d CIDR-PG treatment.

While biological type is inherently confounded by location in the design of this trial, this dataset provides an opportunity to highlight similarities in the effectiveness of the 14-d CIDR-PG protocol across both biological types in their respective production systems. Differences in geographical area, climate, nutritional development practices, and heifer phenotype should be recognized as sources of variance given the different environments. However, given the climate tolerance advantages of *Bos indicus* genetics in subtropical environments and the performance advantages of *Bos taurus* genetics in temperate environments, industry use of these biological types is likewise practically confounded by geographical location and associated nutritional development practices based on regional feed availability. Data relative to biological type are therefore presented relative to cyclicity status, so as to compare protocol effectiveness across biological types while accounting for management differences that influence cyclicity status of heifers. Overall pregnancy rates to FTAI following the 14-d CIDR-PG protocol were lower among *Bos indicus*-influenced compared to *Bos taurus* heifers. However,

differences in FTAI pregnancy rate appear to be explained by differences in pubertal status at protocol initiation, as evidenced by the lack of significant effect due to biological type when RTS was included in the model for FTAI pregnancy rate. When comparing results within RTS (Table 5.4), FTAI pregnancy rates were similar across biological types. Moreover, these results obtained using the 14-d CIDR-PG protocol among *Bos indicus*-influenced heifers are similar to those in a very large field dataset (Locke et al., 2016) of *Bos taurus* heifers in the Missouri Show-Me-Select[®] Replacement Heifer Program (Table 5.5). Note that higher rates of estrous cyclicity (RTS = 4 or 5) at the start of estrus synchronization are observed among the *Bos taurus* heifers in the Show-Me-Select[®] Program (69%; 11,870/17,312) than were observed among the *Bos indicus*-influenced heifers assigned to the 14-d CIDR-PG protocol in this study (26%; 71/277). However, the pregnancy rates for heifers within the respective RTS categories are quite similar, suggesting similar effectiveness of the 14-d CIDR-PG protocol in this biological type when accounting for estrous cyclicity at protocol initiation.

While progestin treatment can effectively induce puberty among a large proportion of peripubertal *Bos indicus*-influenced and *Bos taurus* heifers (Patterson et al., 1990), a common recommendation for *Bos taurus* heifers is that $\geq 50\%$ of heifers have a RTS of 4 or 5 at the time estrus synchronization is initiated (Smith et al., 2011). Management strategies and selection practices that increase the proportion of *Bos indicus*-influenced heifers cycling (RTS = 4 or 5) prior to the start of the breeding season would likely result in AI pregnancy rates more comparable to those typically obtained among *Bos taurus* heifers. The low percentage of *Bos indicus*-influenced heifers that

were pubertal at the time treatments were imposed in this study is in all likelihood related to the postweaning to prebreeding nutritional status of these heifers, as reflected in lower body weights compared to the *Bos taurus* heifers (Table 5.1). Postweaning nutritional treatments were reported (Patterson et al., 1991) to have more immediate effects among *Bos indicus*-influenced heifers than *Bos taurus* heifers, best illustrated by differences in age at puberty onset and pregnancy rates at the end of a 45 day breeding period. In this study, heifers with infantile reproductive tracts (RTS 1) were culled and not exposed for estrus synchronization and AI. Note also that a very sizeable proportion of *Bos indicus*-influenced heifers (Location 3) were culled due to low RTS, as heifers with infantile reproductive tracts are poor candidates for estrus synchronization and AI (Table 5.5). Use of reproductive tract scoring to identify these late-maturing animals provides an opportunity to improve reproductive performance of replacement heifers and to place some level of genetic selection on earlier attainment of puberty.

For both biological types, the relationship between estrous status at FTAI and FTAI pregnancy rate (Table 5.3) mirror results in the literature for *Bos taurus* females, as expression of estrus prior to FTAI is associated with higher pregnancy rates (Richardson et al., 2016). Breeding based upon detected estrus rather than using FTAI would theoretically allow maximum expression of estrus among the synchronized herd. However, breeding programs based on detected estrus generally require a significant labor and time commitment relative to cattle handling and observation, and there may be potential for decreased pregnancy rates among some *Bos indicus*-influenced heifers due to stress induced by frequent animal sorting (Galina et al., 1996; Dobson and Smith,

2000). One alternative may be to use split-time AI (Thomas et al., 2014a,b; Bishop et al., 2016, 2017a,b) rather than fixed-time AI. This approach maximizes the proportion of heifers allowed to express estrus prior to insemination while only requiring a single sort based on estrous status at 66 h after PG. Future work should evaluate the effectiveness and feasibility of using split-time AI following the 14-d CIDR-PG protocol in *Bos indicus*-influenced heifers.

In summary, long-term, CIDR-based protocols are an effective form of estrus synchronization among both *Bos indicus*-influenced and *Bos taurus* beef heifers. In contrast to results previously obtained among mature *Bos taurus* beef cows, pregnancy rate to FTAI was not improved among beef replacement heifers following the 9-d CIDR-PG protocol compared to the 14-d CIDR-PG protocol. However, these results highlight similar pregnancy rates to FTAI following the 14-d CIDR-PG protocol when accounting for differences in estrus cyclicity among *Bos indicus*-influenced and *Bos taurus* beef heifers. These similarities are noteworthy and suggest an opportunity for expanded use of the 14-d CIDR-PG protocol in conjunction with management and selection for decreased age at puberty among *Bos indicus*-influenced beef heifers.

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EstroTECT Inc. (Spring Valley, WI) for providing estrus detection aids; and the University of Missouri South Farm Beef Research and Teaching Center, John Barnhart Cattle Company, and Deseret Ranches of Florida for their support of this project.

Table 5.1. Heifer weight (Wt), reproductive tract score (RTS), and timing of insemination (PG - AI interval), and estrous response based on treatment for *Bos indicus*-influenced and *Bos taurus* heifers.

Treatment ¹	N	Wt, kg	RTS ²	PG - AI, h	Estrous After CIDR Removal		Estrous Prior to FTAI	
					Proportion	%	Proportion	%
14-d CIDR-PG	559	308 ± 2	3.6 ± 0.1	67.5 ± 0.1	492/559	88	301/559	54
<i>Bos indicus</i> -influenced	277	292 ± 2	3.0 ± 0.1	67.5 ± 0.1	220/277	79	127/277	46
<i>Bos taurus</i>	282	323 ± 2	4.1 ± 0.1	67.4 ± 0.1	272/282	96	174/282	62
9-d CIDR-PG	580	306 ± 2	3.6 ± 0.1	67.6 ± 0.1	480/580	83	296/580	51
<i>Bos indicus</i> -influenced	288	289 ± 2	3.0 ± 0.1	67.7 ± 0.1	205/288	71	126/288	44
<i>Bos taurus</i>	292	322 ± 2	4.1 ± 0.1	67.5 ± 0.1	275/292	94	170/292	58

¹Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all heifers concurrent with FTAI.

²RTS (1 to 5 scale, where 1 = immature and 5 = luteal phase) of the heifers, evaluated at CIDR insertion on Day 0.

³Estrous response after CIDR removal, as determined by activation of an estrus detection aid (Estrotest).

⁴Estrous response by 66 h after PG administration, as determined by activation of an estrus detection aid (Estrotest).

Table 5.2. Estrous response¹ rates after CIDR removal based on treatment², biological type, and reproductive tract score³ (RTS) prior to treatment initiation.

Treatment ²	Biological Type	RTS 2		RTS 3		RTS 4		RTS 5	
		Proportion	%	Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	<i>Bos indicus</i> -influenced	84/111	76 ^{ab}	73/95	77 ^b	29/33	88 ^b	34/38	89 ^b
	<i>Bos taurus</i>	19/21	90 ^b	50/57	88 ^b	70/70	100 ^c	133/134	99 ^c
9-d CIDR-PG	<i>Bos indicus</i> -influenced	76/117	65 ^a	72/101	71 ^{ab}	25/33	76 ^{ab}	32/37	87 ^b
	<i>Bos taurus</i>	19/22	86 ^{ab}	50/58	86 ^{ab}	70/70	100 ^c	136/142	96 ^{bc}

¹Estrous response after CIDR removal, as determined by activation of an estrus detection aid (Estrotect) on Day 30.

²Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all heifers concurrent with FTAI.

³RTS (1 to 5 scale, where 1 = immature and 5 = luteal phase) of the heifers, evaluated at CIDR insertion on Day 0.

^{ab,c}Percentages within column with different superscripts differ (P < 0.05).

Table 5.3. Pregnancy rates¹ to fixed-time artificial insemination (FTAI) based on treatment², biological type, and estrous response³ prior to FTAI.

Treatment ²	Overall		Estrous		Non-Estrous	
	Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	262/559	47	181/301	60	81/258	31
<i>Bos indicus</i> -influenced	114/277	41	76/127	60	38/150	25
<i>Bos taurus</i>	148/282	51	105/174	60	43/108	40
9-d CIDR-PG	246/580	42	177/296	60	69/284	24
<i>Bos indicus</i> -influenced	104/288	36	69/126	55	35/162	22
<i>Bos taurus</i>	142/292	49	108/170	64	34/122	28

¹Pregnancy rate to AI determined by transrectal ultrasonography 62 d to 90 d after FTAI.

² Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all heifers concurrent with FTAI.

³Estrous response by 66 h after PG administration, as determined by activation of an estrus detection aid (Estrotect).

