Dose frequency of prostaglandin $F_{2\alpha}$ administration to dairy cows exposed to presynchronization and either 5- or 7-day Ovsynch program durations: Ovulatory and luteolytic risks¹

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ABSTRACT

We hypothesized (1) that neither duration of the Ovsynch program nor dose frequency of $PGF_{2\alpha}$ would change the proportion of cows with complete luteolysis (progesterone <0.4 ng/mL 72 h after $PGF_{2\alpha}$) and (2) that the additional GnRH treatment administered as part of a presynchronization program would not alter the proportion of anovulatory cows starting the timed artificial insemination (AI) program compared with an alternative shorter presynch program including only 1 GnRH treatment. Lactating Holstein cows ($n = 406$) were milked 3 times daily and enrolled in a $2 \times 2 \times 2$ factorial experiment consisting of 8 treatments before the first postpartum AI. Treatments were used to test ovulatory, luteal, and luteolytic outcomes to 3 main effects: (1) 2 GnRH- $PGF_{2\alpha}$ presynchronization programs (PG-3-G vs. Double Ovsynch), (2) 2 Ovsynch program durations [5 d: GnRH (GnRH-1)–5 d- $PGF_{2\alpha}$ –24 h- $PGF_{2\alpha}$ –32 h–GnRH (GnRH-2)–16 h–timed AI; 7 d: GnRH-1–7 d- $PGF_{2\alpha}$ –56 h–GnRH-2–16 h–timed AI], and (3) 2 $PGF_{2\alpha}$ dose frequency treatments (2×25 mg) 24 h apart versus 1 dose (1×50 mg) of $PGF_{2\alpha}$ administered 72 h before timed AI. The presynchronization treatments of PG-3-G and Double Ovsynch had no effect on the proportion of cows with luteal function at the onset of the Ovsynch treatments (87.9 vs. 86.2%). Although ovulatory responses were similar after GnRH-1 ($>60\%$), Double Ovsynch cows tended to have greater ovulatory responses than PG-3-G after GnRH-2 (95.3 vs. 90.6%). The 2×25 -mg doses of $PGF_{2\alpha}$ and the 1×50 -mg dose induced luteolysis in both Ovsynch treatment durations, but the 1×50 -mg dose was less effective in the 5-d program. More pregnancy per AI (P/AI; 49.2%) tended to occur in the PG-3-G cows in

the 7-d program compared with the other treatment combinations (range: 32.4–37.4%; Ovsynch $\times$ presynch interaction). In addition, an Ovsynch $\times$ $PGF_{2\alpha}$ dose frequency interaction resulted in cows receiving the 1×50 -mg dose in the 7-d program having the greatest P/AI (46.1%) and cows receiving the 1×50 -mg dose in the 5-d program having the least P/AI (30.6%). We conclude that complete luteolysis was less effective in the 5-d program when the 1×50 -mg dose was applied, but both $PGF_{2\alpha}$ dose frequencies (1×50 mg and 2×25 mg 24 h apart) effectively induced complete luteolysis in the 7-d program. Treatments producing complete luteolysis tended to be related to subsequent pregnancy outcomes.

Key words: gonadotropin-releasing hormone presynchronization, luteolysis, Ovsynch duration, prostaglandin $F_{2\alpha}$ dose frequency, pregnancy per artificial insemination

INTRODUCTION

Timed AI programs are widely used in the US dairy industry (Caraviello et al., 2006). Two standards include Ovsynch programs of 2 durations: (1) 5-d program (GnRH-1–5 d- $PGF_{2\alpha}$ –24 h- $PGF_{2\alpha}$ –32 h–GnRH-2 + timed AI) and (2) 7-d program (GnRH-1–7 d- $PGF_{2\alpha}$ –56 h–GnRH-2–16 h–timed AI). Limitations of maximizing pregnancy outcomes to timed AI programs for cows submitted for first service include the following observations. Cows that are anovulatory (23%; Bamber et al., 2009) before timed AI or that do not have a corpus luteum (CL) at the time of GnRH-1 are less likely to conceive (Stevenson et al., 2008), have poorer embryo quality (Cerri et al., 2005), and have greater pregnancy loss between 30 and 58 d of gestation than cyclic cows (Santos et al., 2009). Cows are less likely to conceive to timed AI when they fail to ovulate and form new CL in response to GnRH-1 (Galvão et al., 2007; Stevenson et al., 2008), and this ovulation frequency is progesterone dependent (Stevenson and Pulley, 2016). Cows are less likely to conceive to timed AI when complete luteolysis of all luteal structures in response to $PGF_{2\alpha}$ fails to

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occur as evidenced by not achieving concentrations of progesterone <0.3 to 0.5 ng/mL at timed AI (Santos et al., 2010; Ribeiro et al., 2012a; Wiltbank et al., 2015). Cows are less likely to conceive when ovulation of the preovulatory follicle in response to GnRH-2 fails to occur or is not appropriately coincident with fixed-time semen placement in the uterus (Pursley et al., 1998).

In combined studies (Stevenson, 2016) consisting of 2,104 cows, more than 78% of the variation in pregnancy per AI (P/AI) after a timed AI program was explained (multiple regression modeling) by (1) ovulation success after GnRH-2, (2) concentrations of progesterone >0.5 ng/mL before PGF_{2α}, and (3) concentrations of progesterone <0.5 ng/mL by 48 h after PGF_{2α} reflecting complete luteolysis. Other factors of lesser importance in that model were BCS, size of the ovulatory follicle before GnRH-2, and parity. Ovulatory response to GnRH-1 was not retained in the model of 6 significant variables but is likely an important contributor to progesterone concentration before PGF_{2α} when a new or anovulatory CL forms after GnRH-1.

Presynchronization programs before first postpartum AI used in the US dairy industry include combinations of PGF_{2α} and GnRH (i.e., GnRH-PGF_{2α} presynch options). These programs include PG-3-G (PGF_{2α}-3 d-GnRH-7 d-Ovsynch), G-6-G (PGF_{2α}-2 d-GnRH-6 d-Ovsynch), and Double Ovsynch (GnRH-7 d-PGF_{2α}-3 d-GnRH-7 d-Ovsynch). The preceding GnRH-PGF_{2α} presynch programs are alternatives to traditional PGF_{2α} presynch programs (2 injections of PGF_{2α} 14 d apart—10, 11, 12, or 14 d-Ovsynch). Based on a recent meta-analysis (Borchardt et al., 2017), these combinations produce greater P/AI than the traditional PGF_{2α} presynch programs. In the same report, primiparous cows exposed to 1 GnRH-PGF_{2α} presynch program (Double Ovsynch) had greater P/AI at first AI compared with primiparous cows exposed to PGF_{2α} presynch programs, whereas P/AI in multiparous cows did not differ between presynch programs.

Studies demonstrating the effects of decreasing the interval between GnRH-1 and PGF_{2α} treatments from 7 d (7-d program) to 5 d (5-d program) have been described in suckled beef cows (Bridges et al., 2008) and lactating dairy cows (Santos et al., 2010). Although timing of AI after the PGF_{2α} treatment differed for beef cows in the 7-d program and those in the 5-d program, more cows become pregnant in the 5-d program than in the 7-d program in some experiments (Bridges et al., 2008). A 5-d program was compared with the 7-d program in lactating dairy cows (Santos et al., 2010), but unlike the beef cattle studies, in this study dairy cows in the 5-d program received 2 PGF_{2α} treatments, whereas cows in the 7-d program received only 1 PGF_{2α} treatment. Pregnancy risk, however, was reported (Santos

et al., 2010) to be greater for cows in the 5-d program than the 7-d program (39 vs. 34%) for cows that had concentrations of progesterone <1 ng/mL at AI.

