Prostaglandin F_{2α} and gonadotropin-releasing hormone administration improve progesterone status, luteal number, and proportion of ovular and anovular dairy cows with corpora lutea before a timed artificial insemination program¹

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ABSTRACT

The objective of this research was to increase the proportion of cows with at least 1 functional corpus luteum (CL) and elevated progesterone at the onset of the timed artificial insemination (TAI) protocol. Postpartum Holstein cows in one herd were stratified by lactation number at calving (September 2009 through August 2010) and assigned randomly to 2 treatments: 1) Presynch-10 (n = 105): two 25-mg injections of PGF_{2α} (PG) 14 d apart (Presynch); and 2) PG-3-G (n = 105): one 25-mg injection of PG 3 d before 100-μg GnRH (Pre-GnRH) injection, with the PG injection administered at the same time as the second PG injection in the Presynch-10 treatment. Cows were enrolled in a TAI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PG injection with AI 16 to 18 h after GnRH-2] 10 d after the second or only PG injection. Blood samples for progesterone or estradiol analyses were collected on median days in milk (DIM): 36, 39, 50, 53 (Pre-GnRH), 60 (GnRH-1), 67 (PG), 69 (GnRH-2), and 70 (TAI). Ovarian structures were measured by ultrasonography on median DIM 53, 60, 67, 69, and 6 d post-TAI to determine follicle diameters, ovulation response to GnRH, or both. Although progesterone concentration did not differ between treatments before Pre-GnRH injection, the proportion of cows with at least 1 CL tended to be greater for PG-3-G than Presynch-10 cows, and more PG-3-G cows ovulated after Pre-GnRH injection than ovulated spontaneously in Presynch-10. Further, the diameter of follicles that ovulated tended to be smaller in PG-3-G than in Presynch-10 cows after Pre-GnRH injection. At GnRH-1, the proportion of cows with progesterone ≥1 ng/mL, the number of CL per cow, and the proportion of cows with at least 1 CL were

greater for PG-3-G than Presynch-10. Neither follicle diameter nor percentage of cows ovulating after GnRH-1 differed between treatments. At PG injection during the week of TAI, progesterone concentration and the proportion of cows with progesterone ≥1 ng/mL tended to be greater for PG-3-G than Presynch-10, and PG-3-G had more CL per cow than Presynch-10. No ovarian characteristics differed between treatments after GnRH-2, including progesterone concentration, number of CL per cow, and total luteal volume 7 d after GnRH-2. Many of the previous ovarian traits were improved in both ovular and anovular cows after PG-3-G compared with Presynch-10. Pregnancies per AI at d 32 and 60 were only numerically greater for PG-3-G than for Presynch-10 cows, largely because of differences detected during months without heat stress. We concluded that the PG-3-G treatment increased ovulation rate and luteal function 7 d before the onset of Ovsynch, resulting in improved follicular synchrony and predisposing potentially greater pregnancies per AI in lactating dairy cows.

Key words: luteal function, ovulation, presynchronization, progesterone

INTRODUCTION

Timed artificial insemination (TAI) programs facilitate control of reproductive cycles in lactating dairy cattle and provide viable options to AI programs solely based on detection of estrus. Preliminary studies formed the foundation for developing a TAI program when GnRH administered to control follicle waves was followed in 6 d (Twagiramungu et al., 1995) or 7 d by PGF_{2α} (PG) to regress functional luteal tissue in a coordinated fashion (Thatcher et al., 1989; Pursley et al., 1995). According to a survey of 103 Alta Genetics progeny-test herds (Caraviello et al., 2006), development of the Ovsynch protocol [Pursley et al., 1998; injection of GnRH 7 d before (GnRH-1) and 48 h after (GnRH-2) PG with TAI administered 16 h after GnRH-2] led to its adoption in more than 85% of

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these large (>500 cows per herd) US dairy herds. Further, Moeller et al. (2010) recently reported the mean percentage of 231,288 cows inseminated after a TAI protocol was 43.4% and differed among 4 regions of the United States.

Lactating dairy cows treated with the Ovsynch program beginning from d 5 through 12 of the estrous cycle had greater incidences of ovulation and pregnancies per AI (**P/AI**) than cows treated at other stages of the cycle (Vasconcelos et al., 1999). On the basis of the hypothesis that fertility after a TAI program was related to the stage of the estrous cycle (or stage of the first follicular wave), presynchronization of estrous cycles was attempted before the Ovsynch program by using 2 injections of PG administered 14 d apart (**Presynch**). The second Presynch injection given 12 d before the onset of the TAI program (Moreira et al., 2001) resulted in a larger proportion of cows in diestrus at the onset of the TAI program. These cows had greater P/AI than did cows initiating the TAI programs at random stages of the estrous cycle (Moreira et al., 2001), as did cows in subsequent experiments in which estrous cycles were presynchronized after administration of 1 (Cartmill et al., 2001) or 2 presynchronizing injections of PG (El-Zarkouny et al., 2004; Navanukraw et al., 2004). In the latter study, the interval between the second Presynch injection and onset of the TAI program was 14 d rather than 12 d.

Therefore, the interval between the standard second Presynch PG injection and the onset of Ovsynch is important to the stage of estrous cycle or stage of follicular wave in which cows are found at the time of GnRH-1. Assuming that luteolysis occurs from 0 to 5 d after the second Presynch PG injection, intervals of 14 (**Presynch-14**), 12 (**Presynch-12**), 11 (**Presynch-11**), and 10 d (**Presynch-10**) would synchronize most the cows to d 9 to 14, d 7 to 12, d 6 to 11, or d 5 to 10 of the cycle. On the basis of studies in which GnRH was given at various stages of the first follicular wave, intervals of 10 or 11 d (d 5 to 11 of the cycle) should facilitate greater ovulatory responses to GnRH-1 (Vasconcelos et al., 1999; Stevenson, 2011). Moreover, Presynch-14 was concluded to decrease ovulatory responses to the first and second GnRH injections (Galvão and Santos, 2010) and to result in lesser P/AI compared with an 11-d interval (Presynch-11; Galvão et al., 2007).

Other presynchronization schemes tested by injecting PG and GnRH (Peters and Pursley, 2002; Bello et al., 2006) tended to improve P/AI. In addition, applying a nonbreeding Ovsynch protocol before the TAI Ovsynch program (**Double Ovsynch**; Souza et al., 2008) resulted in improved P/AI compared with Presynch-11 in primiparous but not in multiparous cows. Regard-

less of presynchronization scheme, improved P/AI in TAI programs is associated with 1) greater ovulation response to the first GnRH injection of Ovsynch (Vasconcelos et al., 1999; Bello et al., 2006; Chebel et al., 2006), 2) more cows with a functional corpus luteum (**CL**) at the first GnRH injection (Galvão and Santos, 2010), 3) greater ovulatory response to the first and second GnRH injection (Rutigliano et al., 2008), and 4) greater ovulation response after TAI (Galvão and Santos, 2010).

The objectives of the present study were to test which of 2 presynchronization methods administered before a TAI program produced the greatest percentage of cows having a functional CL and elevated progesterone concentrations before enrollment in a TAI program to increase subsequent P/AI. The first treatment selected in which both PG and GnRH were administered before applying the Ovsynch protocol (Peters and Pursley, 2002) is similar to Double Ovsynch, but it excludes the initial GnRH injection of the presynchronization portion of Double Ovsynch. The second treatment represents what should be the best standard Presynch protocol in which the interval from the second Presynch PG injection to the onset of the Ovsynch protocol was decreased from 14 to 10 d. This treatment (Presynch-10) should result in more cows that are earlier in their estrous cycle and first follicular wave (d 5 to 10) at the onset of the Ovsynch protocol than a Presynch-14 program in which most cows would be later (d 9 to 14) in their estrous cycle at the first GnRH (GnRH-1) injection of the TAI program.

