Animal Reproduction Science 204 (2019) 1–9

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Ovarian follicular and luteal characteristics in *Bos indicus*-influenced beef cows using prostaglandin $F_{2\alpha}$ with or without GnRH at the onset of the 5-day CO-Synch + controlled internal drug release (CIDR) protocol

J.O. Scarpa[a,b], M.M. O'Neil[a,b], R.C. Cardoso[b], R.L Stanko[a,c], G.L. Williams[a,b,*]

[a] Animal Reproduction Laboratory, Texas A&M AgriLife Research, Beeville 78102, United States
[b] Department of Animal Science, Texas A&M University, College Station 77843, United States
[c] Department of Animal, Rangeland and Wildlife Sciences, Texas A&M University-Kingsville, Kingsville 78363, United States
[*] Corresponding author at: Animal Reproduction Laboratory, Texas A&M AgriLife Research, Beeville 78102, United States

ARTICLE INFO

Keywords:
GnRH
Prostaglandin $F_{2\alpha}$
CIDR
Bos indicus
Synchronization

ABSTRACT

A modification of the standard 5-day CO-Synch + CIDR procedure (5-day Bee Synch + CIDR; Bee Synch), developed for use in *Bos indicus*-influenced cows, utilizes the addition of prostaglandin F2α (PGF) on Day 0 of the protocol to eliminate mature corpora lutea (CL) and fixed-time AI (FTAI) at 66 h. Objectives were to test the hypothesis that elimination of GnRH on Day 0 (GnRH-1) does not impact significantly the synchronized development of a dominant follicle for presumptive FTAI. Seventy-one estrous cycling Brangus and Brahman x Hereford suckled cows were used in two replicates (35–36/replicate). Following stratification, cows were assigned randomly to a 2 × 3 factorial arrangement of treatments involving two truncated (no FTAI or GnRH-2) versions of Bee Synch (Bee Synch I_t and II_t), each begun 3, 7, and 10 days post-ovulation. Cows in Bee Synch I_t received 100 μg GnRH (GnRH-1), 25 mg PGF, and a CIDR on Day 0, whereas cows assigned to Bee Synch II_t received the same treatment but without GnRH-1. All cows received 50 mg PGF on Day 5 at CIDR removal. Synchronized new follicular wave emergence (NFWE; days 1–4) was observed in 68.6 and 38.9% of Bee Synch I_t and II_t, respectively ($P = 0.01$). This increased to 93.3% and 72.2%, respectively, if days 0–4 were considered. Inclusion of GnRH at CIDR insertion improved synchronized NFWE but size of the largest follicle at 66 h, the normal time of FTAI, did not differ due to treatment or day of the estrous cycle.

1. Introduction

Numerous protocols have been developed to synchronize new ovarian follicle wave emergence (NFWE) and timing of ovulation for FTAI in dairy and beef cows, often involving GnRH (Pursley et al., 1995; Bridges et al., 2007; Lamb et al., 2010; 2016) or estradiol (Martínez et al., 2000; 2005; Colazo et al., 2003; Baruselli et al., 2004; Bó et al., 1993) at the onset of treatment. Applied to *Bos taurus* females, these protocols can consistently yield pregnancy rates ≥ 50% (Lamb and Mercadante, 2016). In the U.S., there are no FDA-approved estrogen products available commercially for this purpose. Importantly, use of either 7-day (Saldarriaga et al., 2007; Zuluaga et al., 2010) or 5-day (Williams et al., 2011, 2013) CO-Synch + CIDR protocols utilizing GnRH (GnRH-1) at treatment onset in *Bos indicus*-influenced mature beef cows have failed to consistently achieve FTAI pregnancy rates above 40%.

E-mail address: glwilliams@tamu.edu (G.L. Williams).

https://doi.org/10.1016/j.anireprosci.2019.02.013
Received 6 December 2018; Received in revised form 13 February 2019; Accepted 25 February 2019
Available online 26 February 2019
0378-4320/

In 2011, Williams et al. published a preliminary report on a modification of the 5-day CO-Synch + CIDR protocol (5-day Bee Synch + CIDR; Bee Synch) involving the addition of PGF at treatment onset. Objectives were to reduce circulating concentrations of progesterone (P4) in a significant proportion of cows by eliminating mature corpora lutea (CL), thus potentially enhancing frequency of LH pulses and rate of follicular maturation during the synchronization period. Results indicated a marked increase in FTAI pregnancy rates to > 50% *in Bos indicus*-influenced mature cows (Williams et al., 2011, 2013).

The original Bee Synch methodology (now termed Bee Synch I) utilizes a 5-day CIDR, GnRH (GnRH-1) and PGF on day 0, double dose of PGF on day 5, and FTAI with GnRH (GnRH-2) at 66 h after CIDR removal. Cruppe et al. (2014) reported that the inclusion of GnRH on day 0 does not contribute appreciably to follicle synchronization in *Bos taurus* beef heifers synchronized with the standard 5-day CO-Synch + CIDR. Similarly, Williams et al. (2015) proposed a modification of Bee Synch I for use in mature *Bos indicus*-influenced cows that eliminates GnRH on day 0 (Bee Synch II). Based on field trials (Williams et al., 2015), it appeared that overall pregnancy rates in Bee Synch I and II were similar when used in these cattle types. However, the timing and pattern of NFWE, growth, and ovulation associated with deletion of GnRH-1 in the Bee Synch protocol have not been critically evaluated in *Bos indicus*-influenced females. Therefore, the objective of this study was to test the hypothesis that elimination of GnRH-1 from Bee Synch I (i.e., Bee Synch II) does not impact significantly the synchronized development of a dominant follicle for presumptive FTAI in *Bos indicus*-influenced beef cows.

2. Materials and methods

All animal-related procedures in this study were approved by the Agricultural Animal Care and Use Committee, Texas A&M AgriLife Research, Texas A&M University System.

2.1. Study location

The experiment was conducted in two replicates from March to June and from September to November 2016, respectively, at the Texas A&M AgriLife Research Station, Beeville, Texas.