In lactating dairy cows enrolled in 2 experiments at a nonpregnant diagnosis, we demonstrated that 50 mg of PGF_{2α} (dinoprost tromethamine) administered on d 6 of a traditional 5-d program (GnRH-1 at d 0) induced luteolysis based on decreased concentrations of progesterone and visual ultrasonic regression of the luteal structures compared with induced luteolysis in cows receiving 25 mg of PGF_{2α} on d 5 and 6 (Stevenson et al., 2013). In a subsequent experiment of lactating dairy cows in 2 herds receiving the same experimental treatments after a nonpregnant diagnosis (Stevenson et al., 2014), P/AI at 30 to 40 d did not differ between cows receiving 25 mg of PGF_{2α} on d 5 and 6 and those receiving 50 mg on d 6 when luteolysis had occurred by d 8 (37.2 vs. 33.3%) in one herd but differed in the other herd (24.7 vs. 19.5%). Other studies demonstrated that a larger dose (200% of normal) of either PGF_{2α} (Ribeiro et al., 2012a) or a PGF_{2α} analog (Ribeiro et al., 2012a) given on d 5 of a 5-d program failed to induce complete luteal regression equal to a 25-mg dose of PGF_{2α} administered on d 5 and 6, resulting in compromised P/AI.

We hypothesized that neither duration of the Ovsynch program nor dose frequency of PGF_{2α} would change the proportion of cows having complete luteolysis. Furthermore, we hypothesized that the additional GnRH injection administered in Double Ovsynch would not alter the proportion of anovulatory cows starting the timed AI program compared with the PG-3-G presynch program. Therefore, we proposed to address (1) whether 1 larger single dose (50 mg) of PGF_{2α} can produce complete luteolysis similar to 2 standard 25-mg doses given 24 h apart, (2) whether complete luteolysis can occur in both the 5- and 7-d Ovsynch programs when similar doses and frequencies of PGF_{2α} injections are administered, and (3) whether an additional GnRH injection in a presynch program (e.g., Double Ovsynch) can decrease the proportion of anovulatory cows compared with a shorter version with only 1 GnRH treatment (e.g., PG-3-G).

MATERIALS AND METHODS

We enrolled 406 lactating Holstein cows in an experiment spanning 20 mo (September 2015 through April 2017) at the Kansas State University Dairy Teaching and Research Center, Manhattan, conducted under the Kansas State University Institutional Animal Care and Use Committee (application no. 3671). Cows were enrolled in weekly clusters, resulting in 80 weekly clusters with the targeted first AI occurring at 73 ± 3 DIM.

Figure 1. Illustration of the $2 \times 2 \times 2$ factorial experiment consisting of 8 treatments. Main effects included presynchronization (Double Ovsynch vs. PG-3-G; $PGF_{2\alpha}$ - 3 d - GnRH - 7 d - Ovsynch), Ovsynch treatment duration (5 vs. 7 d), and dose frequency of $PGF_{2\alpha}$ (2×25 -mg vs. 1×50 -mg doses). Schedule for blood collection (B) and transrectal ovarian ultrasonograms (S) is shown. PrePG or PG = 25 mg of $PGF_{2\alpha}$; 2PG = 50 mg of $PGF_{2\alpha}$; PreG, GnRH-1, or GnRH-2 = 100 μ g of GnRH; PD = pregnancy diagnosis. Color version available online.

Calving difficulty scores (1 = no problems; 2 = slight problem; 3 = needed assistance; 4 = considerable force used; and 5 = extreme difficulty) averaged 1.2 ± 0.5 (mean $\pm$ SD) at calving time. Body condition scores (1 = thin and 5 = obese; Ferguson et al., 1994) averaged 2.8 ± 0.4 (mean $\pm$ SD) at calving and 2.5 ± 0.3 (mean $\pm$ SD) at the onset of the timed AI Ovsynch treatment described hereafter. Parity of enrolled cows ranged from 1 to 5 with an average of 2.0 ± 1 (mean $\pm$ SD).

Cows were housed in freestalls with overhead roofs and fed a TMR twice or thrice (summer) daily 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 cotton seed, ground corn grain, corn-gluten feed, vitamins, and minerals. Cows were milked thrice daily. Daily milk yield was captured electronically to summarize yield during the first 14 wk of lactation.

Treatments

Cows were enrolled in a $2 \times 2 \times 2$ factorial experimental design consisting of 8 treatments when they calved in weekly clusters. This approach was used to test ovulatory, luteal, and luteolytic outcomes to 3 main effects and their potential interactions (Figure 1): (1) 2 presynchronization programs, including PG-3-G ($PGF_{2\alpha}$ -3 d-GnRH-7 d-Ovsynch) and Double Ovsynch

(GnRH-7 d- $PGF_{2\alpha}$ -3 d-GnRH-7 d-Ovsynch); (2) 2 Ovsynch-duration programs, including a 5-d program (GnRH-1-5 d- $PGF_{2\alpha}$ -24 h- $PGF_{2\alpha}$ -32 h-GnRH-2-16 h-timed AI) and a 7-d program (GnRH-1-7 d- $PGF_{2\alpha}$ -56 h-GnRH-2-16 h-timed AI); and (3) 2 dose frequencies of $PGF_{2\alpha}$ administered on d 5 and 6 (5-d program; d 0 = GnRH-1) or on d 7 and 8 (7-d program) for the 2×25 -mg treatment versus the 1×50 -mg treatment of $PGF_{2\alpha}$ on d 5 (5-d program) or d 7 (7-d program), respectively. The products used were 100 μ g of Factrel (GnRH) and 25 or 50 mg of $PGF_{2\alpha}$ (dinoprost tromethamine) from Zoetis Inc. (Kalamazoo, MI).

Blood samples were collected before GnRH-1, before $PGF_{2\alpha}$, and at 24, 48, and 72 h after $PGF_{2\alpha}$ (first or only $PGF_{2\alpha}$ injection) to determine concentrations of progesterone and assess complete luteolysis (blood progesterone <0.4 ng/mL at 72 h after the first or only dose of $PGF_{2\alpha}$). Blood samples were stored on ice and transported to the laboratory for storage at 5°C until serum was harvested by centrifugation ($1,200 \times g$ for 15 min at 5°C). Sera samples were stored at -15°C until assayed for progesterone concentration by RIA using ImmuChem Double Antibody progesterone ^{125}I kits (MP Biomedicals LLC, Orangeburg, NY) previously validated (Hill et al., 2016). Inter- and intra-assay coefficients of variation of 26 assays for a low (1.28 ± 0.07 ng/mL) and high (17.2 ± 1.3 ng/mL) concentration pool were 5.7 and 7.1%, respectively. Calculated assay

sensitivity averaged 16 ± 9 pg/mL, and progesterone standard concentrations in the assay were 0.05, 0.1, 0.2, 0.5, 2.0, 5.0, 10.0, and 25.0 ng/mL.

Transrectal ovarian scans (7.5-MHz linear-array transducer, Aloka 500V; Corometrics Medical Systems Inc., Wallingford, CT) by ultrasonography were conducted before GnRH-1, before PGF_{2α}, and at 48 h after PGF_{2α} (first or only PGF_{2α} injection) to map and measure ovarian structures. Ovulation (single or multiple) was determined by disappearance of follicles previously mapped and recorded (Figure 1). Frozen images of follicles and CL were assessed using electronic calipers and measuring diameters in 2 directions perpendicular to one another. Total volume of luteal tissue was calculated ($4/3 \times r^3 \times \pi$, where r = radius [$W/2 + H/2$]/2, and $\pi = 3.14159$; W = largest width and H = largest height of the structure). When a luteal structure contained a fluid-filled cavity, volume of the cavity was subtracted from the total luteal volume. Although P/AI via transrectal ultrasonography on d 32 and 60 to visualize an embryo or fetus, respectively, was assessed and trends are reported herein, this experiment was neither powered nor designed to evaluate pregnancy risk but rather to focus on (1) proportion of true anovulatory cows before onset of Ovsynch, (2) ovulation responses to GnRH-1 and GnRH-2, and (3) progesterone concentrations and proportion of cows with complete luteolysis.

Statistical Analyses

Anovulation. Postpartum resumption of estrous cycles was assessed by determining the proportion of cows exposed to either Double Ovsynch or PG-3-G having serum progesterone >1 ng/mL and a CL detected via transrectal ultrasonography at GnRH-1. Traits determined at GnRH-1 were analyzed by ANOVA, including the fixed effects of treatment (Double Ovsynch or PG-3-G), parity (primiparous vs. multiparous), and calving season (warm-hot = May through August; cool-cold = September through April) and the random effects of DIM and BCS. Logistic regression using the GLIMMIX procedure (Enterprise 6.1, SAS Institute Inc., Cary, NC) was applied to binomial outcomes (cyclicity, proportion with progesterone >1 ng/mL, and proportions of cows with multiple CL at GnRH-1). Options used in the model statement included LINK = LOGIT, DIST = BINOMIAL, and the least squares means option of ILINK and DIFF. General linear models (MIXED) in SAS were used to analyze continuous variables (progesterone concentration, number of CL, and total luteal volume at GnRH-1) by applying the previously stated model.