MATERIALS AND METHODS

Experimental Cows

Lactating Holstein cows were enrolled at calving from September 2009 through August 2010 at the Kansas State University Dairy Teaching and Research Center (Manhattan). Cows were housed in covered freestalls and fed twice or three times (summer) daily a TMR calculated to meet nutrient requirements for lactating dairy cows producing 50 kg of 3.5% milk (NRC, 2001). The diet consisted of alfalfa hay, corn silage, soybean meal, whole cottonseed, corn or milo grain, corn gluten feed, vitamins, and minerals. Cows were milked every 8 h in a double-6 herringbone milking parlor.

Experimental Design

At calving, 210 cows enrolled in the study were stratified by lactation number (1 vs. 2+) and assigned randomly to receive either of 2 presynchronization

Figure 1. Experimental design of treatments and measurements: at calving, lactating dairy cows were assigned randomly to 2 treatments: 25-mg i.m. injection of PGF_{2α} (PG) 3 d before a 100-μg injection of GnRH (PG-3-G) or 2 injections of PG administered 14 d apart (Presynch-10). Cows received 100 μg of GnRH at Pre-GnRH, GnRH-1, or GnRH-2; 25 mg of PG at Pre-PG, Presynch PG-1, Presynch PG-2, and PG injection. Blood (B) samples were collected from a caudal blood vessel by venipuncture and ovaries were scanned (S) by using transrectal ultrasonography. Positive pregnancy diagnoses required the presence of anechoic uterine fluid and a large corpus luteum or anechoic uterine fluid and presence of a viable embryo. TAI = timed AI. Color version available in the online PDF.

treatments (Figure 1). The first treatment (**PG-3-G**) consisted of a 25-mg i.m. injection of PG (**Pre-PG**; 5 mL of Lutalyse; Pfizer Animal Health, Madison, NJ) 3 d before a 100-μg i.m. injection of GnRH (**Pre-GnRH**; 2 mL of Fertagyl; Merck Animal Health, Whitehouse Station, NJ). The second treatment (Presynch-10) was timed so the second of two 25-mg i.m. injections of PG (5 mL of Lutalyse; Pfizer Animal Health) was administered on the same day as the Pre-PG injection in the PG-3-G treatment (Figure 1). The Ovsynch TAI program was initiated 10 d after either the Pre-PG or Presynch PG-2 injection. Treatment injections were staggered within cluster so that all cows were inseminated on the same day every 2 wk (Figure 1).

At calving, a new breeding cluster of cows was initiated every 2 wk. Body condition scores (Ferguson et al., 1994) were assigned and pregnancies were diagnosed as illustrated in Figure 1. The monthly DHI test-day ECM yield after 60 DIM near the onset of AI was recorded for cows enrolled in the study. Three technicians performed inseminations, with 1 technician conducting more than 85%. Multiple sires were used. Pregnancy diagnosis was conducted by transrectal ultrasonography (5.0 MHz linear-array transducer, Aloka 500V; Corometrics Medical Systems Inc., Wallingford, CT) on d 32 and 60 after TAI. A positive pregnancy outcome required presence of anechoic uterine fluid and a CL ≥ 25 mm in diameter or anechoic uterine fluid and presence of an embryo with a heartbeat.

Ovarian Steroids

Blood samples were collected by puncture of caudal vessels into evacuated tubes as indicated in Figure 1. Concentrations of progesterone in blood serum were measured in all samples by direct quantitative (non-extracted) radioimmunoassay using Coat-A-Count progesterone kits (Catalog no. TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) and validated for bovine serum. The radioligand was 125 I-labeled progesterone (1.5 kBq per tube). Specificity of the antibody (supplied by the manufacturer) was undetectable for androstenediol, estradiol, and pregnane. Specificity of the antibody was less than 3.4% for testosterone, 20 α -dihydroprogesterone, 17 α -hydroxyprogesterone, medroxyprogesterone, 5 β -pregnan-3 α -ol-20-one, 5 β -pregnan-3, 20-dione, 5-pregnen-3 β -ol-20-one-sulfate, pregnenolone, and for 6 other common corticosteroids. Kit standards (0.05, 0.1, 0.5, 2, 10, 20, and 40 ng/mL), unknowns, and assay pools were added (100 μ L each) in duplicate to antibody-coated tubes. One milliliter of 125 I-labeled progesterone was added to each tube, vortexed for 5 s, and incubated overnight at 4°C. The next day, tubes were decanted, blotted on paper towels, and then the radioactivity of each tube was quantified for 1 min in a gamma counter. The recovery of added mass (0.0, 0.05, 0.25, 1.0, 5.0, and 10 ng) added in quadruplicate (50 μ L) to 4 different bovine serum samples (0.07, 0.55, 3.28, and 4.70 ng/mL) averaged

102%. Parallelism was demonstrated by assaying 75- and 100- μ L aliquots of bovine serum in quadruplicate and recovering 91% added mass. Assay sensitivity was 4.8 ± 0.5 pg/mL. Inter- and intraassay coefficients of variation for 14 assays were 5.0 and 4.7%, respectively, for a pooled serum sample that averaged 2.92 ± 0.02 ng/mL ($n = 56$).

Concentrations of estradiol-17 β were measured by RIA (Stevenson, 2011) in samples collected before each of the GnRH injections, before the breeding week PG injection, and at TAI. Assay sensitivity was 0.23 ± 0.03 pg/mL in 4 assays and inter- and intraassay coefficients of variation were 2.5 and 1.9%, respectively, for a pooled serum sample that averaged 8.2 ± 0.1 pg/mL ($n = 16$). Assay sensitivity was 0.12 ± 0.01 pg/mL in 3 additional assays and inter- and intraassay coefficients of variation were 5.8 and 5.5%, respectively, for a different pooled serum sample that averaged 6.2 ± 0.2 pg/mL ($n = 12$).

Ovarian Structures and Ovulation

Ovarian scans were conducted by transrectal ultrasonography (Figure 1) to measure and map all ovarian follicles >5 mm. Location and number of CL were mapped and recorded, except on d 6 post-TAI, when the diameter of all luteal structures was measured. The volume of each CL was calculated [$4/3 \times r^3 \times \pi$, where r = radius ($(W/2 + H/2)/2$, where W = largest width and H = largest height of the structure; and $\pi = 3.14159$]. When a CL contained a fluid-filled cavity, the volume of the cavity was subtracted from the total CL volume.

Measurements

On the basis of serum progesterone concentrations at 36, 39, 50, and 53 DIM, cows were classified as anovulatory when progesterone was <1 ng/mL in all 4 samples; otherwise, they were classified as having ovulated previously (cycling). Luteolysis was assessed after each PG injection (36, 50, and 67 DIM). Luteal regression was defined to occur when progesterone concentration was ≥ 1 ng/mL immediately before PG injections and decreased to <1 ng/mL 72 h later. The proportion of cows that ovulated and ovulation response to Pre-GnRH, GnRH-1, and GnRH-2 were determined 7 d after injections, when a new CL was found at the site of a previously mapped follicle ≥ 10 mm. At the exam before GnRH-2 was administered, the presence or absence of anechoic fluid detected in the uterine lumen was recorded (physiological evidence of potential estrus and effects of elevated estradiol).