2.2. Animal model

Seventy-two mature, suckled Brahman x Hereford (replicate 1; $n = 36$) and Brangus (replicate 2; $n = 36$) beef cows were used in this experiment. All cattle were required to have a minimum BCS of 5 (1–9 scale, 1 = emaciated, and 9 = obese) and be at least 50 days postpartum. Mean (± SEM) BCS and BW were 5.7 ± 0.07, and 628 ± 7.8 kg, respectively. Cows were fed according to recommendations of the National Research Council (NRC, 1996) for lactating beef cows to maintain BCS. To be used in this experiment, cows were required to exhibit evidence of ovarian cyclicity which was defined as the presence of an ultrasonographically-definable CL. During each experimental replicate, cows were placed in pens measuring 25.9 x 9.5 m (6 cow-calf pairs/pen) 15 days before institution of a pre-synchronization procedure.

Estrous cycles were pre-synchronized with two injections of 25 mg PGF (Lutalyse; Zoetis Animal Health, New York, NY), administered 11 days apart (Fig. 1A). Following the second injection of PGF, the ovaries were scanned daily using transrectal ultrasonography until ovulation was confirmed. Ovulation was defined as the sudden disappearance of a large follicle followed by appearance of a CL within 2 days.

2.3. Experimental design and methodology[1]

Following ovulation, cows were assigned randomly to receive truncated versions of Bee Synch I (Bee Synch I_t) or II (Bee Synch II_t) as described below on 1 of 3 days of the estrous cycle (day 3, 7 or 10 post ovulation) in a 3 × 2 factorial arrangement. Because the objectives of the study were to monitor follicular dynamics until natural ovulation, FTAI was not employed in this experiment. Thus, cows did not receive GnRH treatment at 66 h after CIDR removal (GnRH-2) as they would under normal FTAI circumstances.

Bee Synch I_t (Fig. 1B) included the insertion of a CIDR (Zoetis Animal Health, New York, NY), the intramuscular injection of 100 μg GnRH (Factrel; Zoetis Animal Health, New York, NY) and 25 mg PGF on Day 0. On Day 5 of the protocol, CIDRs were removed and cows received 50 mg of PGF. Bee Synch II_t followed the same treatment protocol as Bee Synch I_t except that GnRH on Day 0 (GnRH-1) is omitted. Even though cows estrous synchronized with the Bee Synch II_t did not receive GnRH-1, the double dose (50 mg) of PGF was utilized for both treatments on Day 5 in order to maintain experimental integrity. Normally, a single dose (25 mg) is used for Bee Synch II under a field scenario. Days of the estrous cycle for initiation of treatments represent three putative stages of follicular growth (Ginther et al., 1989, 1997). On day 3 post-ovulation, no dominant follicle is expected to be present, P4 is low but increasing, and ovulation is not expected to occur in response to GnRH. On day 7 post-ovulation, a large, dominant follicle in the growth phase should be present, P4 will be maximally elevated, and ovulatory response to GnRH is expected to be large (> 90%). On day 10 post-ovulation, circulating concentrations of P4 are maximal, approximately 50% of dominant follicles will have begun to regress, and only about 50% will ovulate in response to GnRH (Vasconcelos et al., 1999)

[1] [Note: For the purpose of clarity, “Day” refers to Day of the synchronization protocol and “day” refers to day of the estrous cycle unless otherwise defined.]

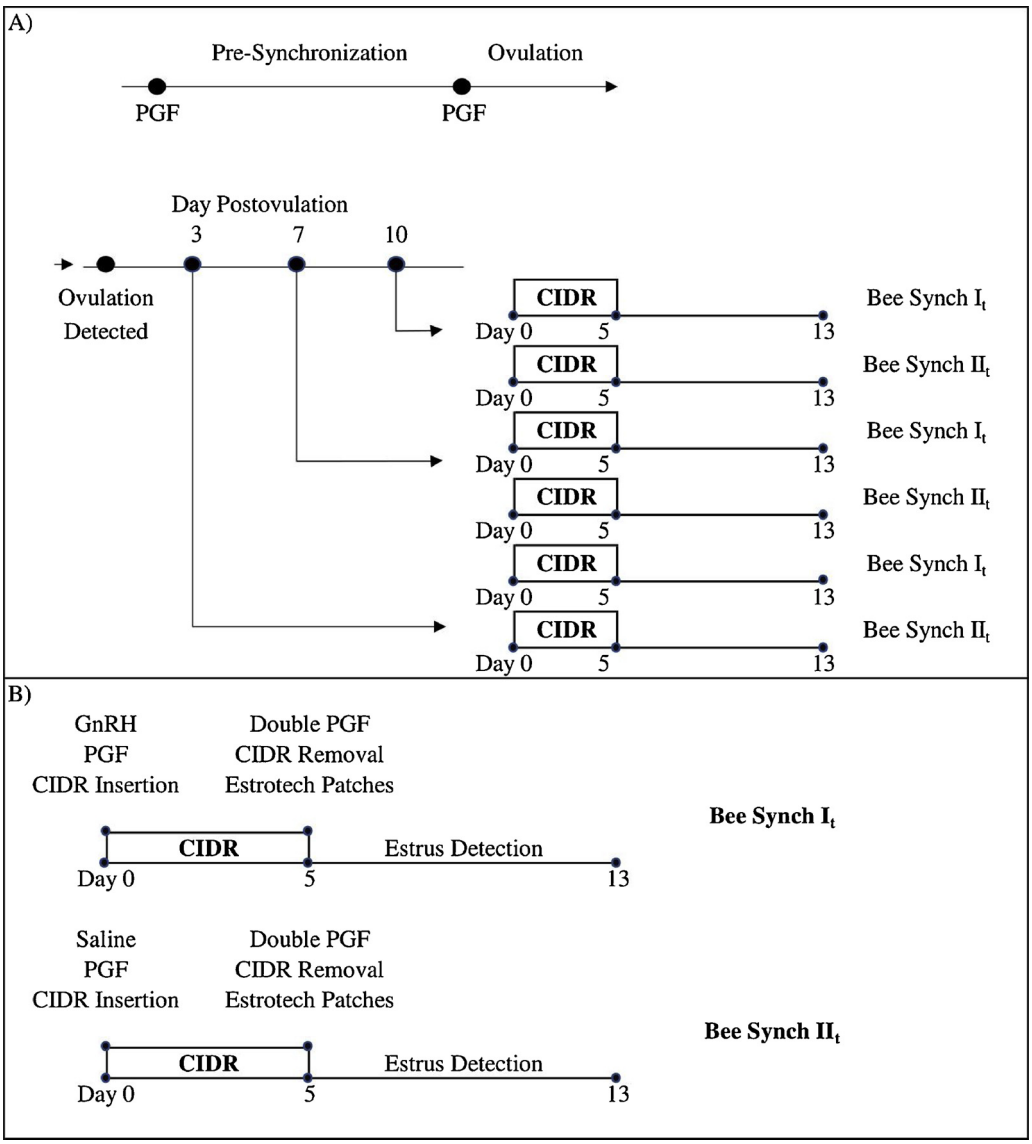

Fig. 1. A) Experimental regimen and B) details of Bee Synch I_t and Bee Synch II_t treatments.