Table 5.4. Pregnancy rates¹ to fixed-time artificial insemination (FTAI) based on treatment², biological type, and reproductive tract score³ (RTS) prior to treatment initiation.

Treatment ²	RTS 2		RTS 3		RTS 4		RTS 5		Overall	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%	Proportion	%
14-d CIDR-PG	44/132	33	65/152	43	54/103	52	99/172	58	262/559	47
<i>Bos indicus</i> -influenced	39/111	35	37/95	39	17/33	52	21/38	55	114/277	41
<i>Bos taurus</i>	5/21	24	28/57	49	37/70	53	78/134	58	148/282	51
9-d CIDR-PG	46/139	33	57/159	36	49/103	48	94/179	53	246/580	42
<i>Bos indicus</i> -influenced	40/117	34	32/101	32	15/33	45	17/37	46	104/288	36
<i>Bos taurus</i>	6/22	27	25/58	43	34/70	49	77/142	54	142/292	49

¹Pregnancy rate to AI determined by transrectal ultrasonography 62 d to 90 d after FTAI.

²Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol received prostaglandin F_{2α} (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) was administered to all heifers concurrent with FTAI.

³RTS (1 to 5 scale, where 1 = immature and 5 = luteal phase) of the heifers, evaluated at CIDR insertion on Day 0.

Table 5.5. Pregnancy rates¹ to fixed-time artificial insemination (FTAI) following the 14-d CIDR-PG protocol²: Data obtained among *Bos indicus*-influenced heifers compared to data from *Bos taurus* heifers in the Show-Me-Select Replacement Heifer Program⁴

Treatment	RTS 1		RTS 2		RTS 3		RTS 4		RTS 5	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%	Proportion	%
<i>Bos indicus</i> -influenced	NA		39/111	35	37/95	39	17/33	52	21/38	55
<i>Bos taurus</i>										
Show-Me-Select	8/97	8	182/572	32	2219/4773	46	2778/5604	50	3249/6266	52

¹Pregnancy rate to AI determined by transrectal ultrasonography 62 d to 90 d after FTAI.

²14-d CIDR-PG protocol consists of a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. PG is administered 16 d after CIDR removal on Day 30, and FTAI is performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100 µg im) is administered to all heifers concurrent with FTAI.

³RTS (1 to 5 scale, where 1 = immature and 5 = luteal phase) of the heifers.

⁴Adapted from Locke et al., 2016.

Figure 5.1

14-d CIDR-PG

9-d CIDR-PG

Figure 5.1. Treatment schedule for the 14-d CIDR-PG and 9-d CIDR-PG protocols. Heifers assigned to the 14-d CIDR-PG protocol received a controlled internal drug release (CIDR) insert [1.38 g progesterone] on Day 0 and CIDR removal on Day 14. Heifers assigned to the 9-d CIDR-PG protocol received prostaglandin $F_{2\alpha}$ (PG) concurrent with CIDR insertion on Day 5 and PG concurrent with CIDR removal on Day 14. In both treatments, PG was administered 16 d after CIDR removal on Day 30, and FTAI was performed on Day 33, 66 h after PG administration. Gonadotropin-releasing hormone (GnRH; 100

CHAPTER 6
EFFECTIVE USE OF SEXEDULTRA™ SEX-SORTED SEMEN FOR TIMED
ARTIFICIAL INSEMINATION OF BEEF HEIFERS

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ABSTRACT

An experiment was designed to evaluate the relative fertility of SexedULTRA™ sex-sorted semen compared to conventional, non-sex-sorted semen when used among beef heifers in conjunction with split-time AI following the 14-d CIDR-PG protocol. Units of conventional semen were generated with 25.0×10^6 live cells per 0.5 ml straw prior to freezing, and units of sex-sorted semen were generated using the SexedULTRA™ Genesis III sorting technology with 4.0×10^6 live cells per 0.25 ml straw prior to freezing. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%. Estrus was synchronized in 851 heifers at four locations using the 14-d CIDR-PG protocol: controlled internal drug release (CIDR) insert (1.38 g progesterone) on Day 0, CIDR removal on Day 14, and administration of prostaglandin $F_{2\alpha}$ (PG; 25 mg im) on Day 30. Estrus detection aids were applied at PG on Day 30 to evaluate estrous response rate, and split-time AI was performed based on estrous response. At 66 h after PG (Day 33), heifers having expressed estrus received timed AI. Heifers failing to express estrus by 66 h received timed AI 24 h later (90 h after PG on Day 34). Heifers failing to express estrus by 90 h were administered gonadotropin-releasing hormone (GnRH; 100 µg im) concurrent with AI. Heifers were preassigned to

treatment (insemination with either conventional or SexedULTRA™ sex-sorted semen), and treatments were balanced within each location based on source, reproductive tract score, and weight. Heifers were exposed for natural service beginning 14 d after AI for the remainder of a 60 d breeding season. Pregnancy rates to AI across locations tended to be higher ($P = 0.09$) for heifers inseminated with conventional semen (60%; 257/429) compared to sex-sorted semen (52%; 218/422). Higher pregnancy rates to AI ($P < 0.0001$) were obtained among heifers that expressed estrus prior to AI than among heifers that failed to express estrus prior to AI at 90 h. Total pregnancy rates at the end of the 60 d breeding season did not differ between heifers that received sex-sorted semen at AI (89%; 376/422) and heifers that received conventional semen at AI (89%; 382/429). In summary, the pregnancy rates observed suggest that SexedULTRA™ sex-sorted semen can be used effectively for timed AI of beef heifers when split-time AI is performed following the 14-d CIDR-PG protocol.

INTRODUCTION

Using a flow cytometer, sperm cells can be effectively sorted based on differences in DNA content between X- and Y- chromosome-bearing sperm cells (Garner et al., 1983; Johnson et al., 1989). This technology has been used successfully to produce sex-sorted units of bovine sperm cells for commercial use (Seidel et al., 1999; Schenk et al., 1999). However, sex-sorted sperm produced using this method have reduced viability and overall quality after cryopreservation and thawing (Seidel and Garner, 2002). Recent research efforts have resulted in the development of optimized equipment and procedures

that decrease the degree of cellular damage occurring to sperm cells during the sex-sorting process (Sharpe and Evans, 2009; Evans, 2010; DeGraaf et al., 2014). Sex-sorted semen produced using this next generation technology is presently available and marketed commercially for both dairy and beef breeds under the trade name SexedULTRATM (Sexing Technologies, Navasota, TX).

In a large trial using semen from eight Holstein bulls for insemination of over 6,000 heifers in 41 commercial locations, pregnancy rates to AI following detected estrus were improved by 4.5 percentage points among dairy heifers inseminated with SexedULTRATM sex-sorted semen (46.1%) compared to sex-sorted semen produced using the previous generation XY technology (41.6%) (Vishwanath, 2015). Furthermore, the improved speed and reduced sperm cell loss associated with the new technology facilitates use of higher sperm cell numbers per unit (Vishwanath, 2015), above the typical 2.0×10^6 concentration that became standard for units of sex-sorted semen produced using the XY method (Seidel et al., 1999; Amann, 1999; DeJarnette et al., 2008). A separate trial using semen from five bulls for insemination of over 6,000 dairy animals based on detected estrus indicated no difference between pregnancy rates to SexedULTRATM semen at a concentration of 4.0×10^6 cells per unit and pregnancy rates to conventional semen at a concentration of 15×10^6 cells per unit (Vishwanath, 2015). However, SexedULTRATM semen has not been evaluated among beef animals or, more critically, in the context of timed AI.

When using protocols designed to facilitate fixed-time artificial insemination (FTAI), insemination is performed for all females at a predetermined time rather than

based on detected estrus. Since FTAI does not require estrus detection, protocols that facilitate FTAI significantly reduce the labor and time commitment associated with carrying out an AI program. This likely enhances adoption of AI in the industry, as time and labor considerations are a barrier to adoption of AI (NAHMS, 2008). However, there is potential for reduced pregnancy rates when using FTAI compared to AI based on detected estrus, as insemination occurs at a less-than-optimal time point for some females. Pregnancy rate to AI is significantly affected by timing of insemination relative to estrus onset and ovulation (Trimberger, 1948; Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001). Females failing to express estrus prior to FTAI achieve much lower pregnancy rates than those having expressed estrus, with a recent meta-analysis (Richardson et al., 2016) finding pregnancy rates were 27% lower for non-estrous beef cows and heifers inseminated with conventional, non-sex-sorted semen.

When using sex-sorted semen produced using the XY technology, pregnancy rates to FTAI were particularly low among beef cows and heifers that had not expressed estrus prior to FTAI (Hall et al., 2010; Meyer et al., 2012; Thomas et al., 2014a). Overall, lower pregnancy rates were obtained when sex-sorted semen was used in FTAI (Hall et al., 2010; Thomas et al., 2014a; Rhinehart et al., 2011; Sá Filho et al., 2012). Therefore, it had generally been recommended that sex-sorted semen not be used in conjunction with FTAI or that use of sex-sorted semen should be restricted to only females expressing estrus prior to FTAI (Seidel, 2003b).