Hourly temperature and relative humidity data were obtained from the Kansas State University Weather Library for the duration of data collection (November 2009 through October 2010). The official weather station is located at 39.2° N latitude, 96.6° W longitude, and at elevation of 336.6 m on the Kansas State University Agronomy Farm <2 km south of where experimental cows were housed. Heat index (temperature-humidity index) was calculated using a standard formula (NOAA, 1976). For each 24-h period, average daily temperature-humidity index values were determined (Figure 2).

Statistical Analyses

Various ovarian binomial characteristics and responses to hormonal injections (GnRH and PGF_{2 α}) were analyzed by logistic regression (procedure LOGISTIC; SAS Institute Inc., Cary, NC). These included the proportions of cows having elevated (≥ 1 ng/mL) or decreased (<1 ng/mL) progesterone concentrations at various time points during the experimental scheme, single or multiple ovulation response to GnRH injections, proportion of cows with an ultrasound-detected CL, and occurrence of luteal regression. The initial model included effects of treatment (PG-3-G vs. Pre-synch-10), lactation number (1 vs. 2+), cycling status (0 vs. 1), test-day ECM yield (greater than or less than the median of 45 kg/d), BCS (greater than or less than the median of 2.25), 2-way interactions of treatment with lactation number and cycling status, and effects of season of year (hot = June through September; moderate and cold = October through May) and calving difficulty score (CDS; 1 = no problems, 2 = slight problem, 3 = needed assistance, 4 = considerable force used, or 5 = extreme difficulty; covariable). The final model produced by backward stepwise selection of independent variables entered or retained in the model was based on a Wald statistic ($P < 0.10$). Odds ratios (OR) and 95% confidence intervals are reported in the text.

Continuous variables were analyzed by ANOVA using a general linear model (procedure GLM; SAS Institute Inc.). Dependent variables included progesterone concentrations, number of CL per cow, follicle diameters, number of follicles ovulating, and CL volume. The model included the effects of treatment, lactation number, cycling status, season, 2-way interactions of treatment with lactation number and cycling status, season, daily ECM yield, BCS, and CDS (covariable). Treatment means were separated by F tests resulting from ANOVA.

Pregnancies per AI at d 32 and 60 and intervening losses were analyzed by procedure GLIMMIX (SAS Institute Inc.). The model included the effects of treat-

Figure 2. Pregnancies per AI (P/AI; bars) assessed at d 32 post-timed AI in the 24 breeding clusters of cows inseminated during the experiment are superimposed over the average heat index (temperature-humidity index, THI; continuous line; NOAA, 1976) during the same period. The broken line (THI = 72) indicates where heat stress begins (mild = 72 to 79; moderate = 80 to 89; severe = 90 to 98; and danger >98). Color version available in the online PDF.

ment, lactation number, season, sire, technician, BCS, CDS, and test-day ECM yield. Additional models included the independent factors of ovulation response to both Pre-GnRH and GnRH-1 or cumulative ovulation responses to all GnRH injections (1, 2, or 3 times), and synchronization rate (progesterone <1 ng/mL at TAI and ovulation success to GnRH-2). All results presented are means $\pm$ standard error.

RESULTS

Presynchronization Luteolysis

More than 80% of the cows had ovulated previously and were considered to be ovular (Table 1). Similar proportions of cows (46%) had progesterone concentrations ≥ 1 ng/mL before the Presynch PG-1 injection or comparable time in PG-3-G cows. As expected, in response to Presynch PG-1, incidence of luteolysis was almost 5 times greater ($P = 0.001$) in the Presynch-10 than PG-3-G cows that did not receive PG (Table 1). No other factors influenced incidence of luteolysis after Presynch PG-1. Fourteen days later (50 DIM), immediately before the Presynch PG-2 injection, more ($P = 0.04$) Presynch-10 than PG-3-G cows had concentrations of progesterone ≥ 1 ng/mL, but incidence of luteolysis did not differ between treatments after injections of Presynch PG-2 or Pre-PG (Table 1).

Luteolysis after Presynch PG-2 and Pre-PG was more ($P = 0.004$; OR = 4.81; CI = 1.66 to 13.9) likely in cows producing less than the median 45 kg of ECM compared with greater than the median ECM (89.2 vs. 70.5%, respectively). Luteolysis after Presynch PG-2 and Pre-PG also was more ($P = 0.022$; OR = 3.45; CI = 1.19 to 10.0) likely to occur during moderate or cold weather than during the summer (85.4 vs. 68.2%, respectively).

Responses to Pre-GnRH

Concentration of progesterone, the proportion of cows having progesterone ≥ 1 ng/mL, and the number of CL per cow did not differ between treatments immediately before the Pre-GnRH injection. In contrast, the proportion of cows with at least 1 CL tended ($P = 0.095$) to be greater in PG-3-G than in Presynch-10 cows (Table 2).

Although diameter of the largest follicle in PG-3-G cows before Pre-GnRH tended ($P = 0.086$) to be smaller, concentrations of estradiol did not differ between treatments (Table 2). The largest ovarian follicle averaged 1.4 ± 0.4 mm smaller ($P = 0.022$) in first- than in multiple-lactation cows.

Ovulatory response to Pre-GnRH in the PG-3-G treatment was greater ($P = 0.001$) than the incidence of spontaneous ovulation in the Presynch-10 treatment.

Table 1. Proportion of cows having estrous cycles and responses to Presynch PGF_{2α} (PG) injections

Item	Treatment ¹				<i>P</i> -value
	n	PG-3-G	n	Presynch-10	
Cyclicity before Pre-GnRH ² (%)	105	85.7	105	81.9	0.455
Proportion with progesterone ≥1 ng/mL before Presynch PG-1 ³ (%)	105	45.7	105	46.7	0.519
Luteal regression after Presynch PG-1 ⁴ (%)	48	14.6	49	67.4	0.001
Proportion with progesterone ≥1 ng/mL before Presynch PG-2 or Pre-PG (%)	105	61.0	105	74.3	0.038
Luteal regression after Presynch PG-2 or Pre-PG (%)	64	84.4	76	76.9	0.164

¹Cows were treated with PG (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

²Cows having serum progesterone ≥1 ng/mL at 36, 39, 50, or 53 DIM.

³At comparable time (36 DIM) for PG-3-G cows.

⁴Progesterone was ≥1 ng/mL before injection and was <1 ng/mL 72 h later or at comparable time (36 to 39 DIM) for PG-3-G cows.

Cows producing more than 45 kg of ECM were less ($P = 0.008$; OR = 0.42; CI = 0.23 to 0.80) likely to ovulate than cows producing less than 45 kg of ECM (59.0 vs. 74.3%, respectively).

Incidence of multiple ovulations and number of follicles that ovulated did not differ between treatments (Table 2). Incidence of multiple ovulations was more ($P = 0.002$; OR = 4.87; CI = 2.14 to 11.1) likely during the summer than during moderate-to-cold weather (43.1 vs. 13.5%). Furthermore, the ovulation rate was greater ($P = 0.001$) during the summer than during cooler weather (1.4 ± 0.07 vs. 1.1 ± 0.07 follicles per cow, respectively).