Transrectal ovarian ultrasonography was performed daily beginning on Day 0 of synchronization treatment and continued until ovulation. Ovarian follicular populations, NFWE, size of the largest and second largest (subordinate) follicle, follicle growth rate, size of the dominant follicle at deviation, and size of the dominant follicle at 24, 48, and 72 h after CIDR removal and at estrus/ovulation were determined. Because the full protocols for Bee Synch I and II utilize 66 h for FTAI, size of the largest follicle at 66 h was estimated in the current study based on the rate of growth between 48 and 72 h. This extrapolation was performed to eliminate additional stress that would be imposed by an additional ultrasound examination, as well as our belief that the trajectory of growth determined between 48 and 72 h provided a reasonable means for estimating follicle size at 66 h.

After CIDR removal, cows were observed for estrus 3 times daily beginning at the time of CIDR removal and continuing until ovulation or for 13 days maximum. Estrous detection was aided by use of Estrotect estrus-detection patches (Estrotect, Spring Valley, WI) and an androgenized cow. Estrus was confirmed when the cow stood to be mounted and/or the Estrotect patch had become fully activated by an undetected standing mount. Upon detection of estrus, each cow was artificially inseminated to facilitate overall management of the herd, but pregnancy outcome was not part of experimental variables associated with this study.

Blood samples were collected daily by caudal venipuncture to monitor serum concentrations of P4. Additional blood samples were obtained on Day 0 of estrous synchronization treatments at 0, 30, 60, 120, and 240 min after GnRH or saline injection for analysis of LH. Samples were placed on ice immediately after collection. Before centrifugation, samples were allowed to clot at room temperature for 3 h. Serum were stored at -20 °C until hormone analysis by RIA.

2.4. Hormone assays

Serum concentrations of P4 and LH were measured by RIA. A coated tube radioimmunoassay (MP Biomedicals (Santa Ana, CA) was modified and validated for assay of bovine serum P4 (i.e., standards made in fetal bovine serum due to a matrix effect) in a single assay. Assay sensitivity was 0.2 ng/mL. Recovery of added mass for high (10 ng/mL), mid (5 ng/mL), and low (1 ng/mL) references was 113.9, 117.7, and 104.6%, respectively. The intraassay CV was 6.7%. For LH, a previously validated RIA (McVey and Williams, 1991) was used except that rabbit anti-ovine LH (AFP-192279; Dr. A.F. Parlow, National Pituitary and Hormone Program, Harbor-UCLA Medical Center, Torrance, CA) was used as the first antibody. The sensitivity of the assay was 0.1 ng/mL, and intra- and inter assay CV were 8.8 and 5.8%, respectively.

2.5. Statistical analyses

One cow was eliminated from the study in the Bee Synch I_t treatment group due to a leg injury sustained in the chute on day 2 after treatment onset. Therefore, the number of cows in this group was reduced to 35. Statistical analysis of data was conducted using JMP Statistical Discovery™ (JMP Pro 12; SAS Inst. Inc., Cary, NC) and Statistical Analysis System Software (SAS Software®; SAS 9.3; SAS Inst. Inc., Cary, NC). Hormone concentrations of LH and P4, and follicle size were analyzed as for a 3 × 2 factorial with repeated measures using PROC MIXED of SAS, with treatment, day post ovulation, replication, and all possible interactions included in the model. Cows were included as a random effect, and time was included as the repeated variable. Non-repeated variables, BW, BCS, DPP, size of follicle ovulating during pre-synchronization; size of the largest follicle on day 0 of treatment within day of cycle; follicle size at NFWE, at CIDR removal, and at 24, 48 and 66 and 72 h were analyzed in JMP by one-way ANOVA, including all appropriate interactions. For all preceding analyses, non-significant interactions were removed from the final statistical model. As noted earlier, follicle size at 66 h was estimated based on growth rate between 48 and 72 h. Interval to NFWE, interval from CIDR removal to ovulation, and daily growth rate of the largest follicle were analyzed in JMP by one-way ANOVA, including all appropriate interactions. *Post-hoc* comparison of means was performed with the Tukey Procedure. The effects of treatment and groups, and their interaction, on categorical data (ovulatory responses to GnRH-1, NFWE, and frequency of estrus) were analyzed in JMP using Fisher's Exact Test.

3. Results

There were no significant treatment x replicate, day x replicate, or treatment x day x replicate interactions for any variable measured. Follicle data are summarized in Table 1. Mean size of the largest follicle did not differ between Bee Synch I_t and II_t at the pre-synchronization ovulation, at onset of treatments, at NFWE, at CIDR removal or at 24, 48 and 66 h after CIDR removal. Daily growth rate of the largest follicle was 1.3 ± 0.1 mm ($P = 0.93$). However, size of the largest follicle was less ($P < 0.05$) in cows in which estrous synchronization began on day 3 across all time points measured in both treatments (Table 1). Size of the largest follicle at 66 h after CIDR removal was 13.5 ± 0.3 and was not affected by treatment or day of the estrous cycle at treatment onset.

Ovarian and reproductive outcomes in response to GnRH-1 or saline on Day 0 are presented in Table 2. Ovulation in response to GnRH-1 was observed in only five cows (14.3%) and was unaffected by treatment or day of the cycle. Follicle regression after GnRH-1 was detected in 63/71 (88.7%) cows and did not differ between treatments or day of estrous cycle. In 4.2% of the cows, no ovarian follicular response was observed.

The frequency of synchronized NFWE was greater in Bee Synch I_t regardless of whether NFWE was considered as occurring between 1 to 4 days after GnRH-1 or saline ($P = 0.01$) or between days 0 to 4 ($P = 0.02$; see Discussion). Mean interval from the onset of treatments to NFWE was 1.8 ± 0.3 (Bee Synch I_t) and 2.2 ± 0.3 days (Bee Synch II_t) and did not differ. Cows treated with Bee Synch II_t on day 3 of the estrous cycle had a greater ($P < 0.05$) interval to NFWE than cows treated with Bee Synch I on day 3. Cows that did not have a synchronized NFWE after GnRH-1 or saline on day 0 eventually ovulated the follicle that was present at

Table 1
Follicle diameter (mm) in Bee Synch I_t ($n = 35$; GnRH on Day 0) and II_t ($n = 36$; no GnRH on Day 0) treatments (TRT) initiated on different days post-ovulation (DPO), including size at the time of presynchronized ovulation (Presynch), time of initiation of treatments (Day 0), day of emergence of the new follicle wave (NFWE), time following CIDR removal, and just prior to the synchronized ovulation (OV).