Cows with progesterone <1 ng/mL were further studied to determine true anovulation after being exposed to the 2 presynch main effects. For cows with progesterone <1 ng/mL at GnRH-1 that either ovulated or failed to ovulate after GnRH-1, progesterone concentrations at 5 or 7 d after GnRH-1 were analyzed as described previously, including the additional fixed independent effect of ovulation response to GnRH-1 (0 or 1). Any cow with progesterone concentrations that never exceeded 1 ng/mL during the entire sampling period was defined to be anovular.

Ovarian Characteristics and Ovulation After GnRH-1 and GnRH-2. Single and multiple ovulatory responses, number of ovarian follicles ≥ 5 mm, and diameter of the largest and second largest follicles at GnRH-1 were analyzed by the MIXED procedure in SAS using the same model as described previously [independent effects of presynch treatment (Double Ovsynch vs. PG-3-G), parity, season, DIM, and BCS]. Single and multiple ovulatory responses were analyzed by the GLIMMIX procedure by using a similar model. When analyzing similar follicular and ovulatory outcomes after GnRH-2, the model included both main effects of presynch treatment, Ovsynch duration (5 vs. 7 d), and dose frequency of PGF_{2α} and their 2- and 3-way interactions.

Ovarian Characteristics at PGF_{2α}. Number of ovarian follicles ≥ 5 mm, number of CL per cow, and diameter of the largest and second largest follicles at GnRH-1 were analyzed by the MIXED procedure in SAS using a similar model as described previously that also included the fixed effects of presynch treatment, Ovsynch duration (5 vs. 7 d), and their interaction.

Luteolytic Responses. Proportions of cows with progesterone <1 ng/mL at 24, 48, and 72 h [and proportions of cows with progesterone <0.4 ng/mL at 72 h (complete luteolysis)] after the first or only dose of PGF_{2α} were calculated and analyzed in a similar model in GLIMMIX described previously but included all 3 main effects [presynch treatment, Ovsynch duration, and dose frequency of PGF_{2α} (2×25 vs. 1×50 mg)] and their 2- and 3-way interaction terms.

P/AI. Pregnancy per AI at d 32 and 60 and intervening pregnancy loss were analyzed using the GLIMMIX procedure as described previously. In addition to the independent effects of season, parity, DIM, and BCS, BCS change from calving to the onset of the timed AI Ovsynch treatment and all 3 main effects and their 2- and 3-way interaction terms were included in the model.

Data are presented as LSM $\pm$ SE where appropriate. Differences among comparisons of either main effects or interactions were considered significant when $P <$

0.05, whereas differences between $0.05 < P \leq 0.10$ were considered to be statistical tendencies.

RESULTS AND DISCUSSION

General

Despite randomization of cows to treatments at calving, mean daily milk yield during the first 14 wk of lactation was greater ($P = 0.03$) in cows exposed to PG-3-G compared with Double Ovsynch (47.9 ± 0.4 vs. 45.9 ± 0.4 kg). Furthermore, cows treated with the 2×25 -mg doses of PGF_{2α} compared with the 1×50 -mg dose had greater ($P = 0.044$) 305-d ECM ($13,529 \pm 117$ vs. $13,197 \pm 117$ kg). These differences in milk traits were not likely related to treatments but rather resulted from other unquantified factors, random chance, or perhaps limited observations. As expected, however, multiparous cows produced more ($P < 0.001$) daily milk (54.3 ± 0.4 vs. 38.8 ± 0.5 kg) and more ($P < 0.001$) 305-d ECM ($14,549 \pm 108$ vs. $12,177 \pm 127$ kg) than primiparous cows.

Ovarian Cyclicity

One objective was to determine the value of the first GnRH injection that is part of Double Ovsynch versus PG-3-G to reduce the number of anovular cows that begin the timed AI program at GnRH-1 (PreG; Figure 1). Anovulation determined at the end of the voluntary waiting period in 5,818 records from 13 studies of 8 herds by assessing circulating progesterone concentrations or by visualization of a CL using transrectal ultrasonography showed an overall prevalence of 23.3% between 37 and 65 DIM (Bamber et al., 2009). These determinations were made approximately 10 d before AI occurred.

In contrast to some of the previously cited reports, cows in the present study had been exposed to either 1

(PG-3-G) or 2 (Double Ovsynch) doses of GnRH before assessing ovulation status. On the basis of progesterone concentrations ≥ 1 ng/mL at GnRH-1 and visual presence of luteal tissue in the present study, the proportions of cows that had initiated estrous cycles at the start of the Ovsynch program (63 ± 3 DIM) did not differ between PG-3-G and Double Ovsynch (Table 1). Concentrations of progesterone in cows at GnRH-1 representing the 8 treatment combinations are illustrated in Figure 2A. Concentrations of progesterone before GnRH-1 did not differ ($P = 0.21$) between PG-3-G and Double Ovsynch cows (3.4 ± 0.2 vs. 3.1 ± 0.2 ng/mL). Proportions of cows in various concentration categories at GnRH-1 did not differ between the 2 presynch treatments (Double Ovsynch and PG-3-G; <0.5 ng/mL: 8.7 vs. 6.9%; ≥ 0.5 and ≤ 3.0 ng/mL: 47.4 vs. 46.5%; >3.0 ng/mL: 43.9 vs. 46.5%). Furthermore, range and mean number of CL per cow, proportions of cows with multiple CL, and luteal tissue volume at GnRH-1 did not differ between PG-3-G and Double Ovsynch cows (Table 1). Characteristics of progesterone and presence of luteal tissue before the onset of Ovsynch (GnRH-1) are consistent with previous reports for cows exposed to PG-3-G (Stevenson et al., 2012) and Double Ovsynch (Herlihy et al., 2012) at similar DIM.

Of 406 cows in the study, 61 cows had concentrations of progesterone <1 ng/mL at GnRH-1 and 43 of 61 (70.5%) ovulated in response to GnRH-1 (65.5% for PG-3-G and 75% for Double Ovsynch). For the 43 cows that ovulated to GnRH-1, progesterone concentrations averaged 0.4 ± 0.1 ng/mL at GnRH-1 and increased ($P < 0.05$) to 1.7 to 3.8 ± 0.3 ng/mL at the time of PGF_{2α} administration depending on the duration of Ovsynch (5 or 7 d later). For 18 cows that failed to ovulate after GnRH-1, progesterone concentrations averaged 0.4 ± 0.1 ng/mL at GnRH-1 and increased ($P < 0.05$) to 1.2 to 3.6 ± 0.3 ng/mL at the time of PGF_{2α} depending on the duration of Ovsynch (5 or 7 d later). The latter 18 cows, which did not ovulate after GnRH-1, consisted of

Table 1. Ovarian responses (LSM $\pm$ SE, except percentages) to presynchronization treatments assessed before initiating either the 5- or 7-d Ovsynch treatment at GnRH-1

Item	Presynchronization ¹		P-value
	DO	PG-3-G	
Cows (no.)	201	205	
Luteal function before GnRH-1			
Progesterone ≥ 1 ng/mL (%)	86.2	87.9	0.63
Presence of luteal tissue (%)	97.5	98.5	0.39
Range in CL ² /cow (no.)	0–4	0–4	
CL/cow (no.)	1.2 ± 0.05	1.3 ± 0.05	0.18
Multiple CL (%)	22.0	24.6	0.53
Luteal tissue volume (cm ³)	6.6 ± 0.3	6.9 ± 0.3	0.56

¹Double Ovsynch (DO) = pre-GnRH-7 d–pre-PGF_{2α}-3 d–pre-GnRH. PG-3-G = PrePGF_{2α}-3 d–PreGnRH.

²Corpus luteum.