Responses to GnRH-1

The proportion of cows with progesterone ≥1 ng/mL, the number of CL per cow, and the proportion of cows having at least 1 CL was greater ($P \leq 0.01$) for PG-3-G than for Presynch-10 cows at the onset of the Ovsynch protocol, but the concentration of progesterone only tended ($P = 0.127$) to be greater in PG-3-G

cows (Table 3). Cows with BCS less than the median of 2.25 had lesser ($P = 0.010$) progesterone concentration than cows of greater BCS (2.9 ± 0.2 vs. 3.7 ± 0.2 ng/mL). More CL per cow were detected during the summer (1.2 ± 0.1 vs. 0.9 ± 0.1) than during cooler weather.

The diameter of the largest follicle before GnRH-1 and concentrations of estradiol did not differ between treatments (Table 3). Ovulatory response to GnRH-1 did not differ between treatments. Multiple ovulations were 2.6 times greater ($P = 0.023$) in PG-3-G cows, whereas the number of follicles in PG-3-G cows that ovulated was only numerically greater (Table 3). Multiple ovulations were more ($P = 0.013$; OR = 5.03; CI = 1.41 to 17.9) likely in multiple-lactation cows than in first-lactation cows (21.2 vs. 5.3%).

Responses to Ovsynch PG

Although number of CL per cow was greater ($P = 0.014$) in PG-3-G than in Presynch-10 cows before the PG injection of Ovsynch, concentrations of progester-

Table 2. Ovarian responses (means ± SE) before and in response to the Pre-GnRH injection

Item	Treatment ¹				<i>P</i> -value
	n	PG-3-G	n	Presynch-10	
Progesterone (ng/mL)	105	0.6 ± 0.1	105	0.9 ± 0.1	0.175
Proportion with progesterone <1 ng/mL (%)	105	78.1	105	78.1	0.823
Corpora lutea (CL) per cow (no.)	105	0.4 ± 0.09	105	0.2 ± 0.08	0.183
Cows with CL (%)	105	48.6	105	36.2	0.095
Estradiol-17β (pg/mL)	102	1.4 ± 0.1	105	1.4 ± 0.1	0.753
Follicle size ² (mm)	105	12.6 ± 0.5	105	13.7 ± 0.4	0.086
Ovulation after Pre-GnRH ³ (%)	105	80.0	105	53.3	0.001
Multiple ovulations after Pre-GnRH (%)	84	22.6	56	26.8	0.603
Follicles ovulated per cow (no.)	84	1.2 ± 0.08	56	1.3 ± 0.09	0.389

¹Cows were treated with PGF_{2α} (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

²Largest Pre-GnRH injection follicle.

³Assessed spontaneous ovulation at a comparable time (after 53 DIM) for Presynch-10 cows.

Table 3. Ovarian responses (means ± SE) before and after the GnRH-1 injection¹

Item	Treatment ²				
	n	PG-3-G	n	Presynch-10	P-value
Progesterone (ng/mL)	105	3.5 ± 0.2	105	3.1 ± 0.2	0.127
Proportion with progesterone ≥ 1 ng/mL (%)	105	90.5	105	76.2	0.003
Corpora lutea (CL) per cow (no.)	105	1.2 ± 0.1	105	0.9 ± 0.09	0.005
Cows with CL (%)	105	94.3	105	76.2	0.001
Estradiol-17β (pg/mL)	104	0.9 ± 0.09	105	0.9 ± 0.09	0.878
Follicle size ³ (mm)	105	12.8 ± 0.5	105	13.5 ± 0.4	0.282
Ovulation after GnRH-1 (%)	105	79.0	105	69.5	0.651
Multiple ovulations after GnRH-1 (%)	83	21.7	73	8.2	0.023
Follicles ovulated (no.)	83	1.2 ± 0.08	73	1.1 ± 0.06	0.161

¹Cows were enrolled in a timed AI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PGF_{2α} (PG) injection with AI 16 to 18 h after GnRH-2] 10 d after the last PG injection (Presynch PG-2 or Pre-PG).

²Cows were treated with PGF_{2α} (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

³Largest pre-GnRH-1 injection follicle.

one ($P = 0.060$) and the proportion of cows having progesterone ≥ 1 ng/mL ($P = 0.099$) only tended to be greater for PG-3-G than Presynch (Table 4). The number of CL per cow was greater ($P = 0.001$) in multiple-lactation than in first-lactation cows (2.0 ± 0.1 vs. 1.5 ± 0.1 CL per cow). Concentrations of progesterone were greater ($P = 0.046$) in cows producing < 45 kg of ECM than in cows producing ≥ 45 kg of ECM (6.1 ± 0.3 vs. 4.8 ± 0.4 ng/mL) and greater ($P = 0.001$) during cool weather than during the summer (6.3 ± 0.3 vs. 4.6 ± 0.4 ng/mL).

Incidence of luteolysis after PG injection did not differ between treatments, but serum estradiol tended ($P = 0.072$) to be less in PG-3-G than in Presynch-10 cows (Table 4). Serum estradiol before PG injection was greater ($P < 0.001$) during the summer than during cooler weather (1.2 ± 0.1 vs. 0.5 ± 0.08 pg/mL).

Responses to GnRH-2 and at TAI

None of the ovarian characteristics assessed before GnRH-2 differed between treatments (Table 5). Mean

concentrations of progesterone were ≤ 0.5 ng/mL and proportions of cows with progesterone < 1 ng/mL ranged from 86 to 91%. Furthermore, ovulatory response to GnRH-2 exceeded 96% in both treatments. The number of follicles ovulated was greater ($P = 0.003$) in multiple- than first-lactation cows (1.6 ± 0.06 vs. 1.3 ± 0.08). The diameter of the preovulatory follicle was greater ($P < 0.001$) during cooler weather than during the summer (14.6 ± 0.3 vs. 13.2 ± 0.4) and larger ($P = 0.010$) in cows with BCS > 2.25 than in cows of lesser BCS (14.3 ± 0.3 vs. 13.4 ± 0.3). Multiple ovulations exceeded 31% and the average number of follicles ovulated ranged from 1.4 to 1.5 ± 0.07 per cow. Multiple ovulations were greater ($P < 0.001$) in multiple-lactation than in first-lactation cows (41.9 vs. 17.7%).

The concentration of estradiol was greater ($P = 0.025$) during the summer than during cooler weather (2.0 ± 0.2 vs. 1.5 ± 0.1 pg/mL) and tended ($P = 0.067$) to be greater in cows with BCS > 2.25 (1.9 ± 0.1 vs. 1.6 ± 0.1). Anechoic fluid in the uterine lumen was

Table 4. Ovarian responses (means ± SE) before and after the Ovsynch PGF_{2α} (PG) injection¹

Item	Treatment ²				
	n	PG-3-G	n	Presynch-10	P-value
Progesterone (ng/mL)	105	5.9 ± 0.3	105	5.0 ± 0.4	0.060
Proportion with progesterone ≥ 1 ng/mL (%)	105	93.3	105	85.7	0.099
Corpora lutea (CL) per cow (no.)	105	2.0 ± 0.1	105	1.5 ± 0.1	0.014
Cows with CL (%)	105	97.1	105	92.4	0.164
Luteal regression (%)	98	92.9	90	87.8	0.183
Estradiol-17β (pg/mL)	101	0.7 ± 0.09	105	0.9 ± 0.09	0.072

¹Cows were enrolled in a timed AI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PG with AI 16 to 18 h after GnRH-2] 10 d after the last PG injection (Presynch PG-2 or Pre-PG).