	Day of cycle	Follicle size (mm)			Follicle size (mm) relative to CIDR removal (h)				
		Pre - Synch	Day 0	NFWE	0	24	48	66	OV
Bee Synch I_t	3	14.6	9.5b	4.8	8.0^{b}	9.3^{cd}	10.9^{bc}	11.5^{c}	15.5
	7	15.2	12.4^{a}	5.1	10.6^{a}	11.6^{bc}	12.9^{ab}	13.5^{bc}	14
	10	15.8	11.6^{a}	5.7	12.0^{a}	13.0^{ab}	14.4^{a}	15.2^{ab}	15.4
Bee Synch II_t	3	14.4	9.3^{b}	5.6	6.8^{b}	8.1^{d}	9.8^{c}	10.5^{d}	13.3
	7	16	12.1^{a}	5.1	11.0^{a}	12.1^{ab}	13.2^{ab}	14.1^{ab}	14.4
	10	14.4	11.8^{a}	5.6	12.9^{a}	14.1^{a}	15.1^{a}	16.1^{a}	15.1
Bee Synch I_t	Mean	15.2	11.1	5.2	10.2	11.3	12.7	13.4	14.9
Bee Synch II_t	Mean	14.9	11.1	5.4	10.6	11.5	12.7	13.6	14.3

Means with different superscripts within each column differ ($P < 0.05$).

Table 2
Ovarian synchronization outcomes for Bee Synch I_t and II_t treatments starting at each day post-ovulation.

Item	Treatments	
Follicle response to GnRH-1 or saline, No. (%):	Bee Synch I_t (n = 35; GnRH Day 0)	Bee Synch II_t (n = 36; No GnRH Day 0)
Ovulation	5 (14.3)	–
Regression	29 (82.8)	34 (94.4)
No response	1 (2.9)	2 (5.6)
New Follicular Wave Emergence (NFWE), No. (%):		
Synchronized NFWE, day 1 – 4	24 (68.6)[a]	14 (38.9)[b]
No synchronized NFWE, days 1 – 4	11 (31.4)[a]	22 (61.1)[b]
Synchronized NFWE, days 0 – 4	33 (94.3)[a]	26 (72.2)[b]
No NFWE, days 0 – 4	2 (5.7)[a]	10 (27.8)[b]
No response to GnRH-1	1 (2.9)	N/A
Interval to NFWE (days) with treatments initiated on different days post-ovulation:		
day 3	3.0[a]	5.0[b]
day 7	1.8	1.1
day 10	0.6	0.4
CL regression after PGF on day 5, No. (%):	35 (100)	36 (100)
Interval from CIDR removal to ovulation (days) with treatments initiated on different days of the estrous cycle:		
day 3	6.6 ± 0.40	6.1 ± 0.36
day 7	4.6 ± 0.38	4.0 ± 0.38
day 10	4.3 ± 0.38	3.2 ± 0.36
Estrus (%)	30 85.7)	34 (94.4)

Means with different superscripts within rows represent a difference ($P < 0.05$).

onset of treatment following CIDR removal.

All cows (n = 71) had serum concentrations of P4 below 1 ng/mL 1 day after PGF treatment at CIDR removal (Day 5). Days from CIDR removal to ovulation did not differ by treatment but differed ($P < 0.0001$) due to day of the estrous cycle at onset of treatment (Table 2). Ovulations detected earlier than 72 h after CIDR removal averaged 5.6% (n = 4/35) in Bee Synch I_t and 15.5% (n = 11/36) in Bee Synch II_t ($P = 0.06$) and all were observed when treatments began on day 10 of the cycle. Frequency of estrus did not differ ($P = 0.21$) due to treatment but the interval to estrus differed due to treatment ($P = 0.05$) and day of the estrous cycle ($P < 0.0001$; Fig. 2).

Serum concentrations of P4 are illustrated in Fig. 3. Mean P4 during the estrous synchronization period and until CIDR removal did not differ ($P = 0.12$) by treatment. An apparent incomplete lysis of the CL after PGF on Day 5 resulted in a rebound in P4 concentrations above 1 ng/mL in 5 cows treated with Bee Synch I_t. These five represented the cows that had ovulations in response to GnRH-1 on Day 0. The effect of this on variability in serum P4 can be seen in Fig. 3 from days 8 to 13 after the onset of treatment (3 days after PGF treatment).

The timing and growth of follicles relative to follicle deviation was assessed in 49 cows (Fig. 4) that had NFWE following

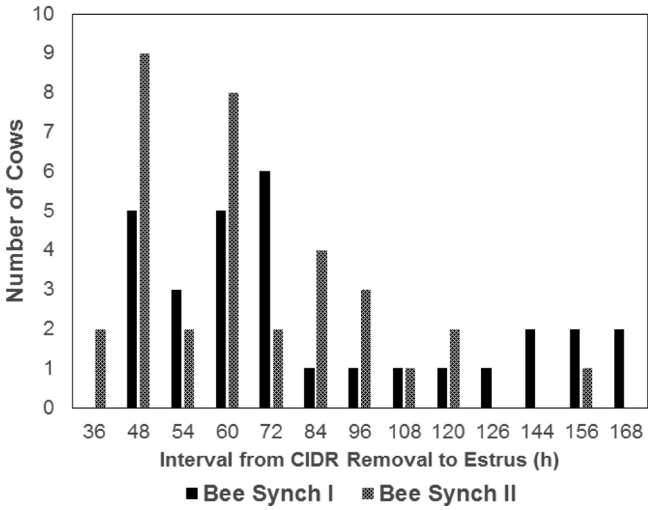

Fig. 2. Interval (h) from controlled internal drug-releasing device (CIDR) removal to detection of behavioral estrus with Bee Synch It (n = 30; GnRH Day 0) and Bee Synch II (n = 34; No GnRH Day 0), respectively.