14 cows that had concentrations of progesterone that averaged 0.4 ± 0.1 ng/mL before GnRH-1 and increased ($P < 0.05$) to concentrations >2.0 ng/mL by 5 to 7 d later (i.e., earlier spontaneous ovulation), whereas the remaining 4 cows (2 in each presynch treatment) had concentrations of progesterone <0.1 ng/mL before and 5 to 7 d after GnRH-1. The latter 4 anovular cows had baseline concentrations of progesterone (<0.1 ng/mL) during the entire 8- to 10-d blood collection period.

Ovulation Response to GnRH-1

Neither single (60–66%) nor multiple (10–11%) ovulatory responses to GnRH-1 differed between PG-3-G and Double Ovsynch treatments (Table 2). Season had differing effects on multiple ovulation after GnRH-1 in cows treated with PG-3-G and Double Ovsynch. During the warm-hot season, multiple ovulation occurred more ($P < 0.05$) frequently in PG-3-G cows than Double

Figure 2. (A) Least squares means ($\pm$ SE) for progesterone concentration of the 8 treatment combinations before GnRH-1 and before PG_{2α} (PG) administration. No presynchronization (PRE) effect was detected before GnRH-1, whereas a tendency ($P = 0.07$) for an effect of Ovsynch (OVS) treatment duration was detected for progesterone concentrations before PG_{2α} administration. (B) Concentrations of progesterone at 24, 48, and 72 h after PG_{2α} (PG) administration. No presynch treatment was detected, but effects of PG_{2α} dose and Ovsynch duration were detected ($P \leq 0.05$) at 48- and 72-h sampling times in addition to a tendency ($P = 0.07$) for an interaction of PG_{2α} dose and Ovsynch treatment duration at 72 h. DO = Double Ovsynch; PG-5-2x25 = 5-d Ovsynch program with two 25-mg doses of PG administered 24 h apart; PG-5-1x50 = 5-d Ovsynch program with one 50-mg dose of PG; PG-7-1x50 = 7-d Ovsynch program with one 50-mg dose of PG. Color version available online.

Table 2. Ovarian responses (LSM $\pm$ SE, except percentages) to GnRH-1 administered 7 d after presynchronization treatments

Item	Presynchronization ¹		<i>P</i> -value
	DO	PG-3-G	
Cows (no.)	201	205	
Single ovulation (%)	65.8	60.2	0.25
Largest follicle diameter (mm)	13.3 $\pm$ 0.2	13.5 $\pm$ 0.2	0.48
Multiple ovulation ² (%)	10.2	11.2	0.80
Largest follicle diameter (mm)	15.4 $\pm$ 1.3	13.6 $\pm$ 1.1	0.26
Second largest follicle diameter ³ (mm)	12.2 $\pm$ 0.9	10.9 $\pm$ 0.8	0.26

¹Double Ovsynch (DO) = pre-GnRH-7 d-pre-PGF_{2α}-3 d-pre-GnRH. PG-3-G = pre-PGF_{2α}-3 d-pre-GnRH.

²Proportion of cows that ovulated more than 1 follicle after GnRH-1.

³Diameter of the second largest follicle that ovulated in multiple-ovulating cows.

Ovsynch cows (17.4 vs. 6.4%), whereas during the cool-cold season, the reverse was detected (8.6 vs. 13.6%). The number of follicles $\geq$ 5 mm in diameter tended ($P = 0.07$) to be greater in PG-3-G cows than Double Ovsynch cows, but diameters of the largest and second largest follicles in single- and multiple-ovulating cows did not differ between presynch treatments (Table 2).

Ovarian Characteristics Before PGF_{2α}

Number of CL per cow before administration of PGF_{2α} ranged from 1 to 5. Cows in PG-3-G and Double Ovsynch treatments had 2 CL per cow before the first or only PGF_{2α} treatment (51.2 vs. 56.7%) with 20.0 and 17.9% of the cows having 3 or more CL, respectively. Similar frequencies of CL per cow were detected between cows exposed to the 5- or 7-d Ovsynch program. Mean number of CL per cow exposed to treatments of presynch and different Ovsynch program durations before the 2 dose frequency treatments of PGF_{2α} averaged almost 2 CL (Table 3) and was not affected by either presynch or Ovsynch duration. Mean number of follicles $\geq$ 5 mm tended (interaction; $P = 0.08$) to be greatest in PG-3-G cows (2.9 $\pm$ 0.1) in the 5-d Ovsynch treatment, whereas that mean number was least in the PG-3-G cows exposed to the 7-d Ovsynch treatment

(2.5 $\pm$ 0.1). The largest ($P = 0.01$) and second largest ($P = 0.01$) follicles were consistently greater in the 7-d compared with the 5-d Ovsynch treatments (Table 3).

Luteolysis Treatment Effects

Concentrations of progesterone in cows representing the 8 treatment combinations before GnRH-1 administration are illustrated in Figure 2A. Among main effects, concentrations of progesterone before the Ovsynch PGF_{2α} administration did not differ between presynch treatments, whereas cows treated with the 5-d versus the 7-d Ovsynch program tended ($P = 0.07$) to have less progesterone (no interactions; Figure 2A; Table 4).

Actual concentrations of progesterone in cows at 24, 48, and 72 h after the first or only dose of PGF_{2α} representing the 8 treatment combinations are illustrated in Figure 2B. Concentrations of progesterone at 24 h were less ($P = 0.01$) in cows treated with the 1 $\times$ 50-mg dose compared with the 2 $\times$ 25-mg doses regardless of Ovsynch duration. At 48 h, 24 h after the second 25-mg dose, both doses (50 mg $<$ 25 mg; $P = 0.01$) and Ovsynch duration (7 d $<$ 5 d; $P = 0.04$) affected progesterone concentration (no interaction). At 72 h, Ovsynch duration ($P = 0.01$), PGF_{2α} dose ($P = 0.01$),

Table 3. Ovarian characteristics (LSM $\pm$ SE, except percentages) at the time of the first or only PGF_{2α} treatment in response to presynchronization (PRE) and Ovsynch (OVS) treatments¹

Item	Double Ovsynch		PG-3-G		<i>P</i> -value		
	5 d	7 d	5 d	7 d	PRE	OVS	PRE $\times$ OVS
Cows (no.)	100	101	102	103			
Largest follicle diameter (mm)	10.2 $\pm$ 0.3	11.1 $\pm$ 0.3	10.5 $\pm$ 0.3	11.5 $\pm$ 0.3	0.24	0.01	0.86
Second largest follicle diameter (mm)	8.0 $\pm$ 0.5	9.7 $\pm$ 0.5	8.6 $\pm$ 0.5	9.6 $\pm$ 0.6	0.66	0.01	0.45
Range in CL ² /cow (no.)	1-4	1-4	1-5	1-4			
CL/cow (no.)	1.9 $\pm$ 0.1	1.9 $\pm$ 0.1	2.0 $\pm$ 0.1	1.8 $\pm$ 0.1	0.84	0.60	0.50

¹Double Ovsynch = GnRH-7 d-PGF_{2α}-3 d-GnRH. PG-3-G = PGF_{2α}-3 d-GnRH. 5 d = GnRH-5 d-PGF_{2α}. 7 d = GnRH-7 d-PGF_{2α}. Both 5- and 7-d programs were initiated 7 d after the end of the presynchronization treatments (Double Ovsynch and PG-3-G).

²Corpus luteum.

and their interaction ($P = 0.07$) affected progesterone concentration.

At 24 h after the first or only PGF_{2α} dose, neither presynch nor Ovsynch treatments influenced the proportion of cows with progesterone concentration <1 ng/mL (Table 4), but a greater ($P < 0.01$) proportion of cows receiving the 1 × 50-mg dose of PGF_{2α} had decreased progesterone at 24 h compared with cows receiving the first of the 2 × 25-mg doses.

In contrast, at 48 and 72 h, more ($P < 0.05$) cows had progesterone <1 ng/mL in the 7- versus 5-d Ovsynch cows and more ($P < 0.01$) cows had progesterone <1 ng/mL when exposed to the 2 × 25-mg dose than the 1 × 50-mg dose (Table 4; no interactions, Figure 3). The same treatment differences were detected at 72 h when the threshold for luteolysis was set at <0.4 ng/mL (Table 4). An interaction ($P = 0.06$) between Ovsynch treatment duration and PGF_{2α} dose, however, was detected at 72 h in which the proportion of cows having progesterone <0.4 ng/mL was least in cows in the 5-d program when treated with the 1 × 50-mg dose (Figure 3).