In an effort to improve pregnancy rates to timed AI, Thomas et al. (2014a) developed a modified timed AI approach in which timing of insemination is delayed for

non-estrous females until 20-24 h after fixed-time AI of cows in estrus. This approach, termed "split-time AI," was found to improve pregnancy rates when using sex-sorted semen among mature beef cows following the 7-d CO-Synch + CIDR protocol. When evaluated using conventional semen, split-time AI was found to improve pregnancy rates among beef heifers following the 14-d CIDR-PG protocol (Thomas et al., 2014b) as well as among mature cows following the 7-d CO-Synch + CIDR protocol (Bishop et al., 2017b), provided that GnRH was administered to non-estrous cows concurrent with the delayed insemination (Bishop et al., 2016). To date, however, split-time AI has not been evaluated using sex-sorted semen among beef heifers following the 14-d CIDR-PG protocol. Moreover, the original split-time AI work with sex-sorted semen (Thomas et al., 2014a) predated the development of the SexedULTRATM sorting process. This experiment was designed to evaluate the relative fertility of SexedULTRATM sex-sorted semen compared to conventional, non-sex-sorted semen when used among beef heifers in conjunction with split-time AI following the 14-d CIDR-PG protocol.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Animals. Individual animal weights and reproductive tract scores (RTS) (Anderson et al., 1991; Holm et al., 2009; Rosenkrans et al., 2003) were obtained for beef replacement heifers (14 to 15 months of age) at four locations. In each location, heifers with abnormal or infantile (RTS 1) reproductive tracts were culled and not exposed for

estrus synchronization and AI (Location 1: n = 3; Location 2: n = 5; Location 3: n = 0; Location 4: n = 2).

Estrus synchronization. Estrus was synchronized using the 14-d CIDR-PG protocol (Figure 6.1). Heifers received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, and CIDRs were removed on Day 14. Prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) was administered on Day 30, and split-time artificial insemination (STAI) was performed at 66 h (Day 33) or 90 h (Day 34) after PG administration based on estrous response. Heifers failing to express estrus by 90 h after PG were administered gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) concurrent with AI. Time of PG administration and time of AI were recorded for each heifer.

Estrus detection. Estrus detection aids (Estroject, Rockway Inc, Spring Valley, WI) were applied at PG administration on Day 30 and evaluated at 66 h after PG on Day 33 and at 90 h after AI on Day 34 to determine rate of estrous response. Estrus was defined as removal of >50% of the scratch-off coating on the Estroject.

Semen collection. Semen was collected from two commercially available two-year-old AI bulls, and units of sex-sorted and conventional semen were produced from contemporaneous ejaculates. Semen was collected over an eight day period (March 18th through March 25th, 2016) for Bull A and over a 22 day period (February 15th through March 7th, 2016) for Bull B. Semen from three separate ejaculates per bull were used, with two ejaculates used for production of sex-sorted semen and one for conventional. All the semen passed the standard quality control criteria use for sex sorted and conventional

semen respectively. Sex sorted semen and conventional semen from all ejaculates within bull were used in equal proportions across locations. Units of conventional semen were generated with 25.0×10^6 live cells per 0.5 ml straw prior to freezing, based on sperm cell concentrations commonly used in industry. Units of sex-sorted semen were generated 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, based on sperm cell concentrations typically used in SexedULTRA™ 4M sex-sorted semen. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%, the typical accuracy level at which SexedULTRA™ sex-sorted semen is produced and marketed.

Artificial insemination. Within each location, heifers were preassigned to balanced treatments (conventional versus sex-sorted semen) based on source, RTS, and weight. Location 1 used Bull A exclusively. Locations 2-4 used Bulls A and B equally, with heifers preassigned to bull within treatment based on source, RTS, and weight. In Locations 1 and 4, AI was performed by a single technician. Three technicians performed AI in Locations 2, and two technicians performed AI in Locations 4. To minimize potential for confounding effects of technician in locations with more than one technician, technicians were preassigned and balanced within treatment and bull on the basis of heifer source, RTS, and weight. Fourteen d after AI, heifers were exposed to fertile bulls for the remainder of the breeding season.

Pregnancy diagnosis. Pregnancy rate to AI was determined by transrectal ultrasonography (Aloka 500V equipped with a 5.0-MHz linear-array transducer; Aloka,

Wallingford, CT) 62 d and 120 d after AI in Location 1, 95 d after AI in Location 2, 89 d after AI in Location 3, and 88 d after AI in Location 4. Pregnancies resulting from AI were distinguished from those resulting from natural service based on fetal size (Curran et al., 1986), as heifers were not exposed to natural service bulls until 14 d after AI. In Locations 2-4, fetal sex was determined for pregnancies resulting from AI.

Statistical analysis. Treatment differences for RTS, weight, and timing of insemination were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to analyze treatment differences in estrous response and final pregnancy rates at the end of the breeding season, as well as differences between AI technicians. Pregnancy rates to STAI were analyzed using a generalized linear model with the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. The error term was specified as the treatment x location interaction. The following variables were tested for inclusion in the model: treatment, location, bull, technician, heifer source, heifer RTS, heifer weight, estrous response (estrous by 66 h, estrous by 90 h, or non-estrous by 90 h), treatment x bull interaction, treatment x estrous response interaction, bull x estrous response interaction, and treatment x bull x estrous response interaction. Variables that did not significantly ($P > 0.10$) affect pregnancy rate to STAI were removed from the model, and the final model contained treatment and estrous response. Least squares means were generated using this model and compared using Fisher's least significant difference.

RESULTS

For heifers expressing estrus prior to 66 h after PG, the interval from PG to AI was 66.0 ± 0.1 h for heifers inseminated with conventional semen and 65.9 ± 0.08 h for heifers inseminated with sex-sorted semen. For the remaining heifers receiving delayed insemination at 90 h after PG, the interval from PG to AI was 90.0 ± 0.09 h for heifers inseminated with conventional semen and 90.0 ± 0.09 h for heifers inseminated with sex-sorted semen. Heifer weight, RTS, and estrous response rates for each treatment and location are reported in Table 6.1. The proportion of heifers expressing estrus prior to 66 h did not differ between treatments, nor did the proportion of heifers expressing estrus during the delay period. Heifer weight and RTS did not differ based on treatment.

In locations with more than one AI technician (Location 2 and 3), pregnancy rates to STAI did not differ based on technician. Pregnancy rates by location, treatment, and bull are presented in Table 6.2. Across locations, pregnancy rates to AI tended to be higher ($P = 0.09$) for heifers inseminated with conventional semen (60%; 257/429) compared to sex-sorted semen (52%; 218/422). Pregnancy rates to AI did not differ between Bull A (58%; 282/487) and Bull B (53%; 193/364), and there was no significant interaction of bull x treatment, bull x estrous response, or bull x treatment x estrous response. Pregnancy rate to AI based on treatment and estrous response are presented in Table 6.3. Pregnancy rates were greater ($P < 0.0001$) for heifers that expressed estrus prior to AI than for heifers that failed to express estrus prior to AI at 90 h.

In Location 1, in which pregnancy diagnosis was performed both at Day 62 and Day 120 after AI, one heifer in each treatment had a pregnancy resulting from AI that was

not present at Day 120. Due to this pregnancy loss, these heifers are reported as not having become pregnant to AI in Tables 2 and 3. Total pregnancy rates at the end of the 60 d breeding season did not differ between heifers that received sex-sorted semen at AI (89%; 376/422) and heifers that received conventional semen at AI (89%; 382/429). For locations in which fetal sex was determined at pregnancy diagnosis (Locations 2-4), fetal sex was determinable for 389 of the 401 heifers that became pregnant to AI. Of these, heifers inseminated with sex-sorted semen carried 96% (171/178) female calves, whereas heifers inseminated with conventional semen carried 49% (104/211) female calves.

DISCUSSION

Generally, one sex of progeny resulting from a planned mating has higher value than the alternative sex, though the sex desired and its relative value differ from operation to operation and mating to mating. Through use of sex-sorted semen in artificial insemination programs, beef and dairy producers can produce calves of the desired sex at accuracies over 90% (Vishwanath, 2015; Johnson, 2000; Seidel, 2014). However, although the direct cost of sex-sorted semen is fairly moderate (generally \$15 - \$30 US more per unit than the cost of conventional semen), indirect costs have limited adoption of this technology, particularly in the beef industry (Seidel, 2003a,b). Indirect costs stem from reduced pregnancy rates obtained when using sex-sorted compared to conventional semen (Hohenboken, 1999; Cooke et al., 2014). Using sex-sorted semen produced with the previous generation technology, prior research has reported pregnancy rates of a factor of .75 relative to the pregnancy rate obtained with conventional semen (Seidel et

al., 1999; Seidel, 2003a,b). For example, a producer would have faced a choice between a 60% first service pregnancy rate to conventional semen and a 45% first service pregnancy rate to sex-sorted semen. Low first service pregnancy rates following the 14-d CIDR-PG protocol would increase days-on-feed in dairy heifer development operations (Claypool et al., 2015), but cost considerations are more complex for beef producers given the seasonal constraints of beef production systems. Early conception significantly increases average age of calves at weaning and therefore pounds of calf weaned (Dzuik and Bellows, 1983), and age at conception also has implications for lifetime productivity of beef heifers (Lesmeister et al., 1973; Kill et al., 2012). Therefore, the indirect cost of low first service AI pregnancy rates is generally quite high in beef cow-calf operations.