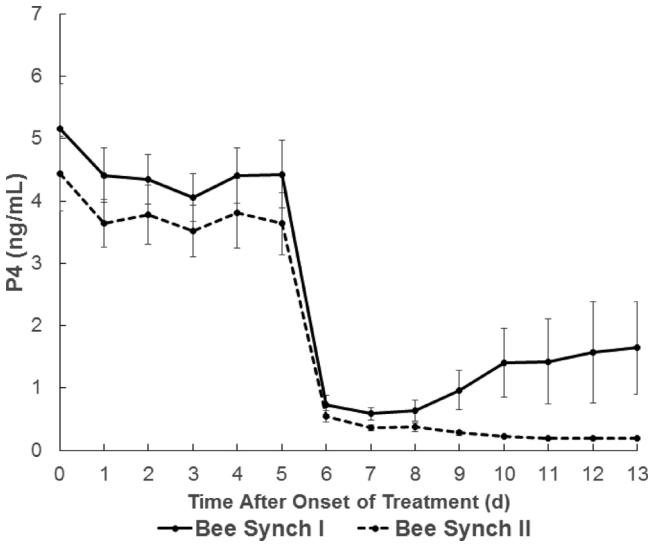

Fig. 3. Serum concentration of P4 in Bee Synch I_t (n = 35; GnRH Day 0) and Bee Synch II_t (n = 36; No GnRH Day 0) from treatment onset (Day 0) until ovulation or a maximum of 13 days, whichever occurred first; Mean P4 did not differ due to treatment.

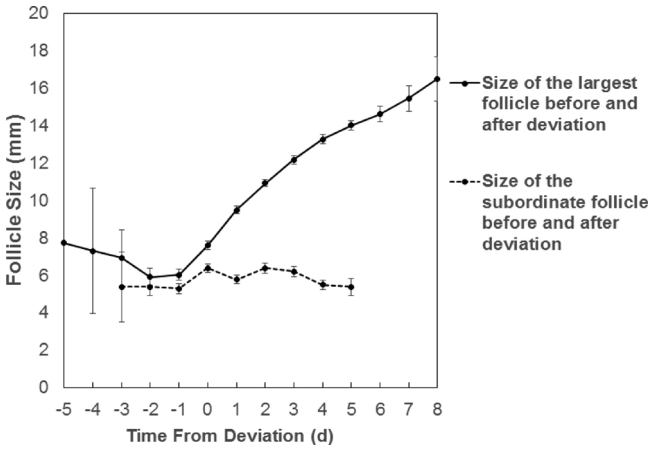

Fig. 4. Largest follicle diameter and largest subordinate follicle diameter normalized at day of deviation.

treatment onset and that had not already established dominance. Because day of deviation did not differ due to treatment, day of estrous cycle, or replicate, data are presented as pooled means. Deviation of the dominant follicle was observed 3.3 ± 0.13 days after emergence and averaged 7.61 ± 0.23 mm the day before and 9.5 ± 0.2 mm on the day of deviation. Size of the largest subordinate follicle on the day before deviation was 6.39 ± 0.23 mm and averaged 5.78 ± 0.22 mm on the day of deviation.

Mean serum concentration of LH following treatment with GnRH-1 (Bee Synch I_t) or saline (Bee Synch II_t) are illustrated in Fig. 5. As expected, mean concentrations were greater ($P < 0.0001$) after GnRH in Bee Synch I_t than for Bee Synch II_t after saline. Peak serum concentrations of LH at 120 min after GnRH injection on day 3 of the estrous cycle were greater (4.28 ± 0.71 ng/mL; $P < 0.05$) than on days 7 (2.70 ± 0.23 ng/mL) and 10 (1.86 ± 0.22 ng/mL) of the cycle, respectively.

4. Discussion

Results of the current experiment indicate that, although GnRH-1 resulted in synchronized NFWE in 68.6% of Bee Synch I_t protocol between 1and 4 days after injection, compared to 38.9% for the Bee Synch II_t protocol, its use failed to increase size of the dominant follicle or frequency of estrus at 66 h after CIDR removal, or to decrease mean interval to ovulation. Furthermore, greater than 90% of Bee Synch I_t-treated cows had a synchronized NFWE if this interval was expanded to include days 0–4 after treatment, compared to 72.2% for Bee Synch II_t.

Results in earlier reports from our group indicate that use of protocols such as Ovsynch (Williams et al., 2002), 7-Day CO-Synch + CIDR (Saldarriaga et al., 2007; Zuluaga et al., 2010), and 5-Day CO-Synch + CIDR (Williams et al., 2011, 2013) fail to yield consistent and acceptable FTAI pregnancy rates in *Bos indicus*-influenced beef cows. This is in contrast to results in straightbred *Bos*

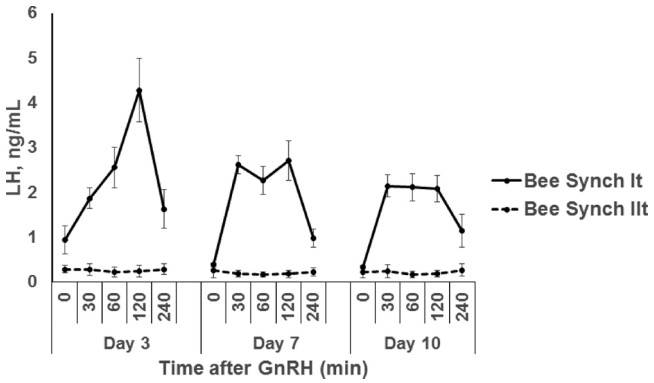

Fig. 5. Serum concentration of luteinizing hormone (LH) at 0, 30, 60, 120, and 240 min after GnRH or saline injection in Bee Synch I_t ($n = 35$; GnRH Day 0) or Bee Synch II_t ($n = 36$; No GnRH Day 0) treatments, respectively; Cows on day 3 post-ovulation had a greater ($P < 0.05$) induced release of LH than cows on days 7 or 10 post-ovulation; Mean concentrations of LH were greater ($P < 0.0001$) in Bee Synch I_t (received GnRH) than Bee Synch II (received saline).