In summary, although mean progesterone concentrations were <0.4 ng/mL at 48 and 72 h (Figure 2B), the single 1 × 50-mg dose was less ($P < 0.01$) effective than the 2 × 25-mg dose in further reducing mean concentrations of progesterone in the 5- versus 7-d Ovsynch-treated cows. Thus, the single larger dose was equally effective as the 2 × 25-mg dose in the 7-d Ovsynch program but less so when applied to cows in the shorter 5-d program. Consistent with results of the present study, applying a second standard dose of PGF_{2α} administered 24 h after the first standard dose to cows before first postpartum AI (7-d program at first service; Wiltbank

et al., 2015), after a nonpregnant diagnosis (5- and 7-d program + progesterone insert; Santos et al., 2016), or after a single larger dose of PGF_{2α} to nonpregnant cows compared with 2 standard doses administered 24 h apart (5-d program ± progesterone insert, Stevenson et al., 2013; 5-d program, Stevenson et al., 2014) maximized complete luteolysis before timed AI.

Luteolysis-Luteal Combination Effects

On the basis of luteal structures identified at GnRH-1 (original CL, if present) plus new CL formed in response to GnRH-1, and the combination of new and original CL identified before PGF_{2α}-induced luteolysis, concentrations of progesterone and their subsequent decrease in response to PGF_{2α} are summarized in Table 5. Seven of the 406 enrolled cows did not have a luteal structure at the time PGF_{2α} was administered (4 anovular cows and 3 cows had premature luteal regression after GnRH-1). Of 24 cows with only new CL at PGF_{2α}, 7 cows (28.2%) had 2 CL. Of 137 cows with only original CL at PGF_{2α}, 51 cows (37.2%) had 2 or more CL. Of 238 cows with new CL and original CL at PGF_{2α}, all had 2 or more CL (24 of 238 cows had 2 new CL). Mean number of CL detected in the new CL and original CL groups was less ($P < 0.05$) compared with the new CL plus original CL group (Table 5). Concentrations of progesterone were greater ($P = 0.001$) in cows with more than 1 CL compared with those with a single CL (5.79 ± 0.3 vs. 3.97 ± 0.2 ng/mL). Concentrations of progesterone increased linearly ($P < 0.001$) at 1.33 ± 0.2 ng/mL for each CL increase per cow (range of 1–5 CL/cow). Consistent with another report (Martins et al., 2017), concentrations of progesterone averaged 4.3

Table 4. Proportion of cows with progesterone concentrations <1 ng/mL at 24, 48, and 72 h after the first or only dose of PGF_{2α} or <0.4 ng/mL at 72 h

Item	Presynchronization ¹		Ovsynch ²		PGF _{2α} dose ³	
	DO	PG-3-G	5 d	7 d	2 × 25 mg	1 × 50 mg
Cows (no.)	201	205	202	204	203	203
Progesterone ⁴ (ng/mL)	5.4 ± 0.2	5.3 ± 0.2	5.0 ± 0.2 ^A	5.6 ± 0.2 ^B	5.4 ± 0.2	5.3 ± 0.2
Cows with progesterone <1 ng/mL (%)						
24 h	78.1	77.0	75.7	79.4	68.5 ^a	86.7 ^b
48 h	97.0	97.1	95.5 ^c	98.5 ^d	99.5 ^a	94.6 ^b
72 h	97.5	96.1	94.6 ^a	99.0 ^b	99.0 ^c	94.6 ^d
Cows with progesterone <0.4 ng/mL (%)						
72 h	93.5	90.7	86.1 ^a	98.0 ^b	96.6 ^a	87.7 ^b

^{a,b}Means differ ($P \leq 0.01$) within main effect category and time after PGF_{2α}.
^{c,d}Means differ ($P \leq 0.05$) within main effect category and time after PGF_{2α}.
^{A,B}Means tended ($P = 0.07$) to differ between Ovsynch treatment durations.
¹Double Ovsynch (DO) = GnRH-7 d-PGF_{2α}-3 d-GnRH. PG-3-G = PGF_{2α}-3 d-GnRH. Both were followed in 7 d by either the 5- or 7-d Ovsynch treatment.
²5 d = GnRH-1-5 d-PGF_{2α}. 7 d = GnRH-1-7 d-PGF_{2α}. Both were initiated 7 d after the end of the presynchronization treatments.
³Two 25-mg doses 24 h apart or one 50-mg dose.
⁴Concentration (LSM ± SE) before first or only PGF_{2α} administration.

Figure 3. Proportion of cows with progesterone <1 ng/mL at 24, 48, and 72 h after the first or only PGF_{2α} dose and the proportion of cows with progesterone <0.4 ng/mL at 72 h. Probability values for the effects of Ovsynch (OVS) treatment program duration and dose delivery of PGF_{2α} (PG) and their interaction are illustrated for each time period after first or only PGF_{2α} dose treatment. Color version available online.

± 0.3 , 5.4 ± 0.2 , and 7.1 ± 0.4 ng/mL for cows bearing 1, 2, or ≥ 3 CL at time of PGF_{2α} administration, respectively.

Concentrations of progesterone differed ($P \leq 0.05$) among CL groups, with the greatest concentration in the new CL plus original CL group (Table 5). Primiparous cows ($n = 170$) had greater ($P = 0.02$) concentra-

tions of progesterone than multiparous cows ($n = 236$; 5.29 ± 0.27 vs. 4.48 ± 0.24 ng/mL). Concentrations decreased by 24, 48, and 72 h after the first or only dose of PGF_{2α} with greater ($P \leq 0.05$) concentrations in the new CL plus original CL group compared with the other 2 groups at 24 h (Table 5). By 48 h, progesterone remained greater ($P \leq 0.05$) in the new CL group plus

Table 5. Concentration of progesterone before PGF_{2α} administration of Ovsynch based on age of luteal structures and subsequent decrease in progesterone associated with luteolysis at 24, 48, and 72 h after PGF_{2α}

Item	Luteal structure ¹		
	New CL	Original CL	New CL + original CL
Cows (no.)	24	137	238
CL/cow (no.)	1.2 ± 0.13^a	1.4 ± 0.05^a	2.3 ± 0.04^b
Range in CL _h /cow (no.)	1	1–4	1–5
Progesterone ² (ng/mL)			
0 h	3.22 ± 0.68^a	5.05 ± 0.27^b	5.77 ± 0.21^c
24 h	0.55 ± 0.17^{ab}	0.54 ± 0.07^a	0.77 ± 0.05^b
48 h	0.34 ± 0.10^{ab}	0.10 ± 0.04^a	0.23 ± 0.03^b
72 h	0.44 ± 0.09^a	0.07 ± 0.03^b	0.16 ± 0.03^c
Cows with progesterone <1 ng/mL (%)			
24 h	83.3 ^a	80.3 ^a	74.8 ^a
48 h	87.5 ^a	99.3 ^b	96.6 ^b
72 h	87.5 ^a	100.0 ^b	95.8 ^a
Cows with progesterone <0.4 ng/mL (%)			
72 h	83.3 ^a	98.5 ^b	92.8 ^c
Pregnancy/AI 32 d after AI (%)	26.1	35.8	40.5

^{a-c}Means within a row differ ($P \leq 0.05$).

¹Age of luteal structures before PGF_{2α} administration: original corpus luteum (CL) was present at GnRH-1 and new CL formed after GnRH-1. Seven of the enrolled 406 cows did not have a luteal structure at PGF_{2α} administration. Where appropriate, values are LSM $\pm$ SEM.

²Concentration of progesterone at various hours after first or only PGF_{2α} administration.

original CL group compared with the original CL group but did not differ from the new CL group. At 72 h, the lowest ($P \leq 0.05$) concentration was detected in the original CL group compared with the other 2 CL groups, which had new, younger CL.