Likewise, lower pregnancy rates to sex-sorted semen limit the degree to which progeny sex ratio can be effectively skewed. For example, inseminating 100 heifers with conventional semen at a 60% pregnancy rate would result in 30 pregnancies of the favored sex, while a 45% pregnancy rate to sex-sorted semen would result in 41 pregnancies of the desired sex (assuming a 50:50 and 92:8 sex ratio for conventional and sex-sorted semen respectively). While a modest increase in the number of desired sex progeny, the relative value of desired sex progeny has not offset the costs in many previous efforts (Seidel, 2003a,b; Hohenboken, 1999; Meyer et al., 2012; Cooke et al., 2014). Moreover, previous attempts to use sex-sorted semen in FTAI treatments have generally resulted in low pregnancy rates relative to conventional semen (Hall et al., 2010; Thomas et al., 2014a; Rhinehart et al., 2011; Sá Filho et al., 2012; Cooke et al., 2014). Increased adoption of advanced reproductive technologies in the beef industry is

likely contingent upon the development of timed protocols that do not require the labor and time commitment of estrus detection (NAHMS, 2008). To be economically viable for widespread adoption in the beef industry, therefore, sex-sorted semen must achieve pregnancy rates comparable to conventional semen and must be suitable for use in timed AI protocols.

The results presented here suggest that SexedULTRA™ sex-sorted semen can be used effectively in timed AI of beef heifers following the 14-d CIDR-PG protocol with STAI. It is noteworthy that the mean pregnancy rates to AI with SexedULTRA™ semen are effectively a factor of .86 relative to conventional semen in this dataset (52% versus 60%). While a much larger dataset would be required to truly evaluate relative equivalence, these results support previous suggestions of improved fertility using the SexedULTRA™ process compared to the previous generation XY technology for sex-sorting of sperm cells (reviewed by Vishwanath, 2015). Moreover, the present results detail effective use of SexedULTRA™ sex-sorted semen in a timed AI approach, which is of significant interest to the beef industry.

Compared to FTAI, STAI enhances use of sex-sorted and conventional semen for several reasons. When using STAI, timing of insemination is more optimally aligned with ovulation among heifers that have not expressed estrus prior to the standard time of FTAI, 66 h after PG (Thomas et al., 2014a,b). Timing of insemination relative to ovulation is critical when using frozen-thawed semen that may not retain fertility for long timespans in the female reproductive tract, such as semen from bulls with lower fertility (Macmillan and Watson, 1975). Efforts evaluating timing of AI with sex-sorted semen have suggested

that AI should take place at a time point close to ovulation for optimal pregnancy rates (Sales et al., 2011; Bombardellia et al., 2016).

The STAI approach used in this study (described by Bishop et al., 2016) also decreases the reliance on GnRH administration for induction of ovulation, instead allowing for maximum estrus expression. Across treatments, 64% (545/851) of heifers expressed estrus prior to 66 h after PG, the recommended time for FTAI following the 14-d CIDR-PG protocol. Due to the delay of insemination when using STAI, non-estrous heifers at this time are afforded an additional 24 h to express estrus prior to AI. This dataset illustrates the resulting improvement in total estrous response, as 69% (212/306) of heifers that had failed to express estrus by 66 h expressed estrus prior to insemination at 90 h, for a total estrous response of 89% (757/851). The estradiol production associated with estrus coordinates several physiological processes critical for establishment and maintenance of pregnancy, including effects on follicular cells, the oocyte, gamete transport, and preparation of the uterine environment (see review by Pohler et al., 2012). Consequently, estrus expression is associated with higher pregnancy rates to timed AI (Richardson et al., 2016). Whereas administration of GnRH to non-estrous females at FTAI likely limits subsequent estradiol production via downregulation of aromatase (Voss and Fortune, 1993), both AI and GnRH administration may be delayed 20-24 h for non-estrous heifers when using STAI. As a result, the total proportion of animals having expressed estrus prior to AI is increased in STAI (Bishop et al., 2016), and this results in corresponding increases in pregnancy rate compared to FTAI (Bishop et al., 2016, 2017b).

Based on bull-to-bull variation in pregnancy rates to sex-sorted semen, it had been suggested that the previous generation XY sorting procedure detrimentally affected fertility of some bulls more than others (Hall and Glaze, 2014). It is unclear at this time whether the newer SexedULTRATM sorting procedure detrimentally affects fertility in a bull dependent manner or to the same degree for a respective bull. However, we have previously suggested that bull-to-bull variation in timed AI pregnancy rates with conventional semen may be explained in part by bull-to-bull differences in pregnancy rates obtained among females that have failed to express estrus prior to FTAI (Thomas and Patterson, 2014). With this hypothesis, one might anticipate an interaction of bull x treatment x estrous status, in which sex-sorted semen from certain bulls may achieve particularly low pregnancy rates among females not expressing estrus prior to timed AI. However, interactions of bull, semen type (conventional versus sex-sorted), and a third variable are difficult to detect even in very large datasets (DeJarnette et al., 2011). Moreover, due to the high estrous response observed when performing split-time AI, numerical differences among non-estrous heifers with respect to bull should be interpreted cautiously.

Lastly, these results do not provide insight into the relative performance of SexedULTRATM sex-sorted semen versus conventional semen in the context of FTAI. Due to higher estrous response rates prior to AI, higher pregnancy rates result from STAI compared to FTAI, even when AI is performed with conventional semen that has not undergone sorting (Thomas et al., 2014a,b; Bishop et al., 2017b). Maximum estrous response prior to AI is likely even more critical when using sex-sorted semen due to the

reduced number of sperm cells per unit and the potential for reduced post-thaw longevity of sorted sperm. Therefore, STAI provides an effective platform for use of sex-sorted semen in timed AI, as considerations related to non-estrous females are mitigated by high estrous response rates.

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Figure 6.1

Figure 6.1. Treatment schedule for the 14-d CIDR-PG protocol. Heifers received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, and CIDRs were removed on Day 14. Prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) was administered on Day 30, and split-time artificial insemination (STAI) was performed at 66 h (Day 33) or 90 h (Day 34) after PG administration based on estrous response. Heifers failing to express estrus by 90 h after PG were administered gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) concurrent with AI at 90 h.

Table 6.1. Heifer weight (Wt), reproductive tract score (RTS), and estrous response by treatment and location.

Treatment ¹		N	Wt, kg	RTS ²	Estrous by 66 h ³		Total Estrous ²	
Location					Proportion	%	Proportion	%
Conventional		429	390 ± 2	4.2 ± 0.1	286/429	67	384/429	90
Location 1		60	358 ± 5	3.6 ± 0.1	41/60	70	52/60	87
Location 2		212	395 ± 2	4.4 ± 0.1	141/212	67	190/212	90
Location 3		60	380 ± 4	4.1 ± 0.1	50/60	83	58/60	97
Location 4		97	404 ± 3	4.4 ± 0.2	54/97	56	84/97	87
Sex-sorted		422	391 ± 2	4.3 ± 0.1	259/422	61	373/422	88
Location 1		61	361 ± 5	3.6 ± 0.1	43/61	70	53/61	87
Location 2		209	396 ± 2	4.5 ± 0.1	128/209	61	182/209	87
Location 3		57	381 ± 4	4.1 ± 0.1	40/57	70	53/57	93
Location 4		95	405 ± 3	4.4 ± 0.1	48/95	51	85/95	89

¹Heifers received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, and CIDRs were removed on Day 14. Prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) was administered on Day 30, and split-time artificial insemination (STAI) was performed at 66 h (Day 33) or 90 h (Day 34) after PG administration based on estrous response. Heifers failing to express estrus by 90 h after PG were administered gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) concurrent with AI at 90 h. Heifers in the conventional treatment were inseminated with conventional, non-sex-sorted semen, and heifers in the sex-sorted treatment were inseminated with sex-sorted semen.

²RTS (1 to 5 scale, where 1 = immature and 5 = luteal phase) of the heifers, evaluated at CIDR insertion on Day 0.

³Estrous response by 66 h after PG administration, as determined by activation of an estrus detection aid (Estrotest).

⁴Total estrous response by 90 h after PG administration, as determined by activation of an estrus detection aid (Estrotest).

Table 6.2. Pregnancy rates¹ to split-time AI (STAI) by location, treatment², and bull.

Location	Treatment	Bull A		Bull B		Overall	
		Proportion	%	Proportion	%	Proportion	%
Location 1	Conventional	37/60	62			37/60	62
	Sex-sorted	37/61	61			37/61	61
Location 2	Conventional	65/107	61	63/105	60	128/212	60
	Sex-sorted	54/104	52	54/105	51	108/209	52
Location 3	Conventional	17/29	59	19/31	61	36/60	60
	Sex-sorted	19/29	66	14/28	50	33/57	58
Location 4	Conventional	32/49	65	24/48	50	56/97	58
	Sex-sorted	21/48	44	19/47	40	40/95	42
Total	Conventional	151/245	62	106/184	58	257/429	60 ^a
	Sex-sorted	131/242	54	87/180	48	218/422	52 ^b

¹Pregnancy rate to AI determined by transrectal ultrasonography 62 d to 95 d after STAI.

²Heifers received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, and CIDRs were removal on Day 14. Prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) was administered on Day 30, and split-time artificial insemination (STAI) was performed at 66 h (Day 33) or 90 h (Day 34) after PG administration based on estrous response. Heifers failing to express estrus by 90 h after PG were administered gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) concurrent with AI at 90 h. Heifers in the conventional treatment were inseminated with conventional, non-sex-sorted semen, and heifers in the sex-sorted treatment were inseminated with sex-sorted semen.