²Cows were treated with PG (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

Table 5. Ovarian responses (means $\pm$ SE) before and after the GnRH-2 injection¹

Item	Treatment ²				<i>P</i> -value
	n	PG-3-G	n	Presynch-10	
Progesterone (ng/mL)	105	0.5 $\pm$ 0.1	105	0.7 $\pm$ 0.1	0.300
Proportion with progesterone <1 ng/mL (%)	105	91.4	105	85.7	0.703
Ovulation after GnRH-2 (%)	105	97.1	105	96.2	0.702
Multiple ovulations (%)	102	31.4	101	33.7	0.742
Follicles ovulated (no.)	102	1.5 $\pm$ 0.07	101	1.4 $\pm$ 0.07	0.700
Estradiol-17 β (pg/mL)	103	1.7 $\pm$ 0.1	104	1.8 $\pm$ 0.1	0.420
Follicle size ³ (mm)	102	13.7 $\pm$ 0.3	105	14.0 $\pm$ 0.3	0.571
Uterine fluid (%)	105	44.8	105	37.1	0.351

¹Cows were enrolled in a timed AI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PGF_{2 α} (PG) with AI 16 to 18 h after GnRH-2] 10 d after the last PG injection (Presynch PG-2 or Pre-PG).

²Cows were treated with PG (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

³Largest pre-GnRH-2 injection follicle.

detected in 44.8% of PG-3-G cows and in 37.1% of Presynch-10 cows. The presence of uterine fluid was more ($P = 0.043$; OR = 1.88; CI = 1.02 to 3.47) likely in multiple than first-lactation cows (45.3 vs. 34.1%).

At TAI, concentrations of progesterone averaged 0.4 to 0.5 $\pm$ 0.1 ng/mL, with a tendency ($P = 0.055$) for more PG-3-G than Presynch-10 cows (93.3 vs. 85.7%) to have progesterone <1 ng/mL. Cows having BCS ≤ 2.25 were less ($P = 0.033$; OR = 0.34; CI = 0.13 to 0.92) likely to have low progesterone concentrations than cows having BCS >2.25 (85.3 vs. 94.1%). Concentrations of estradiol in all cows decreased during the 16 h after the GnRH-2 injection to average 0.5 $\pm$ 0.1 pg/mL. Concentrations of estradiol were greater ($P = 0.002$) during the summer than during cooler weather (0.6 $\pm$ 0.07 vs. 0.4 $\pm$ 0.05 pg/mL, respectively).

Post-TAI Ovarian Characteristics

The proportion of cows with progesterone >1 ng/mL (93.3 vs. 96.1%) and mean concentration of progesterone (2.7 $\pm$ 0.2 vs. 2.9 $\pm$ 0.2 ng/mL) did not differ between PG-3-G and Presynch-10 cows 6 d after TAI. The number of CL per cow (1.4 $\pm$ 0.06 vs. 1.3 $\pm$ 0.06) and total luteal volume (6.4 $\pm$ 0.4 vs. 6.3 $\pm$ 0.4 cm³) also did not differ between treatments, respectively. The number of CL per cow tended ($P = 0.06$) to be greater in multiparous cows than in first-lactation cows (1.5 $\pm$ 0.06 vs. 1.3 $\pm$ 0.08). The number of CL per cow was greater ($P = 0.010$) during the summer than during cooler weather (1.5 $\pm$ 0.05 vs. 1.2 $\pm$ 0.07).

Pregnancies per AI

Pregnancies per AI (d 32 post-TAI) for clusters of cows inseminated during the experiment are superim-

posed on the average daily heat index (Figure 2). The P/AI ranged from 0 to 78.6% during the 24 breeding clusters. During 8 breeding clusters from June to late September, P/AI averaged <10% and was clearly related to the elevated heat index. In the summer months of June to September, P/AI was less ($P = 0.001$) than during the remaining months (8.2 vs. 51.8%).

Pregnancy per AI did not differ between PG-3-G and Presynch-10 cows at d 32 or 60 (Table 6). No interaction was detected between treatment and cycling status at either time. Ovular cows had numerically greater P/AI at d 32 than anovular cows (39.8 vs. 20.6%) and differed ($P = 0.04$) at d 60 (36.9 vs. 12.5%). At both pregnancy diagnoses, P/AI in ovular cows was numerically greater in the PG-3-G than Presynch-10 cows (d 32: 43.3 vs. 36.0% and d 60: 40.0 vs. 33.7%, respectively). Pregnancy losses were <9% between d 32 and 60 (Table 6). Summer accounted for 2 of the 6 total pregnancy losses (summer vs. cooler weather: 33.3 vs. 5.6%).

Cumulative Responses to Hormone Injections

Significant factors that influenced ovulation responses to all GnRH injections included treatment, serum progesterone concentration before GnRH injection, and BCS. Cows in the PG-3-G compared with Presynch-10 treatment (85.4 vs. 73.0%) were more likely ($P = 0.001$; OR = 2.13; CI = 1.43 to 3.21) to ovulate to all 3 injections. Cows with greater ($P = 0.055$; OR = 1.48; CI = 0.99 to 2.20) BCS (≥ 2.25 vs. <2.25; 82.23 vs. 76.5%) and those in which serum progesterone ≥ 1 ng/mL versus <1 ng/mL (81.3 vs. 69.2%) before GnRH injection ($P = 0.022$; OR = 1.75; CI = 1.09 to 2.81) were more likely to ovulate in response to each injection of GnRH.

The overall incidence of multiple ovulations in response to all 3 GnRH injections was not affected by

Table 6. Pregnancy outcomes after timed AI (TAI)

Item	Treatment ¹				P-value
	n	PG-3-G	n	Presynch-10	
Pregnancies per AI, d 32 (%)	105	40.0	105	33.3	0.146
Moderate to cold ²	66	59.1	71	45.7	
Hot ²	39	7.7	34	8.8	
Pregnancies per AI, d 60 (%)	103 ³	35.9	105	30.5	0.226
Moderate to cold	64	54.7	71	42.3	
Hot	39	5.1	34	5.9	
Pregnancy loss (%)	40	7.5	35	8.6	0.916

¹Cows were enrolled in a TAI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PGF_{2α} (PG) with AI 16 to 18 h after GnRH-2] 10 d after the last PG injection (Presynch PG-2 or Pre-PG). Cows were treated with PG (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

²Moderate to cold = October through May, and hot = June through September.

treatment ($P = 0.39$), but was more likely to occur in multiple- than first-lactation cows (36.1 vs. 19.1%; $P = 0.001$; OR = 2.82; CI = 1.66 to 4.81) and during the summer than during cooler months (41.3 vs. 22.5%; $P = 0.001$; OR = 2.85; CI = 1.72 to 4.72).

Combined luteal regression in response to Pre-PG or Presynch PG-2 and ovulation after Pre-GnRH or spontaneous ovulation in Presynch-10 cows was greater ($P = 0.007$) in PG-3-G (71.9%) than in Presynch-10 cows (52.6%). For cows producing more than 45 kg of ECM to have combined luteal regression and ovulation was less ($P = 0.001$; OR = 0.30; CI = 0.14 to 0.52) likely (48.5 vs. 73.0%) than for those producing <45 kg ECM.