taurus cows where FTAI pregnancy rates of 50% or greater have been reported consistently, as reviewed by Lamb and Mercadante (2016). Thus, modifications were made in the 5-Day CO-Synch + CIDR regimen to address this issue and initially included the addition of PGF on Day 0 of the protocol (Bee Synch I; Williams et al., 2011, 2013) to presumptively lower overall circulating P4 in a majority of cycling cows during the estrous synchronization period. We had hypothesized that *Bos indicus*-influenced cattle might be more sensitive to the negative feedback effects of P4 on secretion of LH, thus delaying maturation of the dominant follicle. Use of this strategy (Bee Synch I) resulted in FTAI pregnancy rates of 50% or greater relatively consistently in these types of cattle using a CIDR in combination with GnRH and PGF (Williams et al., 2011). Those studies were followed by preliminary field trials in which Bee Synch I_t was modified further by eliminating GnRH on Day 0. By default, the latter obviated the need for the double dose of PGF at Day 5 (Bee Synch II; Williams et al., 2015), since no new CL were induced by GnRH on Day 0. The basis of this modification was from earlier observations (Saldarriaga et al., 2007; Zuluaga et al., 2010) that ovulation rates to GnRH in mature, randomly estrous-cycling *Bos indicus*-influenced cows were often low and highly variable. Therefore, we tested the hypothesis that FTAI pregnancy rates would be similar between Bee Synch I (full protocol) and Bee Synch II (modified to eliminate GnRH on Day 0). This hypothesis was similar to that of Cruppe et al. (2014) in which elimination of GnRH on Day 0 in the standard 5-day CO-Synch + CIDR regimen using straight *Bos taurus* heifers resulted in pregnancy rates that did not differ from the original regimen that included GnRH. Although no statistically significant differences in FTAI pregnancy rates between Bee Synch I and II protocols have been observed in preliminary field trials, intensive studies to examine ovarian events associated with onset of treatments relative to stage of the follicle wave/estrous cycle have not been performed. Indeed, the inclusion of GnRH-1 in the current study resulted in measurable effects on synchrony of NFWE during the first 4 days after treatment onset. However, at 66 to 72 h after CIDR removal, the time at which mature cows estrous synchronized with both Bee Synch I and II are inseminated in a FTAI setting, there were no measurable differences due to treatment in follicle characteristics known to affect FTAI pregnancy rates.

Research of Kojima et al. (2000); DeJarnette et al. (2001), and Saldarriaga et al. (2007) inferred that synchronization of NFWE is related to ovulation response after GnRH-1. In the research of Saldarriaga et al. (2007) with *Bos indicus*-influenced beef cows, ovulation rate was 40% and estrous synchronization rate was greater in cows having ovulations after GnRH-1 administration (88%) compared to those not having ovulations (42%). Vasconcelos et al. (1999) suggested that ovulation rates following GnRH in growing, dominant, and regressing phases of dominant follicles (days 3, 7, and 10 of the cycle in the present experiment) vary because of differences in ovulatory capacity related to the number of LH receptors. In this previous experiment, administration of GnRH resulted in ovulation in 23%, 96%, 54% and 77% of cows when treatment was administered on days 1–4, 5–9, 10–16, and 17–21 of the estrous cycle, respectively. Sartori et al. (2001) made similar observations, reporting a low rate of ovulation for day 3 (0%) and a high rate of ovulation for day 6 (100%) of the estrous cycle, respectively. Conversely, in *Bos taurus* beef heifers, Martinez et al. (1999) administered GnRH on days 3, 6 and 9 of the estrous cycle and obtained ovulation in 89%, 56% and 22%, respectively. Martinez et al. (1999) indicated that all heifers with a dominant follicle larger than 9 mm ovulated in response to GnRH, which supports more recent work by Ginther et al. (2016) in which follicle deviation was shown to occur at a diameter of 8.5 mm, and by Sartori et al. (2001), where ovulatory capacity was achieved immediately after deviation. In our study, mean largest follicle size on Day 0 of treatment onset for Bee Synch I_t (GnRH-1) was 11.1 mm ± 0.2 and deviation of the synchronized NFW in both treatments was achieved at 9.5 ± 0.2 mm. Therefore, ovulation after GnRH-1 treatment was expected to occur in cows with follicles larger than 9.5 mm in diameter already present at treatment onset.

Sartori et al. (2001) has also indicated that ovulatory capacity is dependent upon both the amount of LH released and diameter of the dominant follicle. The mean peak concentration of LH observed at 120 min after GnRH-1 on day 10 of the estrous cycle in Bee Synch I_t-treated cows in the current study was 1.86 ng/mL compared to 4.3 ng/mL on day 3 of the estrous cycle. Such differences are consistent with observations by Stevenson and Pulley (2016) where follicle size, presence of a CL, and concentrations of estradiol and P4 had major effects on the magnitude of GnRH-induced release of LH in lactating dairy cows. In addition, maximum concentrations of LH observed following GnRH in the current study were less than overall mean values observed previously (Saldarriaga et al.,

2007), which may explain the relatively lesser ovulation response after GnRH-1 in this study. Interestingly, the lack of any ovarian response to GnRH-1 in the current study was 10-fold less (2.9%) than that reported by Zuluaga et al. (2010; ˜29%), as well as Saldarriaga et al. (2007) and Barros et al. (2000) where about 21% of *Bos indicus*-influenced cows failed to have any follicular response to GnRH-1 on Day 0 of 7-day CO-Synch + CIDR protocols. Nonetheless, estrous-synchronized NFWE after GnRH-1 or saline in the present experiment was quite high (72% for days 1–4 and 94.3% for days 0–4 after treatment), which is consistent with the findings of Twagiramungu et al. (1995) and Martínez et al. (2000), both of which indicated that both follicle regression and ovulation are equally effective for inducing NFWE.

Mean size of the dominant follicle at 66 h after CIDR removal for cows in the present study was always greater than 11.5 mm except for individuals starting treatment on day 3 of the cycle, regardless of treatment. Sartori et al. (2001) reported that 11.5 mm is the apparent threshold size for optimal fertility after ovulation in Holstein cows. This observation is consistent with the findings of Perry et al. (2005), who reported that induction of ovulation in follicles smaller than 11 mm resulted in a significant lowering of pregnancy rates in *Bos taurus* females. Lowered fertility as a consequence of smaller follicles occurs because the oocyte is physiologically-immature, resulting in increased embryonic loss. Moreover, because smaller follicles generate smaller CL, concentrations of P4 are reduced during the luteal phase. Thus, based on dominant follicle size, it is possible to infer that cows in the present experiment could have been induced to ovulate with GnRH at 66 h after CIDR removal without negative effects on ovulation rate or fertility, except for cows starting Bee Synch II treatment on day 3 of the estrous cycle.