^{ab}Overall pregnancy rates to AI tended to be higher (P = 0.09) among heifers inseminated with conventional compared to sex-sorted semen.

Table 6.3. Pregnancy rates¹ to split-time artificial insemination (STAI) based on treatment² and estrous response during STAI.

Treatment ²	Overall		Estrous by 66 h ³		Estrous 66 h - 90 h ⁴		Non-Estrous ⁵	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%
Conventional	257/429	60 ^a	177/286	62	67/98	68	13/45	29
Bull A	151/245	62	99/161	61	43/57	75	9/27	33
Bull B	106/184	58	78/125	62	24/41	59	4/18	22
Sex-sorted	218/422	52 ^b	136/259	53	68/114	60	14/49	29
Bull A	131/242	55	81/150	54	38/61	62	12/31	39
Bull B	87/180	48	55/109	50	30/53	57	2/18	11

¹Pregnancy rate to AI determined by transrectal ultrasonography 41 d to 95 d after STAI.

²Heifers received an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Madison, NJ) on Day 0, and CIDRs were removal on Day 14. Prostaglandin F_{2α} (PG; 25 mg im; Lutalyse, Zoetis, Madison, NJ) was administered on Day 30, and split-time artificial insemination (STAI) was performed at 66 h (Day 33) or 90 h (Day 34) after PG administration based on estrous response. Heifers failing to express estrus by 90 h after PG were administered gonadotropin-releasing hormone (GnRH; 100 µg im; Cystorelin, Merial, Athens, GA) concurrent with AI at 90 h. Heifers in the conventional treatment were inseminated with conventional, non-sex-sorted semen, and heifers in the sex-sorted treatment were inseminated with sex-sorted semen.

³Expression of estrus by 66 h after PG administration, as determined by activation of an estrus detection aid (Estroject). Insemination was performed at 66 h after PG administration.

⁴Expression of estrus between 66 h and 90 h after PG administration, as determined by lack of activation of an estrus detection aid at 66 h but activation at 90 h. Insemination was performed at 90 h after PG administration.

⁵Failure to express estrus by 90 h after PG, as determined by lack of activation of an estrus detection aid both at 90 h after PG administration.

^{ab}Overall pregnancy rates to AI tended to be higher (P = 0.09) among heifers inseminated with conventional compared to sex-sorted semen.

CHAPTER 7
EVALUATION OF FERTILITY OF SEXEDULTRA™ SEX-SORTED SEMEN IN
TIMED ARTIFICIAL INSEMINATION PROGRAMS FOR MATURE BEEF
COWS

ABSTRACT

An experiment was designed to compare fertility of SexedULTRA™ sex-sorted semen and conventional, non-sex-sorted semen following either fixed-time artificial insemination (FTAI) or split-time artificial insemination (STAI) of mature suckled beef cows. Units of sex-sorted and conventional semen were produced using contemporaneous ejaculates from three commercially available AI sires. Units of conventional semen were generated with 25.0×10^6 live cells per 0.25 ml straw prior to freezing, and units of sex-sorted semen were generated using the SexedULTRA™ Genesis III sorting technology with 4.0×10^6 live cells per 0.25 ml straw prior to freezing. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%. Cows (n = 922) across seven locations were treated with the 7-d CO-Synch + CIDR protocol [administration of gonadotropin-releasing hormone (GnRH) and insertion of a progesterone insert (CIDR) on Day -10, followed by administration of prostaglandin F_{2α} (PG) and removal of CIDR inserts on Day -3]. Cows were preassigned based on age, body condition score, and days postpartum to one of the following four treatments: FTAI with SexedULTRA™ sex-sorted semen, FTAI with conventional semen, STAI with SexedULTRA™ sex-sorted semen, or STAI with conventional semen. On Day -3, estrus

detection aids (Estroject[®]) were applied. For cows in FTAI treatments, AI was performed on Day 0 at 66 h after PG administration and CIDR removal, and 100 µg GnRH was administered concurrent with AI. For cows in STAI treatments, AI was performed on either Day 0 or 1, at 66 or 90 h after PG administration and CIDR removal, based on timing of estrus expression. On Day 1 at 90 h after PG administration and CIDR removal, 100 µg GnRH was administered concurrent with AI to any STAI-treated cows that had failed to express estrus. Total estrous response prior to AI was greater ($P < 0.001$) among cows receiving STAI (90%) compared to those receiving FTAI (80%) due to the additional 24 h for potential estrus expression when performing STAI. Greater pregnancy rates ($P < 0.0001$) were obtained among cows inseminated with conventional semen (68%; 315/464) compared to cows inseminated with SexedULTRA[™] sex-sorted semen (51%; 235/458). However, pregnancy rates did not differ between cows inseminated using a FTAI versus STAI approach, regardless of whether insemination was performed with conventional semen (69% versus 67%) or SexedULTRA[™] sex-sorted semen (50% versus 53%). Pregnancy rates to AI were affected ($P = 0.001$) by the interaction of bull and semen type. Greater pregnancy rates were obtained with conventional semen versus SexedULTRA[™] sex-sorted semen when using from Bull A (69% [111/161] versus 38% [59/157]; $P < 0.0001$) and Bull B (75% [121/161] versus 58% [92/157]; $P < 0.01$), whereas pregnancy rates to AI did not differ between conventional and SexedULTRA[™] sex-sorted when using semen from Bull C (58% [82/142] versus 58% [84/144]). In summary, greater pregnancy rates were obtained among cows receiving timed AI with conventional semen compared to SexedULTRA[™] sex-sorted semen, although the

relative fertility of SexedULTRATM sex-sorted semen and conventional semen varied across bulls. Although likely resulting in lower average pregnancy rates than those obtained with conventional semen, use of SexedULTRATM sex-sorted semen for timed AI of mature beef cows may result in pregnancy rates of a satisfactory level for some production settings.

INTRODUCTION

Technologies that allow for predetermination of progeny sex have long been of interest to commercial animal agriculture due to inherent value differences between male and female livestock. Although which sex is more valuable differs across animal industries and within various segments of each industry, cost-effective preselection of progeny sex presents an opportunity to enhance the profitability, sustainability, and rate of genetic progress in animal agriculture. Currently, flow-cytometric cell sorting can be used effectively to generate sorted populations of X- or Y-chromosome-bearing bovine sperm cells for use in commercial artificial insemination (AI) programs (Garner et al., 1983; Johnson et al., 1989; Seidel et al., 1999; Schenk et al., 1999). However, fertility of frozen-thawed sex-sorted semen has generally been reduced relative to conventional, non-sex-sorted semen (Seidel and Garner, 2002). Reduced fertility of sex-sorted semen has been attributed to several factors, namely the reduced number of sperm cells placed in units of sex-sorted semen and the fact that sperm cells undergo cellular damage during the sorting, cryopreservation, and thawing processes. Timing of insemination relative to ovulation affects fertility with conventional semen, likely in a bull dependent manner

(Trimberger, 1948; Macmillan and Watson, 1975; Dransfield et al., 1998; Saacke et al., 2000; Dalton et al., 2001). As a result of damage occurring to sperm cells during the sorting process, the timing window for optimal fertility is likely narrower and closer to ovulation when using sex-sorted semen (Sales et al., 2011; Bombardellia et al., 2016). Therefore, use of sex-sorted semen in fixed-time artificial insemination (FTAI) programs has generally been discouraged (Seidel, 2003a,b).

Recently, sex-sorted semen produced using an enhanced proprietary sex-sorting technology has become commercially available for both dairy and beef breeds under the trade name SexedULTRA™ (Sexing Technologies, Navasota, TX). Improvements made to the technologies and processes used to produce sex-sorted semen now allow units of SexedULTRA™ sex-sorted semen to be generated economically at 4.0×10^6 cells per unit, as compared to the 2.0×10^6 cells per unit that had become the industry standard dose for sex-sorted semen (Seidel et al., 1999; Amann, 1999; DeJarnette et al., 2008). Extensive evaluation of SexedULTRA™ semen has indicated improved fertility in comparison to sex-sorted semen produced using previous generation technology (see review by Vishwanath, 2015). Research efforts also suggest improved comparability between pregnancy rates obtained using SexedULTRA™ sex-sorted semen and those obtained using conventional semen (see review by Vishwanath, 2015). However, these results are based on AI following detected estrus; further research efforts are needed to evaluate use of SexedULTRA™ sex-sorted semen in timed AI settings.

In addition to basic research efforts to improve the quality of sex-sorted semen, translational research efforts in estrous cycle control may also result in strategies that

improve results when using sex-sorted semen in timed AI of beef cows and heifers. For example, strategies that maximize estrous response and enhance synchrony of estrus expression may offer improvements in timed AI pregnancy rates specifically with sex-sorted semen. In other publications, pregnancy rates following FTAI with sex-sorted semen have been particularly low among beef females that have not expressed estrus prior to FTAI (Hall et al., 2010; Meyer et al., 2012; Thomas et al., 2014a). The disparity between FTAI pregnancy rates of estrous and non-estrous females is also observed with conventional semen (see meta-analysis by Richardson et al., 2016), although we have previously proposed the degree to which pregnancy rates of estrous and non-estrous females differ may be bull dependent and may be exacerbated by the sex-sorting process (Thomas and Patterson, 2014).