More ($P < 0.001$) PG-3-G than Presynch-10 cows ovulated after both the Pre-GnRH (or spontaneous ovulation for Presynch-10 cows) and GnRH-1 injections (67.6 vs. 37.1%); however, a significant treatment by lactation number interaction was detected ($P = 0.039$). Among first-lactation cows, more PG-3-G than Presynch-10 cows ovulated both times (58.5 vs. 43.9%), but the treatment difference was even greater (73.4 vs. 32.8%) among older cows. Because more than 96% of cows in both treatments ovulated after GnRH-2, the proportions of cows ovulating after all 3 GnRH injections were almost identical to those ovulating after both Pre-GnRH and GnRH-1 injections. For cows that ovulated after Pre-GnRH and GnRH-1, P/AI at d 32 tended ($P = 0.085$) to be greater for PG-3-G than Presynch-10 (43.7 vs. 38.5%), but less so for cows that did not ovulate after both injections (32.4 vs. 30.3%).

A tendency ($P = 0.114$) was detected for more PG-3-G than Presynch-10 cows to have concentrations of progesterone <1 ng/mL at TAI and to ovulate in response to GnRH-2 (synchronization rate: 90.5 vs. 83.8%). Cows with BCS ≤ 2.25 were less likely ($P = 0.034$; OR = 0.39; CI = 0.16 to 0.93) to have a synchronized ovulation than for cows of greater BCS (82.6 vs. 92.1%).

Selected Outcomes in Ovular and Anovular Cows

Pre-GnRH. Only 34 of 210 (16.2%) of the cows were anovular before treatments were imposed. Ovulation response in ovular cows and anovular cows was greater in PG-3-G than in Presynch-10 cows, respectively, but differences were only detected between treatments for ovular cows (Table 7). Overall, ovular cows were more likely ($P = 0.032$; OR = 2.42; CI = 1.08 to 5.41) to ovulate in response to Pre-GnRH than were anovular cows (69.9 vs. 50%). Ovular-cycling cows were more ($P = 0.001$; OR = 6.92; CI = 2.33 to 20.5) likely to have a CL than were anovular cows (48.3 vs. 11.8%, respectively).

GnRH-1. More ($P < 0.05$) ovular and anovular cows had at least 1 CL in the PG-3-G than the Presynch-10 treatment. The number of CL per cow was greater for PG-3-G than Presynch-10 cows among both ovular and anovular cows (Table 7). The proportion with progesterone ≥ 1 ng/mL was greater in ovular than anovular cows, respectively, in the PG-3-G versus Presynch-10 treatments, and differed ($P < 0.05$) among ovular cows (Table 7). Ovular cows were more ($P = 0.001$; OR = 10.0; CI = 4.20 to 24.0) likely to have progesterone concentrations ≥ 1 ng/mL than anovular cows (89.2 vs. 47.1%) because a greater ($P = 0.001$; OR = 9.6; CI = 3.7 to 25.2) proportion of ovular cows had detectable CL (90.9 vs. 55.9%) than did anovular cows. Among ovular cows, more PG-3-G than Presynch-10 cows ovulated, whereas fewer PG-3-G than Presynch-10 anovular cows ovulated in response to GnRH-1 (interaction between treatment and cycling status; $P = 0.014$; Table 7).

Ovsynch PG. The number of CL per cow was greater ($P < 0.05$) for PG-3-G than Presynch-10 cows among ovular cows, but not among anovular cows (Table 7). Mean CL per cow was greater ($P < 0.001$) for ovular PG-3-G than Presynch-10 cows (2.3 ± 0.2 vs. 1.8 ± 0.1 CL per cow) and for anovular cows (1.6 ± 0.2 vs. $1.3 \pm$

Table 7. Selected outcomes in ovular and anovular cows after treatment

Item	Cycling status	Treatment ¹		Cycling mean
		PG-3-G	Presynch-10	
Cows (no.)	Anovular	15	19	34
	Ovular	90	86	176
Ovulation after Pre-GnRH ² (%)	Anovular	66.7 ^a	36.8 ^a	50.0 ^A
	Ovular	82.2 ^a	56.9 ^b	69.9 ^B
GnRH-1				
Cows with CL (%)	Anovular	80.0 ^a	36.8 ^b	55.9 ^A
	Ovular	96.7 ^a	84.9 ^a	90.0 ^B
Corpora lutea per cow (no.; means $\pm$ SE)	Anovular	1.0 $\pm$ 0.2 ^a	0.5 $\pm$ 0.2 ^b	0.7 $\pm$ 0.1 ^A
	Ovular	1.5 $\pm$ 0.1 ^a	1.2 $\pm$ 0.1 ^b	1.4 $\pm$ 0.1 ^B
Proportion with progesterone $\geq$ 1 ng/mL (%)	Anovular	60.0 ^a	36.8 ^a	47.1 ^A
	Ovular	95.6 ^a	82.6 ^b	89.2 ^B
Ovulation after GnRH-1 ³	Anovular	82.2	66.3	73.5 ^A
	Ovular	60.0	84.2	74.4 ^A
Ovsynch PG				
Corpora lutea per cow (no.; means $\pm$ SE)	Anovular	1.6 $\pm$ 0.2 ^a	1.3 $\pm$ 0.2 ^a	1.5 $\pm$ 0.1 ^A
	Ovular	2.3 $\pm$ 0.2 ^a	1.8 $\pm$ 0.1 ^b	2.0 $\pm$ 0.1 ^B
GnRH-2				
Multiple ovulations (%)	Anovular	53.3 ^a	52.6 ^a	52.9 ^A
	Ovular	27.6 ^a	29.3 ^a	28.4 ^B

^{a,b}Means within rows with different lowercase superscript letters differ ($P \leq 0.05$).

^{A,B}Cycling means within trait with different uppercase superscript letters differ ($P \leq 0.05$).

¹Cows were enrolled in a timed AI protocol [Ovsynch; injection of GnRH 7 d before (GnRH-1) and 56 h after (GnRH-2) PGF_{2α} (PG) with AI 16 to 18 h after GnRH-2] 10 d after the last PG injection (Presynch PG-2 or Pre-PG). Cows were treated with PG (Pre-PG) 3 d before GnRH (PG-3-G) or with 2 PG injections (Presynch PG-1 and Presynch PG-2) 14 d apart (Presynch-10). The Presynch PG-2 injection was given at the same time as the Pre-PG injection.

²Assessed spontaneous ovulation at a comparable time (after 53 DIM) for Presynch-10 cows.

³Interaction between treatment and cycling status ($P = 0.014$).

0.2 CL per cow, respectively). The proportion of cows with progesterone $\geq$ 1 ng/mL was more ($P = 0.003$; OR = 4.4; CI = 1.69 to 11.5) likely in ovular than anovular cows (92.6 vs. 73.5%).

GnRH-2. The number of follicles that ovulated after GnRH-2 was not only greater ($P = 0.005$) in anovular than ovular cows (1.6 ± 0.09 vs. 1.3 ± 0.04), but the percentage of multiple ovulations was less ($P = 0.019$) frequent in ovular than anovular cows in both treatments (Table 7).