New follicular wave emergence is associated closely with the stage of the estrous cycle (Sirois and Fortune, 1988). As indicated by results from the present experiment, cows that had regression of the largest follicle on Day 0 of treatment onset had follicle wave characteristics that were consistent with those described by Sirois and Fortune (1988) and Ginther et al. (1989). Based on these previous observations, NFWE occurs approximately on the day of ovulation and again on about the 10th day of the estrous cycle in cows with two follicular waves and on days 0, 9, and 15 of the estrous cycle for cows with three follicular waves. Cows with initiation of Bee Synch I_t or II_t treatments 3 days after ovulation had a delayed occurrence of NFWE at approximately 4.3 ± 0.3 days, which represents day 7.3 ± 0.3 of the estrous cycle. These responses are markedly consistent with the timing of the natural second follicular wave. The same pattern was observed for cows beginning both treatments on day 7, where NFWE occurred on average at 1.4 ± 0.2 days after treatment onset. This coincides with day 8.4 ± 0.2 of the estrous cycle. For day 10 of the estrous cycle, average interval to NFWE was 0.5 days.

Ovulation after CIDR removal was detected in 21% of all cows (15/71) before 72 h (average of 68 ± 4 h), of which 80% (12/15) occurred in cows starting treatment on day 10 of the estrous cycle and 83.3% (10/12) of these were observed in Bee Synch II_t-treated cows. This suggests that treatment with GnRH at treatment onset in Bee Synch I_t reduced the incidence of earlier ovulation after CIDR removal, which could have a negative impact on fertility by loss of oocyte viability if FTAI is conducted at 72 h. This risk can potentially be decreased at the proposed FTAI at 66 h after CIDR removal.

Overall mean concentrations of serum P4 during CIDR insertion for both treatments were similar, followed by a decline to below 1 ng/mL in all cows after CIDR removal and PGF. Because either naturally-occurring ovulations or ovulations induced by GnRH on Day 0 of 5- day protocols result in CL that do not respond to standard doses of PGF on Day 5, studies have been conducted to determine efficacy of different doses and split application. Nascimento et al. (2014) tested single (25 mg), double (50 mg), and double-split (two 25-mg injections 8 h apart) injection of PGF on Day 5 in Holstein cows. Results showed a dramatic superiority in efficacy of either the double or double-split doses of PGF compared to the single dose for regressing 5-day CL. In field trials, Rabaglino et al. (2010) observed no difference in pregnancy rate of dairy heifers estrous-synchronized using either single or double doses of PGF at Day 5 with the standard 5-day protocol. However, incomplete luteolysis has also been reported even when the double dose (50 mg of PGF) is applied (Nascimento et al., 2014). In the current study, because there was no induction of ovulation with GnRH after CIDR removal, most cows did not ovulate for 4–7 days after CIDR removal. Thus, serum concentrations of P4, on average, remained low during the blood sampling period following CIDR removal and leading up to spontaneous ovulation. However, serum P4 in the five cows that ovulated after GnRH-1 in the Bee Synch I_t group, while decreasing to < 1 ng/ml initially, had a resurgence beginning 2 days after CIDR removal and PGF. Thus, as observed in Fig. 3, it appears that a suboptimal response to PGF in these 5-day-old CL resulted in incomplete functional luteolysis (Lauderdale et al., 1974; Macmillan, 1983). Nonetheless, the temporary 3-day suppression of P4 in those 5 cows permitted final maturation of the dominant follicle and subsequent ovulation of an ovulatory follicle. Therefore, cows in this category would actually bear 2 CL following the synchronized ovulation, both of which would be maintained if cows were bred and became pregnant.

In summary, results of the present experiment indicate that greater synchronization of NFWE and a reduced incidence of early ovulations with use of the Bee Synch I_t protocol provide a mechanistic basis for improved fertility at FTAI using Bee Synch I vs. II. However, in spite of these observations, GnRH-1 did not enhance the synchronized development of a dominant follicle at 66 h after CIDR removal, the time at which FTAI is employed for both Bee Synch I and II in *Bos indicus*-influenced beef cows. Consistent with these findings, no differences in FTAI pregnancy rates between Bee Synch I and II have been observed to date. A full report of field data comparing the two methodologies is in preparation (Williams and Stanko, unpublished).

Conflict of interest statement

Authors declare no conflicts of interest.

Acknowledgements

We acknowledge the support of Zoetis Animal Health, Estrotect, Select Sires and Texas A&M AgriLife Research. The assistance of Randle Franke and Ernest Soto is also gratefully acknowledged.

References

Barros, C.M., Moreira, M.B.P., Figueiredo, H.A., Teixeira, A.B., Trinca, L.A., 2000. Synchronization of ovulation in beef cows (*Bos indicus*) using GnRH, PGF2 alpha and estradiol benzoate. Theriogenology 53, 1121–1134.

Baruselli, P.S., Reis, E.L., Marques, M.O., Nasser, L.F., Bó, G.A., 2004. The use of hormonal treatments to improve reproductive performance of anestrus beef cattle in tropical climates. Anim. Reprod. Sci. 82-83, 479–486.

Bó, G.A., Adams, G.P., Nasser, L.F., Pierson, R.A., Mapletoft, R.J., 1993. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 40, 225–239.

Bridges, G.A., Helser, L.A., Grum, D.E., Mussard, M.L., Gasser, C.L., Day, M.L., 2007. Decreasing the interval between GnRH and PGF2a from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 69, 843–851.

Colazo, M.G., Kastelic, J.P., Mapletoft, R.J., 2003. Effects of estradiol cypionate (ECP) on ovarian follicular dynamics, synchrony of ovulation, and fertility in CIDR-based, fixed-time AI programs in beef heifers. Theriogenology 60, 855–865.

Cruppe, L.H., Day, M.L., Abreu, F.M., Kruse, S., Lake, S.L., Bielh, M.V., Cipriano, R.S., Mussard, M.L., Bridges, G.A., 2014. The requirement of GnRH at the beginning of the five-day CO-synch + controlled internal drug release protocol in beef heifers. J. Anim. Sci. 92, 4198–4203.

DeJarnette, J.M., Day, M.L., House, R.B., Wallace, R.A., Marshall, C.E., 2001. Effect of GnRH pretreatment on reproductive performance of postpartum suckled beef cows following synchronization of estrus using GnRH and $PGF_2\alpha$. J. Anim. Sci. 79, 1675–1682.

Ginther, O.J., Knopf, L., Kastelic, J.P., 1989. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J. Reprod. Fertil. 87, 223–230.

Ginther, O.J., Kot, K., Kulick, L.J., Wiltbank, M.C., 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. Theriogenology 48, 75–87.