The proportion of cows with progesterone <1 ng/mL at 24 h after the first or only dose of PGF_{2α} did not differ among CL groups. At 48 h, a greater ($P \leq 0.01$) proportion of cows bearing original CL or the combination of original and new CL had concentrations of progesterone <1 ng/mL compared with cows bearing new CL. At 72 h, all cows in the original CL group had concentrations <1 ng/mL, which differed from the proportions of cows in the other 2 CL groups. When the threshold was set at <0.4 ng/mL, all 3 CL groups differed ($P \leq 0.05$) in the proportion of cows at that lower threshold, with the greatest proportion in the original CL group. Poorer complete regression of new CL in the present study is consistent with reports that CL <10 d old are resistant to complete regression after a single standard dose of PGF_{2α} (Momont and Seguin, 1984). Day 5 luteal structures in nonlactating dairy cows are equally resistant to two 25-mg doses (administered 8 h apart on d 5) or one 50-mg dose of PGF_{2α} (administered on d 5; Nascimento et al., 2014). Consistent with the latter reports, more than 96% of cows in the present study had progesterone concentrations <1 ng/mL by 48 h after PGF_{2α}, indicating earlier and more complete luteolysis compared with cows having only a new, younger CL.

That more cows with both older and new CL had more complete luteolysis than cows with only a new, younger CL is consistent with our previous report (Stevenson, 2016). Earlier luteolysis of new CL is also consistent with the report that human chorionic gonadotropin (hCG)-induced luteal structures or CL on d 10 of the estrous cycle were responsive to PGF_{2α} when the new CL or hCG-induced luteal structures were less than 5 d old when they were in the presence of an older CL (Howard and Britt, 1990). In that study, the authors concluded that CL induced by treatment with hCG on d 10 of the estrous cycle regress after exogenous PGF_{2α} by 2 d after ovulation, whereas spontaneously formed CL respond to PGF_{2α} only after 5 d. The latter response of new CL to PGF_{2α} could be unique to CL that form in response to hCG in the presence of a more mature CL. Regression of younger CL in the presence of a more mature CL is intriguing because high-affinity PGF_{2α} receptors were identified in CL recovered from plasma membranes of CL of heifers on d 2, 4, 6, or 10 (d 0 = ovulation), suggesting that the reported lack of responsiveness to PGF_{2α} in the early CL may not be attributable to a deficiency of high-affinity PGF_{2α} receptors (Wiltbank et al., 1995).

One avenue by which the younger CL regresses more successfully in the presence of a more mature CL could be attributed to the luteal oxytocin enhancement of uterine PGF_{2α} secretion generated by the larger and more mature CL, thus inhibiting progesterone synthesis via a low-sensitivity state of the PGF_{2α} receptor and, at the same time, releasing more oxytocin in a closed loop fashion (McCracken, 1998).

Progesterone profiles differed between cows with a mature CL and those with a new plus mature CL at the time PGF_{2α} was administered, as we observed in an earlier report of combined studies consisting of 2,104 cows (Stevenson, 2016). Although no differences were detected in pregnancy risk between those latter 2 groups of cows in the present study (Table 5), with sufficient observations in the latter publication, pregnancy risk was greater in cows having ovulated in response to GnRH-1 (mature CL plus new CL; 42.4%) compared with cows having only a mature CL (37.0%). This improvement in pregnancy risk coupled with a small increase in the concentration of progesterone (6.0 ng/mL) in the former compared with the latter group (5.3 ng/mL) before the administration of PGF_{2α} as observed in the present study (5.8 vs. 5.0 ng/mL; Table 5) was associated with positive benefits of the elevated progesterone environment for the growth of the pre-ovulatory follicle or oocyte, uterine function, or both. Pregnancy risk was the least in cows bearing only a new CL in the present study (26.1%) and in Stevenson (2016; 31.1%) in addition to having lesser progesterone concentration at PGF_{2α}. Taken together, small to significant increases in progesterone concentration and likely improved synchronization of the developing preovulatory follicle and its oocyte seemed to facilitate the greater pregnancy risk in Stevenson (2016), as observed in the present study (Table 5) for cows having both the new CL plus more mature CL compared with cows having only a mature luteal structure or only a new CL. Because the cows with a new CL also had a synchronized developing preovulatory follicle as in the cows with both a new CL and a more mature CL, the lower concentrations of progesterone emphasize the importance of a greater progesterone milieu during follicular development to the subsequently greater pregnancy outcome.

Follicular and Ovulation Responses to GnRH-2

The mean number of follicles ≥ 5 mm in single- ($P = 0.05$) and multiple-ovulating ($P = 0.05$) cows at 48 h after the first or only PGF_{2α} administration was greater in PG-3-G cows (2.5 ± 0.1 and 3.4 ± 0.1) compared with Double Ovsynch cows (2.2 ± 0.1 and 2.8 ± 0.1). The second largest follicle in multiple-ovulating cows

Table 6. Ovarian characteristics (LSM $\pm$ SEM, except percentages) in response to GnRH-2 after presynchronization (PRE) and Ovsynch (OVS) treatments¹

Item	Double Ovsynch		PG-3-G		<i>P</i> -value		
	5 d	7 d	5 d	7 d	PRE	OVS	PRE $\times$ OVS
Cows (no.)	100	101	102	103			
Single ovulation (%)	95.2	95.3	89.2	91.9	0.06	0.65	0.72
Largest follicle diameter (mm)	13.0 $\pm$ 0.3	13.4 $\pm$ 0.5	12.9 $\pm$ 0.2	13.6 $\pm$ 0.3	0.90	0.02	0.62
Multiple ovulation ² (%)	14.3	13.7	30.1	10.3	0.27	0.01	0.03
Largest follicle diameter (mm)	12.2 $\pm$ 0.7	12.6 $\pm$ 0.7	12.6 $\pm$ 0.5	13.0 $\pm$ 0.8	0.53	0.57	0.98
Second largest follicle ³ (mm)	11.0 $\pm$ 0.4	12.4 $\pm$ 0.5	11.0 $\pm$ 0.4	11.8 $\pm$ 0.5	0.50	0.01	0.49

¹Double Ovsynch = GnRH-7 d-PGF_{2α}-3 d-GnRH. PG-3-G = PGF_{2α}-3 d-GnRH. 5 d = GnRH-5 d-PGF_{2α}. 7 d = GnRH-7 d-PGF_{2α}. Both 5- and 7-d programs were initiated 7 d after the end of the presynchronization treatments.

²Proportion of cows that ovulated more than 1 follicle after GnRH-1.

³Diameter of the second largest follicle that ovulated in multiple-ovulating cows.

was greater ($P = 0.01$) in 7-d than 5-d Ovsynch-treated cows in the present study (Table 6). The largest follicle in single-ovulating cows was greater ($P = 0.02$) in 7-d compared with 5-d Ovsynch treatments (Table 6), consistent with a previous report in which the largest follicle and concentrations of plasma estradiol also were greater in cows exposed to a 7-d versus 5-d program (Santos et al., 2010).

A tendency ($P = 0.06$) occurred for a greater percentage of Double Ovsynch cows to single ovulate after GnRH-2 compared with PG-3-G cows (95.3 vs. 90.6%). Neither duration of Ovsynch (5 vs. 7 d) nor the interaction between presynch and Ovsynch main effects altered single ovulation response to GnRH-2. Single ovulatory response to GnRH-2 tended ($P = 0.07$) to be greater during the cooler-cold season than during the warmer-hot season (94.2 vs. 88.3%), whereas season $\times$ presynch interaction effect occurred for multiple ovulation after GnRH-1. No such effect was detected after GnRH-2.

An interaction ($P = 0.05$) of presynch and Ovsynch was detected for multiple ovulation after GnRH-2 in which an unexpected greater proportion of PG-3-G cows treated in the 5-d Ovsynch program had multiple ovulations compared with the remaining treatment combinations (Table 6). Increased incidence of multiple ovulation was restricted to the PG-3-G cows exposed to the 5-d Ovsynch treatment duration (interaction; $P = 0.03$; Table 6). Season had no effect on the frequency of multiple ovulation. Duration of Ovsynch did not affect the incidence of either single or double ovulation response to GnRH-2 (Santos et al., 2010).