DISCUSSION

One objective of the present study was to increase the percentage of cows having a functional CL and elevated progesterone concentrations before enrollment in a TAI program. This objective was tested by 2 presynchronization treatments, one of which (Presynch-10) should provide the greatest potential for maximal ovulatory response to GnRH-1 for cows in early diestrus (d 5 to 10 of the cycle) compared with other presynchronization intervals of 11 to 14 d. Assuming that luteolysis occurs between 0 and 5 d after the Presynch PG-2 injection, intervals of 14, 12, 11, and 10 d would synchronize most the cows to d 9 to 14, d 7 to 12, d 6 to 11, or d 5 to 10 of

the cycle at the onset of the TAI protocol. On the basis of studies in which GnRH was given at various stages of the first follicular wave, intervals of 10 or 11 d (d 5 to 11 of the cycle) should facilitate the greatest ovulatory responses to GnRH-1 (Vasconcelos et al., 1999; Galvão et al., 2007). Ovulation responses to GnRH-1 were greater in cows 1) without a CL, 2) with follicles >19 mm, and 3) not presynchronized (Galvão and Santos, 2010). Greater ovulatory response to GnRH-1 produced better embryo quality (Cerri et al., 2009) and greater P/AI (Chebel et al., 2006; Stevenson et al., 2007a; Rutigliano et al., 2008).

The second treatment, PG-3-G, was chosen because of its potential to ovulate follicles in cows before the onset of the TAI program (Bello et al., 2006; Souza et al., 2008), particularly in anovular cows. The previous studies described treatments, known as G-6-G and Double Ovsynch, respectively, which both included a pre-GnRH injection, preceded 2 or 3 d earlier by a PG injection. In theory, most ovular cows to which G-6-G or Double Ovsynch is applied should be between d 4 and 6 of the cycle and greatly responsive to a GnRH injection (Vasconcelos et al., 1999). In anovular cows, this pre-GnRH injection provides one more opportunity to induce ovulation before the Ovsynch protocol is ap-

plied 6 d (Bello et al., 2006) to 7 d (Souza et al., 2008) later.

Differing positive characteristics between treatments existed before the Pre-GnRH injection of the PG-3-G treatment. Although tendencies for more PG-3-G cows to have at least 1 CL and a smaller-size follicle compared with Presynch-10 were observed, 80% of PG-3-G cows ovulated in response to the Pre-GnRH injection compared with only 53% spontaneous ovulation in Presynch-10 cows. The proportion of cows ovulating after the Pre-GnRH injection was identical to the reported 80% response in similar treatments (G-6-G; Bello et al., 2006). Furthermore, these differences occurred in the current study despite no difference in luteal regression after the preceding Presynch PG-2 or Pre-PG injection, but with more than 70% of the cows ovulating in response to both injections of PG (i.e., luteolysis) and Pre-GnRH (i.e., ovulation). This combined response matched the reported 71% response in cows in which presynchronization included a PG injection followed in 2 d by a Pre-GnRH injection (Bello et al., 2006).

Presynchronization of key ovarian factors were apparent as cows began the Ovsynch protocol. The PG-3-G cows had more CL per cow, more cows had at least 1 CL, and more cows had progesterone ≥ 1 ng/mL. Compared with cows at random stages of the estrous cycle, more cows to which the presynchronization PG-3-G treatment was applied had progesterone ≥ 1 ng/mL at the time of GnRH-1 (93 vs. 56%; Peters and Pursley, 2002). When Ovsynch was applied to cows as a presynchronization in Double Ovsynch, the only reported significant difference detected before GnRH-1 was fewer cows with progesterone < 1 ng/mL compared with Presynch-12 (9.4 vs. 33.3%; Souza et al., 2008), which is analogous to our PG-3-G and Presynch-10 cows having 9.5 versus 23.8% (calculated from Table 3), respectively. Ovulation responses to GnRH-1 in the current (69 to 79%) and latter study (67 to 72%; Souza et al., 2008) were similar, but we observed 2.5 times more double ovulation in the PG-3-G than Presynch-10 treatment (equally distributed in both ovular and anovular cows). In addition, 68% of cows in the PG-3-G treatment ovulated after both Pre-GnRH and GnRH-1 compared with only 37% of Presynch-10 cows.

The responses to GnRH-1 (greater progesterone concentration and more CL per cow) resulting from presynchronization effects of PG-3-G carried over for cows when they received their Ovsynch PG injection. A trend toward a greater proportion of cows with progesterone ≥ 1 ng/mL (93 vs. 86%) for PG-3-G compared with Presynch-10 before the Ovsynch PG was similar to that reported for PG-3-G versus Ovsynch cows (95 vs. 83%; Peters and Pursley, 2002). Increased progesterone

in the current study for PG-3-G compared with Presynch-10 cows (5.9 vs. 5.0 ng/mL) before the Ovsynch PG corroborates a similar increase for Double Ovsynch cows compared with cows treated with Presynch-11 (4.2 vs. 3.2 ng/mL; Souza et al., 2008).

Greater concentrations of progesterone during follicle development after GnRH-1, ovulatory response to GnRH-1, or both, may be more important to overall pregnancy outcomes. Concentrations of progesterone tended to be greater in PG-3-G cows before GnRH-1 and were greater before the Ovsynch PG injection during which time the preovulatory follicle developed before AI. Further, when cycles of lactating dairy cows were synchronized so a second-wave follicle or a first-wave follicle (plus supplemental progesterone during the last 5 d before the Ovsynch PG injection) was induced to ovulate after GnRH-1, P/AI were greater than in cows in which a first-wave follicle was induced to ovulate (Denicol et al., 2012). Further, cows induced to ovulate follicles derived from the first follicular wave produced embryos of poorer quality because of exposure to progesterone concentrations < 2 ng/mL during follicle growth (Rivera et al., 2011).

Treatments with Presynch and incorporation of an intravaginal progesterone insert during 7 d after Presynch PG-1 (Chebel et al., 2006; Bicalho et al., 2007) or applying Double Ovsynch (Souza et al., 2008) decreased the proportion of cows with progesterone < 1 ng/mL at the time of GnRH-1, and presumably decreased the proportion of anovular cows to which the subsequent TAI protocol was applied. We detected only 16.2% of the cows in the present study to be anovular. We expected more positive effects of PG-3-G than Presynch-10 treatment to occur in anovular cows because of the Pre-GnRH injection. The PG-3-G treatment was clearly superior to Presynch-10 in response detected in anovular cows by increasing ovulatory responses to GnRH (except after GnRH-1) and increasing proportions of anovular cows with progesterone ≥ 1 ng/mL before GnRH-1, thus resulting in more cows with at least 1 CL and a greater mean number of CL at GnRH-1 and PG injection. Our results are consistent with one treatment in a study by Galvão et al. (2007) in which cows treated with Presynch-11 also were given a GnRH injection 4 d after Presynch PG-2 (Presynch-11 + GnRH). Compared with a Presynch-14 control, applying the GnRH injection 4 d after the Presynch PG-2 increased ovulation response to GnRH-1 (44.7 vs. $> 61\%$) and the proportion of cows with a CL at GnRH-1 (37 to $> 54\%$). Notably, none of these factors in the Presynch-11 + GnRH treatment differed from Presynch-11 except for the lesser proportion of cows with a CL at GnRH-1 compared with the Presynch-11

+ GnRH treatment (74 vs. 88%). We observed that 90.5% of our PG-3-G cows had a CL at GnRH-1 compared with 76.2% in the Presynch-10 treatment with the greater response among anovular cows (80.0 vs. 36.8%, respectively). These results not only reiterate the superiority of shortening the time from Presynch PG-2 to the onset of the TAI protocol to 10 or 11 d from 14 d, but also illustrate that the PG-3-G treatment may be a better treatment choice for herds with greater proportions of anovular cows.