Previous work from our lab led to the development of a timed AI approach known as split-time artificial insemination (STAI), in which AI is delayed by 20-24 h for females failing to express estrus prior to the standard time of FTAI. This approach was first found to improve pregnancy rates with sex-sorted semen among mature beef cows following the 7-d CO-Synch + CIDR protocol (Thomas et al., 2014a). Subsequent work with conventional semen demonstrated similarly improved fertility among beef heifers following the 14-d CIDR-PG protocol (Thomas et al., 2014b) and among beef cows following the 7-d CO-Synch + CIDR protocol (Bishop et al., 2017b). Research efforts were undertaken to evaluate the effectiveness and optimal timing of administration of gonadotropin-releasing hormone (GnRH) when performing STAI (Bishop et al., 2016, 2017a). Together, these experiments and subsequent similarly-designed experiments from

other labs (Markwood et al., 2014; Hill et al., 2016a,b) largely support the conclusion that STAI is an effective opportunity for producers to optimize pregnancy rates to timed AI.

In a recent experiment, Thomas et al. (2017c) evaluated use of SexedULTRA™ sex-sorted semen in comparison to conventional semen when performing STAI among beef heifers following the 14-d CIDR-PG protocol. Although pregnancy rates were greater among heifers inseminated with conventional semen (60%), the authors noted the pregnancy rates observed among heifers inseminated with SexedULTRA™ sex-sorted semen (52%) were well within the range of what many producers may consider acceptable in commercial production settings. To date, however, use of SexedULTRA™ sex-sorted semen has not been evaluated for timed AI of mature beef cows, nor has the relative effectiveness of STAI versus FTAI when using SexedULTRA™ sex-sorted semen. Therefore, the present study was designed as a two-by-two factorial to compare pregnancy rates following either FTAI or STAI with either conventional or SexedULTRA™ sex-sorted semen.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Semen collection. Semen was collected from three commercially available AI bulls (age 26-29 months at the time of collection), and units of SexedULTRA™ sex-sorted and conventional semen were produced from contemporaneous ejaculates. Semen was collected over an 11 d period (May 16th through 26th, 2017) for Bull A, a 15 d period

(June 2nd through June 16th, 2017) for Bull B, and an 11 d period (June 6th through 16th, 2017) for Bull C. For Bull A, semen from four ejaculates was used, with two ejaculates used for production of sex-sorted semen and two for conventional. For Bull B, semen from six ejaculates was used, with four ejaculates used for production of sex-sorted semen and two for conventional. For Bull C, semen from five ejaculates was used, with four ejaculates used for production of sex-sorted semen and one for conventional. All the semen passed the standard quality control criteria use for sex-sorted and conventional semen respectively. Units of conventional semen were generated with 25.0×10^6 live cells per 0.25 ml straw prior to freezing, based on sperm cell concentrations commonly used in industry. Units of sex-sorted semen were generated 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, based on sperm cell concentrations typically used in SexedULTRATM sex-sorted semen. Sex-sorted units were sorted to contain X chromosome-bearing sperm cells at an accuracy level of >90%, based on the accuracy level at which SexedULTRATM sex-sorted semen is typically produced and marketed.

Animals and treatments. Estrus was synchronized for 922 suckled beef cows of varying age and parity across seven locations. Estrus synchronization was performed using the 7-d CO-Synch + CIDR protocol as follows: administration of 100 µg gonadorelin diacetate tetrahydrate (GnRH; Cystorelin, Merial, Athens, GA) and insertion of a 1.38 g progesterone insert (CIDR; Zoetis, Madison, NJ) on Day -10, followed by administration of 500 µg cloprostenol sodium (PG; Estrumate, Merck Animal Health, Madison, NJ) and removal of CIDR inserts on Day -3. On Day -3, estrus detection aids

(Estroject[®], Rockway Inc, Spring Valley, WI) were applied to determine estrous status of animals at AI. Estrus expression was defined as removal of >50% of the rub-off coating on the estrus detection aid.

Within each location, cows were preassigned to balanced treatments based on age, days postpartum, and an assessment of body condition score (BCS) using a 1 to 9 scale (1 = emaciated and 9 = obese) on Day -3 (Richards et al., 1986). Cows were assigned to one of the following four treatments: FTAI with SexedULTRA[™] sex-sorted semen (FTAI-SS), FTAI with conventional semen (FTAI-CON), STAI with SexedULTRA[™] sex-sorted semen (STAI-SS), or STAI with conventional semen (STAI-CON). For cows in FTAI treatments, AI was performed on Day 0 at 66 h after PG administration and CIDR removal, and 100 µg GnRH was administered concurrent with AI. For cows in STAI treatments, AI was performed on either Day 0 or 1, at 66 or 90 h after PG administration and CIDR removal, based on timing of estrus expression. Cows having expressed estrus by 66 h after PG administration and CIDR removal received timed AI at that time, whereas insemination was delayed by 24 h until 90 h for cows failing to express estrus by 66 h. On Day 1 at 90 h after PG administration and CIDR removal, 100 µg GnRH was administered concurrent with AI to any STAI-treated cows that had failed to express estrus. A diagram of the AI approaches used in the FTAI versus STAI treatments is presented in Figure 7.1.

Artificial insemination. Artificial insemination was performed with either conventional or SexedULTRA[™] sex-sorted semen based on treatment. A single technician, blinded to semen type, performed AI across all locations. Sires were used

proportionally across all treatments in all locations and were preassigned to cows based on age, DPP, and BCS. Fourteen d after AI, cows were exposed to fertile bulls for the remainder of a 60 d breeding season.

Pregnancy diagnosis. Pregnancy rate to AI was determined by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA). Pregnancies resulting from AI were distinguished from those resulting from natural service based on fetal size (Curran et al., 1986), as cows were not exposed to natural service bulls until 10 d after AI. Ultrasonography was performed 84-92 d after AI in each location.

Statistical analysis. Potential treatment differences for age, DPP, and BCS were analyzed using the TTEST procedure of SAS (SAS Inst. Inc., Cary, NC). Chi-square contingency tables (PROC FREQ; SAS Inst. Inc., Cary, NC) were used to analyze treatment differences in estrous response rates and final pregnancy rate at the end of the breeding season. Pregnancy rates to AI were analyzed using a generalized linear model via the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC) using the binomial distribution, link logit function. The error term was specified as the interaction of location x bull x AI approach x semen type. Variables tested for inclusion in the model were location, bull, AI approach, semen type, age, DPP, BCS, estrous response at 66 h, and the following interactions: bull x AI approach, bull x semen type, AI approach x semen type, bull x AI approach, bull x AI approach x semen type, location x bull, location x AI approach, location x semen type, semen type x estrous response at 66 h, and approach x estrous response at 66 h. Variables that did not significantly ($P > 0.10$) affect pregnancy

rate to AI were removed from the model, so that the final model for pregnancy rate to AI consisted of location, estrous response at 66 h, bull, semen type, and bull x semen type interaction. Least squares means were generated using this model and compared using Fisher's least significant difference.

RESULTS

Cow age, DPP, and BCS are presented for each treatment and location in Table 7.1. Treatments did not differ with respect to mean cow age, DPP, and BCS. Cows receiving FTAI were inseminated 66.6 ± 0.1 h after PG administration and CIDR removal. Cows receiving STAI and expressing estrus prior to this time point were inseminated 66.7 ± 0.1 h after PG administration and CIDR removal, and STAI-treated cows failing to express estrus by this time point were inseminated 89.7 ± 0.1 h after PG administration and CIDR removal. Rates of estrous response are presented for each treatment and location in Table 7.2. Treatments did not differ with respect to the proportion of cows expressing estrus prior to 66 h after PG administration and CIDR removal. However, due to the additional 24 h for potential estrus expression prior to AI when STAI is performed, total estrous response prior to AI was increased ($P < 0.001$) among cows in STAI treatments (90%; 416/460) compared to FTAI treatments (80%; 368/462).

Across treatments, pregnancy rates to AI were affected by estrous response at 66 h after PG administration and CIDR removal. Cows expressing estrus prior to this time achieved greater ($P = 0.01$) pregnancy rates to AI (62%; 448/726) than cows failing to

express estrus prior to this time (52%; 102/196). However, the AI approach used to carry out timed AI did not affect pregnancy rates to AI, and there was no effect of an interaction of AI approach and estrous status or of AI approach and another variable. Pregnancy rates to AI did not differ between cows inseminated using a STAI versus FTAI approach, regardless of whether insemination was performed with conventional semen (69% versus 67%) or SexedULTRA™ sex-sorted semen (50% versus 53%). Locations tended to differ ($P = 0.07$) with respect to pregnancy rates following AI; however, there was no effect associated with the interaction of location and another variable.