Pregnancy Outcomes

Seasonal effects were detected for P/AI, reflecting the expected nadirs in fertility during summer and early autumn in both years of the study (Figure 4). As

expected, P/AI tended ($P = 0.06$) to be greater during the cooler-cold months compared with the warmer-hot season (40.2 vs. 30.0%). No overall differences, however, were detected in P/AI between any of the main effects: presynch, Ovsynch duration, or PGF_{2α} dose frequencies (Table 7). Two interaction trends, however, were detected for P/AI (Figure 5A). The greatest P/AI was observed in the PG-3-G cows in a 7-d program compared with the other treatment combinations (interaction of presynch and Ovsynch treatment duration; $P = 0.07$). Furthermore, because of the differences in the effectiveness of PGF_{2α} dose frequencies to induce complete luteolysis in the 2 Ovsynch duration programs (decreasing progesterone after PGF_{2α} during the timed AI week), an interaction ($P = 0.07$) between Ovsynch duration and PGF_{2α} dose frequency also was detected for P/AI (Figure 5B). Cows receiving the 1 $\times$ 50-mg dose in the 7-d program had the greatest P/AI, and the least P/AI occurred in the cows receiving the 1 $\times$ 50-mg dose in the 5-d program.

Cows that maintained or gained BCS ($n = 226$) between calving and the onset of the Ovsynch programs had greater ($P < 0.05$) P/AI (41.6 vs. 32.3%) than those that lost BCS ($n = 170$), but P/AI in cows with BCS ≤ 2.5 ($n = 266$) at timed AI did not differ from that of cows with BCS > 2.5 ($n = 129$; 33.7 vs. 40.2%). The negative effect on P/AI was greater for cows that lost body condition during the first 3 wk postpartum compared with those that either maintained or gained BCS (Carvalho et al., 2014). Furthermore, multiparous cows ($n = 55$) losing 7 to 17% more BW than average between the first and fifth weeks postpartum had poorer P/AI for the combined first through third inseminations compared with 37 multiparous cows that lost 3 to 6% of BW (Zachut and Moallem, 2017). The pattern of postpartum BW loss in the previous study across several lactations indicated an inherent trait with a genetic basis (Friggens et al., 2007).

Table 7. Pregnancy per AI (%; 32 d after AI) in lactating dairy cows according to presynchronization treatment, Ovsynch treatment duration, and dose frequency of PGF_{2α} and their interactions^{1,2}

Treatment	5-d Ovsynch		7-d Ovsynch		DO vs. PG-3-G ³
	2 × 25	1 × 50	2 × 25	1 × 50	
DO	38.7	35.0	30.0	39.1	35.6 (n = 194)
PG-3-G	39.4	25.2	44.0	52.1	40.0 (n = 201)
Ovsynch ⁴	34.3 (n = 196)		41.1 (n = 199)		

¹Eleven of the 406 enrolled cows were culled before pregnancy diagnosis because of disease, mastitis, or low production. Interaction tendencies ($P = 0.07$) were detected for presynchronization by Ovsynch treatment and Ovsynch treatment by PGF_{2α} dose frequency (see Figure 5).

²Double Ovsynch (DO) = GnRH-7 d-PGF_{2α}-3 d-GnRH. PG-3-G = PGF_{2α}-3 d-GnRH. 5 d = GnRH-5 d-PGF_{2α}. 7 d = GnRH-7 d-PGF_{2α}. Both 5- and 7-d programs were initiated 7 d after the end of the presynchronization treatments. Comparison of PGF_{2α} dose frequency main effects ($P = 0.92$): two 25-mg doses 24 h apart (2 × 25 mg = 37.9%; n = 198) versus one 50-mg dose (1 × 50 mg = 37.4%; n = 197).

³Main effect comparison of DO versus PG-3-G ($P = 0.42$).

⁴Main effect of 5-d versus 7-d Ovsynch ($P = 0.20$).

General Discussion

One objective was to determine whether the additional GnRH treatment (PreG; Figure 1) in the presynchronization portion of Double Ovsynch reduced the proportion of anovulatory cows compared with the PG-3-G presynch program. The advantages of these GnRH-PGF_{2α} presynch programs compared with standard presynch PGF_{2α} programs include inducing ovulation in anovulatory cows and increasing P/AI (Borchardt et al., 2017). In addition, GnRH is efficacious at inducing ovulation in anovular cows (Gümen et al., 2003). No

differences were detected between the 2 GnRH-PGF_{2α} presynch programs tested in the present study for the proportion of ovular and anovular cows beginning the Ovsynch program (Table 1). What was unknown in the present study was the incidence of anovulation before treatments were applied. Regardless of the magnitude of anovulation before presynchronization treatments, cows in the present study had been exposed to either 1 (PG-3-G) or 2 (Double Ovsynch) doses of GnRH before assessing ovulation status. That only 4 cows were truly anovular, having basal progesterone concentrations (<0.2 ng/mL) during the entire sampling period

Figure 4. Pregnancies per AI (P/AI; vertical bars) assessed at d 32 post-timed AI in the 80 breeding clusters of cows inseminated during the experiment superimposed over the maximum daily heat index (temperature-humidity index; continuous line; NOAA, 1976) during the same period. Broken line (black) represents the P/AI trend line (fifth-order polynomial). Color version available online.

despite receiving 3 or 4 GnRH treatments, could be interpreted that both presynchronization treatments were equally effective in initiating estrous cycles or that the incidence of anovulation was very low. According to previous reports, the incidence of anovulation in 19 herds ranged from 7.3 to 41.7% and was assessed before the end of the voluntary waiting periods, ranging from 37 to 65 DIM (Bamber et al., 2009).

The remaining objectives were to determine the efficacy of applying 1 larger single dose (50 mg) of $PGF_{2\alpha}$

to produce complete luteolysis (<0.4 ng/mL) similar to 2 standard 25-mg doses given 24 h apart in cows exposed to either the 5-d or the 7-d Ovsynch program. It is clear that when the 7-d Ovsynch program was used, use of 1 larger single dose of $PGF_{2\alpha}$ produced complete luteolysis as effectively as 2 standard doses administered 24 h apart. In contrast, when applying the 5-d Ovsynch program, complete luteolysis assessed at 72 h after the first or only $PGF_{2\alpha}$ treatment was compromised in cows receiving the single larger dose

Figure 5. Pregnancy per AI (P/AI) at d 32 after AI. (A) Interaction tendency ($P = 0.07$) between presynchronization (PRE) treatments (Double Ovsynch vs. PG-3-G; $PGF_{2\alpha}$ – 3 d – GnRH – 7 d – Ovsynch) and Ovsynch (OVS) treatment duration (5 vs. 7 d). (B) Interaction tendency ($P = 0.07$) between Ovsynch treatment duration (5 vs. 7 d) and $PGF_{2\alpha}$ (PG) dose frequency (2 $\times$ 25-mg doses vs. 1 $\times$ 50-mg dose). No 3-way interaction was detected. Eleven cows were culled before pregnancy diagnosis. Color version available online.

(85%) compared with the other 3 combinations of dose and Ovsynch duration (>96%; Figure 3). More important, P/AI also tended to be compromised in the cows receiving the single larger dose and exposed to the 5-d Ovsynch program (Figure 5B).

Our results for complete luteolysis corroborate those in a previous study (Ribeiro et al., 2012a) in which a single larger dose of cloprostenol was administered (1×1 mg vs. 2×0.5 mg) to cows exposed to a 5-d Ovsynch program before first service. Luteolysis (progesterone <1 ng/mL 72 h after PGF_{2α}) in the latter study was greater in cows receiving the 2 doses versus 1 dose of cloprostenol (95.9 vs. 72.2%), but especially in cows whose estrous cycles were presynchronized with GnRH and PGF_{2α} (96.2 vs. 61.7%). In a second experiment (Ribeiro et al., 2012a) using dinoprost, cows exposed to a 5-d Ovsynch program and receiving 2×25 -mg doses of PGF_{2α} compared with a 1×50 -mg dose had greater P/AI on d 35 (44.5 vs. 36.4%). No study was found in which the 2 PGF_{2α} dose frequencies were tested in postpartum first-service cows exposed to either the 5-d or 7-d Ovsynch program.