Incidence of multiple ovulations in response to Pre-GnRH (22.6 to 26.8%), GnRH-1 (8.2 to 21.7%), and GnRH-2 (31.4 to 33.7%) injections for PG-3-G and Presynch-10 cows, respectively, likely reflects the presence of co-dominant follicles. Incidence of co-dominance was greater in the first than the second follicular wave (Kulick et al., 2001) during a period when endogenous progesterone concentrations were less. More frequent multiple ovulations have been demonstrated experimentally during a lesser progesterone environment (Denicol et al., 2012). It is possible that decreased progesterone concentrations result in greater LH release preceding deviation of the largest from next largest follicle during the first follicular wave (Kulick et al., 2001). Suggestions that LH secretion is increased during deviation of follicles in twinning beef cows (Echternkamp et al., 1990) are not consistent with studies in heifers (Ginther et al., 2001). Incidence of multiple ovulations in response to GnRH was greater in lactating cows having more follicles ≥ 5 mm and in those in early diestrus (Stevenson et al., 2007a). In the present study, multiple ovulations were more likely to occur in older cows and during the summer. Our results in Table 3 indicate that although progesterone concentration was numerically less in the PG-3-G than in the Pre-10 treatment, double ovulation was actually greater (21.7 vs. 8.2%), suggesting that the greater incidence of multiple ovulations may be a phenomenon associated with the first- versus second-follicular wave rather than the actual progesterone environment in which the follicle developed.

Increased multiple ovulations are generally associated with increased P/AI after first AI (Stevenson et al., 2007a; Rutigliano et al., 2008) because of the resulting increased progesterone and potentially more ova for fertilization. Among cows with twin pregnancies, CL number was associated negatively with embryo reduction and pregnancy loss (Silva-del-Río et al., 2009). Lesser progesterone concentration is associated with increased steroid metabolism in cows producing greater quantities of milk (Wiltbank et al., 2006) and may predispose cows to greater early embryo loss before d 30 of pregnancy (Stevenson et al., 2007b).

No further ovarian differences detected between treatments after the Ovsynch PG injection indicated that the PG-3-G presynchronization effects were effective in staging cows to the most optimal stage of a follicular wave to maximize the responses to Ovsynch. Although P/AI on d 32 and 60 in the present study were only numerically greater for PG-3-G than Presynch-10 cows, this difference included poor P/AI (<9%) observed during summer. Pregnancy outcome for cows inseminated during months without heat stress was quite remarkable (59.1 vs. 45.7%; PG-3-G vs. Presynch-10 cows), albeit representing a smaller sample size.

Cows in the present study were housed in open 30-yr-old barns with covered freestalls retrofitted with some heat abatement features (shade over the feed bunks and overhead sprinklers). Most TAI studies in which fertility is assessed avoid summer to maximize potential differences among treatments. The P/AI in the current study during months of heat stress (Figure 2) averaged 8.2 vs. 51.8% during cooler months. These results are consistent with another report of 8.6 vs. 39.2% for cows to which a TAI program was applied from February through November in California (Rutigliano et al., 2008). Cows exposed to heat stress in that study were 5.8 times more likely to have poorer P/AI than those not exposed to heat stress, and were 7.4 times more likely to lose an established pregnancy between 28 and 56 d of gestation (Rutigliano et al., 2008). Compared with winter conditions, chronic seasonal heat stress or hyperthermia alters follicular steroidogenesis, which leads to formation of suboptimal CL and decreased progesterone concentration (Wolfenson et al. 2000), factors that likely decrease synchronization efficiency.

Season accounted for several factors that may have contributed directly or indirectly to poorer fertility during heat stress in the present study. Those factors that were affected by heat stress compared with cows that were not heat-stressed included 1) 3 times more multiple ovulations after Pre-GnRH injection, 2) 1.3 times greater ovulation rates after GnRH injections, 3) reduced (by 20%) regression of CL after Presynch PG-2, 4) decreased (by 27%) progesterone concentration at the time of the Ovsynch PG injection, 5) decreased (by 10%) size of the preovulatory follicle at GnRH-2, 6) 1.3 to 2.4 times greater concentration of estradiol secreted by the preovulatory follicle, and 7) 1.25 times greater number of CL by 6 d post-AI.

We did not detect a treatment by parity interaction in P/AI as reported for Double Ovsynch cows (Souza et al., 2008). The previous report detected greater P/AI in primiparous cows treated with Double Ovsynch compared with Presynch-12 (65.2 vs. 45.2%), whereas

no benefit was detected in multiparous cows (37.5 vs. 39.3%, respectively). In our small sample, if any real difference occurred, the P/AI favored older cows (43.8 vs. 34.4%) more than primiparous cows (34.1 vs. 31.7%), for PG-3-G versus Presynch-10 cows, respectively.

Fertility advantages are documented in cows inseminated at estrus after earlier postpartum prebreeding injections of PG (Benmrad and Stevenson, 1986). Presynchronization with standard Presynch PG injections has the potential to follow suit. Actual fertility tests in uterine-compromised cows may (LeBlanc et al., 2002) or may not (Dubuc et al., 2011) have uterine health benefits after PG-induced CL regression and induced estrus. Because only PG is applied in Presynch programs, the PG would likely be less effective in anovular cows (Moreira et al., 2001; El-Zarkouny et al., 2004).

Fertility after a TAI program is enhanced when 1) more cows have a CL before PG injection (Chebel et al., 2006), 2) when cows have greater concentrations of progesterone at PG injection (Bello et al., 2006), and 3) a greater proportion of cows ovulate after GnRH-1 and GnRH-2 (Vasconcelos et al., 1999; Galvão and Santos, 2010); of these, ovulation after GnRH-2 is most critical to pregnancy outcome (Galvão and Santos, 2010). Because of these 3 factors and potential benefits in anovular cows, P/AI was numerically greater in PG-3-G than in Presynch-10 cows at d 32 and 60.

An earlier report (Peters and Pursley, 2002) compared PG-3-G with a standard Ovsynch and failed to detect an increase in P/AI; however, in first-lactation cows, P/AI was 53% in 73 PG-3-G cows compared with 44% in 69 Ovsynch cows (similar to the effects of Double Ovsynch vs. Presynch-12 in primiparous cows; 65.2 vs. 45.2%; Souza et al., 2008). Overall, in 209 PG-3-G cows (41.5%), P/AI did not differ from 219 Ovsynch-treated cows (38.3%; Peters and Pursley, 2002). A recent report (Ribeiro et al., 2011a) of grazing Holstein (31 kg/d) and Jersey (22 kg/d) cows producing less milk than cows in the present study showed no differences in pregnancy rates at d 30 or 65 for cows in Presynch-11 compared with G-6-G (similar to PG-3-G where PG is injected 2 d before GnRH but followed by Ovsynch in 6 rather than 7 d in PG-3-G). The TAI protocol used in the previous study was a 5-d Cosynch in which an additional PGF injection, given 24 h after the single PG injection, was applied 5 d after GnRH-1 and GnRH-2 was injected at the time of AI (72 h after the first PG injection). Another report also detected no difference in P/AI at d 35 or 60 in grazing dairy cows treated with Double Ovsynch versus Presynch-10 when a 5-d Cosynch or Ovsynch TAI protocol was used (Ribeiro et al., 2011b). Further comparisons are warranted in multiple herds to verify any real effects on pregnancy

outcomes of the PG-3-G treatment in cows receiving the standard 7-d Ovsynch protocol.

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