Ginther, O.J., Baldrighi, J.M., Siddiqui, M.A.R., Araujo, E.R., 2016. Complexities of follicle deviation during selection of a dominant follicle in *Bos taurus* heifers. Theriogenology 86, 2012–2019.

Kojima, F.N., Wood, S.L., Smith, M.F., Patterson, D.J., 2000. Does pretreatment with GnRH prior to a GnRH-$PGF_2\alpha$ (PG) protocol improve synchronization of estrus in beef cattle? J. Anim. Sci. 78 (Suppl. 1), 210.

Lamb, G.C., Mercadante, V.R., 2016. Synchronization and artificial insemination strategies in beef cattle. Vet. Clin. North Am. Food Anim. Pract. 32, 335–347.

Lamb, G.C., Dahlen, C.R., Larson, J.E., Marquezini, G., Stevenson, J.S., 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. J. Anim. Sci. 88 (Suppl. 13), E181–92.

Lauderdale, J.W., Seguin, B.E., Stellflug, J.N., Chenault, J.R., Thatcher, W.W., Vincent, C.K., Loyancano, A.F., 1974. Fertility of cattle following PGF2 alpha injection. J. Anim. Sci. 38, 964–967.

Macmillan, K.L., 1983. Prostaglandin responses in dairy herd breeding programmes. N. Z. Vet. J. 31, 110–113.

Martinez, M.F., Adams, G.P., Bergfelt, D.R., Kastelic, J.P., Mapletoft, R.J., 1999. Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. Anim. Reprod. Sci. 57, 23–33.

Martínez, M.F., Adams, G.P., Kastelic, J.P., Bergfelt, D.R., Mapletoft, R.J., 2000. Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology 54, 757–769.

Martínez, M.F., Kastelic, J.P., Bó, G.A., Caccia, M., Mapletoft, R.J., 2005. Effects of oestradiol and some of its esters on gonadotrophin release and ovarian follicular dynamics in CIDR-treated beef cattle. Anim. Reprod. Sci. 86, 37–52.

McVey, W.R.Jr., Williams, G.L., 1991. Mechanical masking of neurosensory pathways at the calf-teat interface: endocrine, reproductive and lactational features of the suckled anestrous cow. Theriogenology 35, 931–941.

Nascimento, A.B., Souza, A.H., Keskin, A., Sartori, R., Wiltbank, M.C., 2014. Lack of complete regression of the day 5 corpus luteum after one or two doses of PGF2a in nonlactating Holstein cows. Theriogenology 81, 389–395.

NRC, 1996. Nutrient Requirements of Beef Cattle, 7th ed. Nat. Acad. Press, Washington, DC.

Perry, G.A., Smith, M.F., Lucy, M.C., Green, J.A., Parks, T.E., MacNeil, M.D., Roberts, A.J., Geary, T.W., 2005. Relationship between follicle size at insemination and pregnancy success. Proc. Natl. Acad. Sci. U. S. A. 102, 5268–5273.

Pursley, J.R., Mee, M.O., Wiltbank, M.C., 1995. Synchronization of ovulation in dairy cows using PGF2a and GnRH. Theriogenology 44, 915–923.

Rabaglino, M.B., Risco, C.A., Thatcher, M.J., Kim, I.H., Santos, J.E.P., Thatcher, W.W., 2010. Application of one injection of prostaglandin F2a in the five-day Co-synch plus CIDR protocol for estrous synchronization and resynchronization of dairy heifers. J. Dairy Sci. 93, 1050–1058.

Saldarriaga, J.P., Cooper, D.A., Cartmill, J.A., Zuluaga, J.F., Stanko, R.L., Williams, G.L., 2007. Ovarian, hormonal and reproductive events associated with synchronization of ovulation and timed appointment breeding of *Bos indicus* influenced cattle using intravaginal progesterone. J. Anim. Sci. 85, 151–162.

Sartori, R., Fricke, P.M., Ferreira, J.P.C., Ginther, O.J., Wiltbank, M.C., 2001. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol. Reprod. 65, 1403–1409.

Sirois, J., Fortune, J.E., 1988. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol. Reprod. 38, 308–317.

Stevenson, J.S., Pulley, S.L., 2016. Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. J. Dairy Sci. 99, 3003–3015.

Twagiramungu, H., Guilbault, L.A., Dufour, J.J., 1995. Synchronization of ovarian follicular waves with a gonadotropin-releasing hormone agonist to increase the precision of estrus in cattle: a review. J. Anim. Sci. 73, 3141–3151.

Vasconcelos, J.L.M., Silcox, R.W., Rosa, G.J., Pursley, J.R., Wiltbank, M.C., 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 55, 1067–1078.

Williams, S.W., Stanko, R.L., Amstalden, M., Williams, G.L., 2002. Comparision of three approaches for synchronization of ovulation for timed artificial insemination in *Bos indicus*-influenced cattle managed on the Texas gulf coast. J. Anim. Sci. 80, 173–1178.

Williams, G.L., Stanko, R.L., Allen, C.C., Cardoso, R.C., Presotto, L.D., Thorson, J.F., Amstalden, M., 2011. Evidence that luteal regression at the onset of a 5-Day CO-Synch + CIDR synchronization protocol markedly improves fixed-time AI pregnancy rates in *Bos indicus*-influenced cattle. J. Anim. Sci. 89, 251 (Abstr.).

Williams, G.L., Stanko, R.L., Amstalden, M., 2013. Bee synch for successful fixed-time AI of Brahman-influenced cattle. Proc. 59th Ann. Texas A&M Beef Cattle Shortcourse. pp. G6–G9.

Williams, G.L., Stanko, R.L., Amstalden, M., 2015. Evolving concepts in the development and application of synchronization protocols for fixed-time AI in *Bos indicus*-influenced cattle. Bee Synch I and II. Proc. 61st Ann. Texas A&M Beef Cattle Shortcourse. pp. E18–E21.

Zuluaga, J.F., Saldarriaga, J.P., Cooper, D.A., Cartmill, J.A., Williams, G.L., 2010. Presynchronization with gonadotropin-releasing hormone increases the proportion of *Bos indicus*-influenced females ovulating at initiation of synchronization but fails to improve synchronized new follicular wave emergence or fixed-time artificial insemination conception rates using intravaginal progesterone, gonadotropin-releasing hormone, and prostaglandin F2a. J. Anim. Sci. 88, 1663–1671.