A recent study tested the ability of the 50-mg dose versus 1 or 2 standard doses of PGF_{2α} in a 7-d program to maximize complete luteolysis in cows exposed to a timed AI Ovsynch program after a nonpregnant diagnosis (Barletta et al., 2018). In their experiment, the proportion of cows with complete luteolysis (progesterone <0.4 ng/mL at 56 h after PGF_{2α}) was not improved overall by their treatments, but the orthogonal comparison between the 1×50 -mg and 2×25 -mg doses differed significantly (88 vs. 94%). Pregnancy risk at d 39 after AI did not differ among treatments, but the comparison between one 50-mg dose and two 25-mg doses tended to differ in magnitude. No comparisons were made between the 1 standard 25-mg dose and 1 larger dose of PGF_{2α} in that study. In another study of similar design of nonpregnant cows treated with the 5-d program in which 2 standard 25-mg doses 24 h apart were compared with the larger single 50-mg dose of PGF_{2α}, complete luteolysis (progesterone <0.5 ng/mL) by 72 h after the first or only dose of PGF_{2α} was greater in cows receiving the 2 doses (93 vs. 78%), and resulting pregnancy risk was less in cows of 2 herds receiving the 1×50 -mg dose of PGF_{2α} (Stevenson et al., 2014).

Responses of luteal structures to PGF_{2α} to complete luteolysis are essential to improve P/AI. The literature seems to support the application of 2 standard doses of PGF_{2α} in both the 5- and 7-d programs to maximize complete luteolysis, but the subsequent increase in P/AI in the 12 herds studied was marginal from 34.4 to 37.6% in first-service cows (7-d program) and only significant in second- and third-lactation cows (Wiltbank et al., 2015). The greatest enhancement in

fertility in the previous report occurred in cows having the greatest progesterone concentration before PGF_{2α} and not in cows with low progesterone (<2 ng/mL) before the first of the 2 PGF_{2α} treatments, consistent with our previous findings (Stevenson, 2016), whereas the greatest improvement in luteolysis in cows exposed to 2 standard doses of PGF_{2α} occurred in cows with progesterone concentrations between 2 and 4.8 ng/mL. In another study of similar design in which previously inseminated nonpregnant cows were enrolled, a greater proportion of cows receiving 2 standard doses of PGF_{2α} in the 7-d program had concentrations of progesterone ≤0.4 ng/mL at GnRH-2 compared with cows receiving 1 standard dose in the 7-d program (Santos et al., 2016). Pregnancy per AI was increased from 36 to 41% in cows receiving the additional dose of PGF_{2α}. A third study (Colazo and Ambrose, 2015) compared the 5-d program with 2 PGF_{2α} treatments with the 7-d program with 1 PGF_{2α} treatment (both programs include CIDR inserts for 5 or 7 d) but did not detect any difference in P/AI between treatments (34.1 vs. 38.7%). A fourth study compared a 5-d and 7-d program in lactating dairy cows (Santos et al., 2010), but unlike the beef cattle studies (Bridges et al., 2008), only cows in the 5-d program received 2 PGF_{2α} treatments, whereas cows in the 7-d program received only 1 PGF_{2α} treatment. Although P/AI was greater for cows in the 5-d program (37.9 vs. 30.9%), duration of Ovsynch and frequency of PGF_{2α} treatment was confounded because the 2 standard doses were tested only in cows exposed to the 5-d Ovsynch program and not in cows also exposed to the 7-d Ovsynch program. Because a single 50-mg dose was effective in causing luteolysis in the 7-d cows but less so in the 5-d cows, when applying the shorter 5-d program, the two 25-mg doses administered 24 h apart are recommended to maximize complete luteolysis.

Because progesterone concentrations at or near baseline at the final GnRH treatment influence the characteristics of GnRH-induced luteinizing hormone release and subsequent P/AI (Stevenson and Pulley, 2016), achieving complete CL regression is important to maximize subsequent P/AI. Nevertheless, the greatest improvement in CL regression in response to 2 treatments of PGF_{2α} compared with 1 treatment (67 to 95%) was observed in cows with lesser concentrations of progesterone at the time of PGF_{2α} (Wiltbank et al., 2015), indicating a potential nonluteolytic role for the second PGF_{2α} treatment. A recent study (Ambrose et al., 2015) consisting of 2 experiments demonstrated increased P/AI in lactating dairy cows exposed to 10 mg but not 5 mg of PGF_{2α} administered at the time of an Ovsynch timed AI. Apart from its primary luteolytic role, PGF_{2α} has multiple actions on the female reproductive system in cattle (Weems et al., 2006), including

altered oviductal and uterine motility in addition to known post-luteinizing hormone surge effects of $PGF_{2\alpha}$ in the ovary and preovulatory follicle, such as lysosomal enzyme activity, contraction of ovarian smooth muscle, and increased follicular pressure (Espey, 1994).

Potential advantages of these GnRH- $PGF_{2\alpha}$ presynch programs include inducing ovulation in anovulatory cows in addition to decreasing the percentage of cows with lower circulating progesterone concentrations (<0.50 ng/mL) at GnRH-1 (Ayres et al., 2013), increasing the percentage of cows with medium progesterone concentrations ($0.50 < \text{progesterone} \leq 3.0$ ng/mL) at GnRH-1, and increasing the proportion of cows with a CL at GnRH-1 (Stevenson, 2016). In addition, these GnRH- $PGF_{2\alpha}$ presynch programs increase the percentage of cows with high progesterone (>3.0 ng/mL) at the $PGF_{2\alpha}$ treatment that immediately precedes timed AI and tend to increase average circulating progesterone at $PGF_{2\alpha}$ (Ayres et al., 2013; Stevenson et al., 2014). These observations are consistent with greater progesterone concentration in cows of the present study that had an original CL plus new CL for both luteal regression and greater P/AI compared with cows with lower progesterone before $PGF_{2\alpha}$ and likely poorer complete luteal regression and poorer P/AI (Table 5; Stevenson, 2016). Furthermore, elevated progesterone during the period of follicular development before $PGF_{2\alpha}$ -induced luteolysis is critical to uterine function because the stage of the estrous cycle and progesterone play important roles in modulating AA in the histotroph, a potentially critical factor for early embryonic or conceptus survival (Mullen et al., 2014).

Reducing the period of ovulatory follicle dominance by shortening the interval from follicle recruitment to luteal regression in the 5-d versus 7-d program resulted in less plasma estradiol at AI but increased pregnancy risk in the cows exposed to the 5-d versus 7-d program only when progesterone was <1 ng/mL at AI (Santos et al., 2010). Follicle size 24 h before AI was larger in cows exposed to the 7-d program in the present study with the expectation of greater concentrations of estradiol (Santos et al., 2010), and pregnant cows have greater concentration of estradiol at AI than nonpregnant cows (Ribeiro et al., 2012b). Regardless of follicle size before AI in the present study, pregnancy outcome was associated with $PGF_{2\alpha}$ dose frequency to initiate complete luteolysis and prior presynch treatment (Figure 5). Cows in the 5-d program had reduced P/AI when proestrus was restricted to 58 compared with 72 h when presynchronization included only $PGF_{2\alpha}$ rather than $PGF_{2\alpha}$ and GnRH (Ribeiro et al., 2012b).

In summary, we were unable to detect any benefit to the additional GnRH injection (PreG; Figure 1) that is part of Double Ovsynch compared with PG-3-G pre-

synch treatments. The 2×25 -mg doses and the 1×50 -mg dose induced complete luteolysis in both Ovsynch treatment durations, but the 1×50 -mg dose was less effective in the 5-d program. More P/AI tended to occur in the PG-3-G cows in the 7-d program compared with the other treatment combinations (Ovsynch $\times$ presynch interaction). In addition, an Ovsynch $\times$ $PGF_{2\alpha}$ dose frequency interaction resulted in cows receiving the 1×50 -mg dose in the 7-d program having the greatest P/AI, and the least P/AI occurred in cows receiving the 1×50 -mg dose in the 5-d program. Therefore, complete luteolysis was less effective in the 5-d program when the 1×50 -mg dose was applied, but both $PGF_{2\alpha}$ dose frequencies effectively induced complete luteolysis in the 7-d program. Treatments producing complete luteolysis tended to be related to subsequent P/AI.

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