Pregnancy rates to AI were also affected by semen type ($P < 0.0001$), bull ($P < 0.01$), and the interaction of semen type and bull ($P = 0.001$). Across bulls, greater pregnancy rates ($P < 0.0001$) were obtained among cows inseminated with conventional semen (68%; 315/464) compared to cows inseminated with SexedULTRA™ sex-sorted semen (51%; 235/458). Across semen types, greater pregnancy rates were obtained when using semen from Bull B (67%; 213/318) compared to Bull A (53% [170/318]; $P < 0.01$) or Bull C (58% [166/286]; $P = 0.04$). Greater pregnancy rates were obtained with conventional semen versus SexedULTRA™ sex-sorted semen when using semen from Bull A (69% [111/161] versus 38% [59/157]; $P < 0.0001$) and Bull B (75% [121/161] versus 58% [92/157] $P < 0.01$), whereas pregnancy rates to AI did not differ between conventional and SexedULTRA™ sex-sorted when using semen from Bull C (58% [82/142] versus 58% [84/144]). Final pregnancy rate at the end of a 60 d breeding season did not differ between cows that had received conventional semen at AI (94%; 436/464) and cows that had received SexedULTRA™ sex-sorted semen (94%; 429/458).

DISCUSSION

In the vast majority of publications involving use of sex-sorted semen, pregnancy rates following AI with sex-sorted semen have been found to be reduced in comparison to pregnancy rates following AI with conventional semen. Specifically, publications reporting FTAI results using sex-sorted semen highlight the unique challenges associated with use of this product in timed AI programs (Hall et al., 2010; Thomas et al., 2014a; Rhinehart et al., 2011; Sá Filho et al., 2012; Cooke et al., 2014). Expanded use of sex-sorted semen in the beef industry will likely only occur if sufficiently high pregnancy rates can be obtained when using sex-sorted semen in timed AI systems, as the labor and time commitments associated with estrus detection-based programs present a significant challenge for many beef producers (NAHMS, 2008).

In the present study, pregnancy rates to timed AI were reduced among beef cows inseminated with SexedULTRATM sex-sorted semen. Reduced pregnancy rates to timed AI are effectively an indirect cost to producers when using sex-sorted semen (Hohenboken, 1999; Cooke et al., 2014). Depending on the degree to which pregnancy rates are reduced, this reduction in pregnancy rates may in fact be the largest cost associated with use of sex-sorted semen. However, the magnitude of the pregnancy rates obtained with SexedULTRATM sex-sorted semen in this trial should be noted (51%; 235/458). In a recent publication evaluating use of SexedULTRATM sex-sorted semen for timed AI of beef heifers following the 14-d CIDR-PG protocol (Thomas et al., 2017c), pregnancy rates observed following STAI with SexedULTRATM sex-sorted semen (52%; 218/422) were nearly identical to those observed in the present study among cows

receiving SexedULTRATM sex-sorted semen. Although lower than the pregnancy rates that may be obtained when using conventional semen, pregnancy rates in this range when using sex-sorted semen may still be commercially acceptable in certain production settings when the value difference between male and female calves is sufficiently large. Further work is needed to evaluate the economic implications of use of SexedULTRATM sex-sorted semen for timed AI across different segments of the beef industry.

In contrast to previous work comparing STAI and FTAI approaches, no difference in pregnancy rates was observed between cows in STAI treatments and cows in FTAI treatments. However, this lack of effect may be due to the exceptionally high rates of estrous response observed by 66 h after PG administration and CIDR removal (80% across FTAI treatments). In addition to limiting statistical power to detect treatment differences among cows failing to express estrus prior to 66 h, high rates of estrous response by this time likely limit any potential improvement in pregnancy rate associated with use of STAI. In previously published data (Thomas et al., 2014b; Bishop et al., 2016, 2017b), we have noted that improved pregnancy rates associated with STAI are primarily associated with increased total rates of estrous response, as cows that fail to express estrus by 66 h after PG administration are afforded an additional 20-24 h to potentially express estrus prior to AI. Although the total rate of estrous response prior to AI was enhanced in the present data through use of STAI rather than FTAI (90% versus 80%), the magnitude of this improvement is rather moderate compared to that observed in previous work with STAI. For example, Bishop et al. (2017b) found that total estrous response prior to AI was enhanced from 60% among FTAI treated cows to 86% among

STAI treated cows. Pregnancy rates for cows inseminated at 66 h that exhibited estrus did not differ between treatments (FTAI = 58%; STAI = 58%; $P = 0.93$); however, pregnancy rates among cows that failed to express estrus by 66 h were improved in STAI treatments when insemination and GnRH administration were postponed by 24 h (FTAI = 35%; STAI = 51%; $P = 0.01$). Consequently, total AI pregnancy rate tended to be higher for cows that received STAI (FTAI = 49%; STAI = 56%; $P = 0.06$). In contrast, the potential for any improvement from STAI is much more limited in the present data set due to the high rates of estrous response observed by 66 h after PG administration and CIDR removal.

In addition, pregnancy rates to FTAI may be highly influenced by variation in the fertile lifespan of sperm cells from different bulls or different ejaculates. The degree to which the sex-sorting process negatively impacts fertility of sperm cells appears to vary based on bull (Hall and Glaze, 2014). Routine semen analysis can easily identify collections that are particularly negatively affected, such as those with very low percent live or motile cells. Effective screening of collections in this manner can virtually eliminate use of obviously low fertility sex-sorted semen. However, identification of moderately affected collections is considerably more challenging and requires use of more complex analytical techniques relative to semen quality. We have previously proposed (Thomas and Patterson, 2014) that bull or ejaculate variability may contribute to variability in pregnancy rates specifically in the context of timed AI, as these systems inherently involve more variability in estrous status at AI and in timing of insemination relative to ovulation. Unfortunately, this experiment lacks statistical power to detect a

bull x treatment x estrous status interaction due to the high rates of estrous response observed by 66 h after PG administration and CIDR removal. However, variability in pregnancy rates of bulls in this trial is apparent and raises questions as to whether bull or ejaculate variability may influence the degree to which pregnancy rates are improved when using STAI rather than FTAI. One may speculate, for example, that sires or ejaculates of particularly high semen quality may perform equally well in FTAI and STAI systems, whereas those of lower semen quality may greatly benefit from the higher rates of estrous response and more forgiving timing of AI when using STAI. This could offer an explanation as to why previous evaluations of STAI versus FTAI demonstrated more marked improvements in pregnancy rates when using sex-sorted semen (Thomas et al., 2014a). Alternatively, the recent improvements made in the SexedULTRATM sex-sorting process may have mitigated some damage negatively impacting the fertile lifespan of sorted sperm produced with previous technologies.

In summary, pregnancy rates to timed AI among mature cows following the 7-d CO-Synch + CIDR were reduced when using SexedULTRATM sex-sorted semen compared to conventional semen. However, as in a previous evaluation of SexedULTRATM sex-sorted semen for timed AI of beef heifers following the 14-d CIDR-PG protocol, pregnancy rates to timed AI with SexedULTRATM sex-sorted semen exceeded 50% and as such may be commercially acceptable in some beef production settings. Future work should evaluate economic implications of use of SexedULTRATM sex-sorted semen for timed AI of beef females, and further translational research is needed to develop timed AI approaches specifically tailored for use of sex-sorted semen.

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Table 7.1. Cow age, days postpartum (DPP), and body condition score (BCS) by treatment and location.

Treatment ¹				
Location	N	Age	DPP	BCS ²
FTAI Conventional	230	4.7 ± 0.2	86.6 ± 1.1	5.7 ± 0.1
Location 1	23	2.0 ± 0.0	121.0 ± 4.3	5.6 ± 0.1
Location 2	24	3.2 ± 0.1	77.9 ± 2.3	5.7 ± 0.1
Location 3	17	7.4 ± 0.6	85.9 ± 1.0	5.6 ± 0.1
Location 4	43	6.3 ± 0.3	83.1 ± 1.9	6.1 ± 0.1
Location 5	43	2.9 ± 0.3	82.9 ± 1.3	5.6 ± 0.1
Location 6	50	5.9 ± 0.3	87.9 ± 0.7	5.5 ± 0.1
Location 7	30	4.5 ± 0.3	73.6 ± 1.9	5.7 ± 0.1
FTAI SexedULTRA	232	4.6 ± 0.2	86.9 ± 1.1	5.7 ± 0.1
Location 1	23	2.0 ± 0.0	124.0 ± 4.1	5.6 ± 0.1
Location 2	26	3.3 ± 0.1	78.0 ± 2.1	5.8 ± 0.1
Location 3	20	7.0 ± 0.5	85.4 ± 0.9	5.6 ± 0.1
Location 4	41	6.3 ± 0.3	86.0 ± 1.2	6.0 ± 0.1
Location 5	43	2.8 ± 0.2	81.0 ± 1.6	5.6 ± 0.1
Location 6	49	5.9 ± 0.3	87.9 ± 0.9	5.6 ± 0.1
Location 7	30	4.5 ± 0.2	73.9 ± 2.3	5.7 ± 0.1
STAI Conventional	234	4.6 ± 0.1	86.5 ± 1.0	5.7 ± 0.1
Location 1	23	2.0 ± 0.0	118.3 ± 4.6	5.6 ± 0.1
Location 2	23	3.2 ± 0.1	77.0 ± 2.3	5.6 ± 0.1
Location 3	19	7.1 ± 0.4	87.2 ± 0.8	5.6 ± 0.1
Location 4	43	6.4 ± 0.3	85.7 ± 1.5	6.1 ± 0.1
Location 5	44	2.8 ± 0.2	81.4 ± 1.4	5.6 ± 0.1
Location 6	52	5.6 ± 0.3	87.6 ± 0.6	5.6 ± 0.1
Location 7	30	4.5 ± 0.2	75.0 ± 2.5	5.6 ± 0.1
STAI SexedULTRA	226	4.5 ± 0.1	86.8 ± 1.1	5.7 ± 0.1
Location 1	22	2.0 ± 0.0	121.7 ± 4.2	5.6 ± 0.1
Location 2	24	3.2 ± 0.1	77.0 ± 2.3	5.6 ± 0.1
Location 3	19	7.0 ± 0.5	85.6 ± 0.8	5.4 ± 0.1
Location 4	39	5.8 ± 0.2	84.2 ± 1.9	6.1 ± 0.1
Location 5	44	2.8 ± 0.2	81.9 ± 1.2	5.6 ± 0.1
Location 6	48	6.0 ± 0.3	87.3 ± 1.2	5.5 ± 0.1
Location 7	30	4.4 ± 0.3	73.9 ± 2.1	5.7 ± 0.1

¹Cows received either conventional or SexedULTRA™ sex-sorted semen in either a split-time artificial insemination (STAI) or fixed-time artificial insemination (FTAI) approach. See Figure 7.1 for a graphical depiction of timing of AI in STAI and FTAI treatments.

²Body condition score (mean ± SE) of cows at treatment assignment on Day 0 (1 to 9 scale, where 1 = emaciated and 9 = obese).

Table 7.2. Estrous response based on treatment and location.

Treatment ¹ Location	Estrous Response Prior to FTAI Time ²		Estrous Response During 24 h Delay ³		Total Estrous Response ⁴	
	Proportion	%	Proportion	%	Proportion	%
FTAI Conventional	181/230	79			181/230	79 ^a
Location 1	19/23	83			19/23	83
Location 2	23/24	96			23/24	96
Location 3	11/17	65			11/17	65
Location 4	35/43	81			35/43	81
Location 5	29/43	67			29/43	67
Location 6	42/50	84			42/50	84
Location 7	22/30	73			22/30	73
FTAI SexedULTRA	187/232	81			187/232	81 ^a
Location 1	20/23	87			20/23	87
Location 2	23/26	88			23/26	88
Location 3	17/20	85			17/20	85
Location 4	31/41	76			31/41	76
Location 5	34/43	79			34/43	79
Location 6	39/49	80			39/49	80
Location 7	23/30	77			23/30	77
STAI Conventional	178/234	76	28/234	12	206/234	88 ^b
Location 1	20/23	87	0/23	0	20/23	87
Location 2	20/24	83	0/24	0	20/24	83
Location 3	12/19	63	6/19	32	18/19	95
Location 4	30/43	70	8/43	19	38/43	88
Location 5	34/44	77	3/44	7	37/44	84
Location 6	40/52	77	7/52	13	47/52	90
Location 7	23/30	77	4/30	13	27/30	90
STAI SexedULTRA	180/226	80	28/226	12	208/226	92 ^b
Location 1	20/22	91	0/22	0	20/22	91
Location 2	23/24	96	1/24	4	24/24	100
Location 3	13/19	68	4/19	21	17/19	89
Location 4	33/39	85	4/39	10	37/39	95
Location 5	34/44	77	4/44	9	38/44	86
Location 6	37/48	77	7/48	15	44/48	92
Location 7	20/30	67	8/30	27	28/30	93

¹See Table 7.1 for a description of treatments.

²Estrous response prior to 66 h after CIDR removal and PG administration.

³Estrous response occurring during the 24 h delay period from 66-90 h after CIDR removal. and PG administration.

⁴Total cumulative estrous response.

Table 7.3. Pregnancy rates¹ to timed artificial insemination based on treatment² and estrous response³.

Treatment	Overall		Estrous by 66 h		Estrous by 90 h		Non-Estrous	
Location	Proportion	%	Proportion	%	Proportion	%	Proportion	%
FTAI	158/230	69 ^a	129/181	71			29/49	59
Conventional								
Location 1	14/23	61	12/19	63			2/4	50
Location 2	22/24	92	21/23	91			1/1	100
Location 3	11/17	65	8/11	73			3/6	50
Location 4	34/43	79	27/35	77			7/8	88
Location 5	29/43	67	21/29	72			8/14	57
Location 6	28/50	56	24/42	57			4/8	50
Location 7	20/30	67	16/22	73			4/8	50
FTAI	116/232	50 ^b	97/187	52			19/45	42
SexedULTRA								
Location 1	14/23	61	12/20	60			2/3	67
Location 2	18/26	69	15/23	65			3/3	100
Location 3	9/20	45	8/17	47			1/3	33
Location 4	19/41	46	14/31	45			5/10	50
Location 5	20/43	47	17/34	50			3/9	33
Location 6	21/49	43	18/39	46			3/10	30
Location 7	15/30	50	13/23	57			2/7	29
STAI	157/234	67 ^a	125/178	70	20/28	71	12/28	43
Conventional								
Location 1	13/23	56	13/20	65	0/0	0	0/3	0
Location 2	17/23	74	14/19	74	0/0	0	3/4	75
Location 3	16/19	85	11/12	92	5/6	83	0/1	0
Location 4	31/43	72	20/30	67	6/8	75	5/5	100
Location 5	24/44	55	20/34	59	1/3	33	3/7	43
Location 6	38/52	73	32/40	80	6/7	86	0/5	0
Location 7	18/30	60	15/23	65	2/4	50	1/3	33
STAI	119/226	53 ^b	97/180	54	18/28	64	4/18	22
SexedULTRA								
Location 1	11/22	50	11/20	55	0/0	0	0/2	0
Location 2	17/24	71	16/23	70	1/1	100	0/0	0
Location 3	8/19	42	6/13	46	2/4	50	0/2	0
Location 4	20/39	51	18/33	55	1/4	25	1/2	50
Location 5	21/44	48	19/34	56	2/4	50	0/6	0
Location 6	28/48	58	19/37	51	6/7	86	3/4	75
Location 7	14/30	47	8/20	40	6/8	75	0/2	0

¹Pregnancy rate to AI determined by transrectal ultrasonography 84-92 d after STAI.

²See Table 7.1 for a description of treatments.

³Estrous response as determined by activation of an estrus detection aid (Estrotect).

Table 7.4 Pregnancy rates¹ to artificial insemination based on treatment², bull, and estrous response³ during STAI.

Treatment ² Bull	Overall		Estrous by 66 h		Estrous 66 - 90 h		Non-Estrous	
	Proportion	%	Proportion	%	Proportion	%	Proportion	%
FTAI								
Conventional	158/230	69 ^a	129/181	71			29/49	59
Bull A	55/80	69	47/64	73			8/16	50
Bull B	60/80	75	47/61	77			13/19	68
Bull C	42/70	60	34/56	61			8/14	57
FTAI								
SexedULTRA	116/232	50 ^b	97/187	52			19/45	42
Bull A	24/79	30	22/68	32			2/11	18
Bull B	44/78	56	38/63	60			6/15	40
Bull C	48/75	64	37/56	66			11/19	58
STAI								
Conventional	157/234	67 ^a	125/178	70	20/28	71	12/28	43
Bull A	56/81	69	41/55	75	11/15	73	4/11	36
Bull B	61/81	75	50/65	77	5/7	71	6/9	67
Bull C	40/72	56	34/58	59	4/6	67	2/8	25
STAI								
SexedULTRA	119/226	53 ^a	97/180	54	18/28	64	4/18	22
Bull A	35/78	45	30/63	48	5/11	45	0/4	0
Bull B	48/79	61	36/63	57	9/9	100	3/7	43
Bull C	36/69	52	31/54	57	4/8	50	1/7	14

¹Pregnancy rate to AI determined by transrectal ultrasonography 84-92 d after STAI.

²See Table 7.1 for a description of treatments.

³Estrous response as determined by activation of an estrus detection aid (Estrotest).

Figure 7.1

7-d CO-Synch + CIDR with FTAI

7-d CO-Synch + CIDR with STAI

Figure 7. 1. Treatment schedule for the 7-d CO-Synch + CIDR protocol with fixed-time AI (FTAI) or split-time AI (STAI). Cows received a 1.38 g progesterone (CIDR) insert and GnRH administration (100 µg im) on Day -10. On Day -3, CIDR inserts were removed, PG (25 mg im) was administered, and estrus detection aids (Estrotect) were applied. On Day 0, 66 h after PG administration, cows in the FTAI treatments received GnRH administration and AI was performed. Cows in the STAI treatments received AI at this time if detection aids were activated. On Day 1, 90 h after PG administration, all remaining cows in the STAI treatment received AI, and GnRH was administered to cows with non-activated estrus detection aids.

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VITA

Jordan Matthew Thomas was born in Columbia, Missouri on July 4th, 1990 to Wally and Elizabeth Thomas. He was raised in eastern Boone County adjacent to a farm owned and operated by his grandparents, Everett “Bud” and Ruth Little, who inspired his interest in beef cattle production. Jordan was primarily home-educated until enrolling at the University of Missouri in 2008. He completed his Bachelor of Science in Animal Sciences, graduating *summa cum laude* in December of 2011. In January 2012, he began a Master of Science program under the guidance of Dr. David J. Patterson in the Division of Animal Sciences of the College of Agriculture, Food and Natural Resources at the University of Missouri. Following completion of his M.S. program in December of 2013, he became employed in the Division of Animal Sciences as a Senior Research Specialist. Concurrent with this ongoing employment, he began coursework and research in pursuit of a Ph.D. in January of 2015. This dissertation is the result of that work and was defended in November of 2017.