Adding a second prostaglandin $F_{2\alpha}$ treatment to but not reducing the duration of a PRID-Synch protocol increases fertility after resynchronization of ovulation in lactating Holstein cows

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ABSTRACT

Our objective was to evaluate the effect of a second $PGF_{2\alpha}$ treatment and duration of an Ovsynch protocol that included a progesterone-releasing intravaginal device (PRID) on progesterone (P4) concentrations and pregnancies per artificial insemination (P/AI) after resynchronization of ovulation and timed artificial insemination (TAI). Lactating Holstein cows ($n = 821$) were assigned randomly at a nonpregnancy diagnosis (d 0) to 3 resynchronization protocols: (1) GnRH, d 0; $PGF_{2\alpha}$, d 7; GnRH, d 9.5 (7D1PGF); (2) GnRH, d 0; $PGF_{2\alpha}$, d 7; $PGF_{2\alpha}$, d 8; GnRH, d 9.5; (7D2PGF); or (3) GnRH, d 2; $PGF_{2\alpha}$, d 7; $PGF_{2\alpha}$, d 8; GnRH, d 9.5 (5D2PGF). All cows received a PRID at the first GnRH treatment of the resynchronization protocol, which was removed at the first $PGF_{2\alpha}$ treatment, and all cows received TAI approximately 16 h after the second GnRH treatment. Blood samples were collected from a subgroup of cows at each treatment of the resynchronization protocols. At 32 d after TAI, cows receiving a second $PGF_{2\alpha}$ treatment (7D2PGF + 5D2PGF cows) had more P/AI (42.6 vs. 35.7%) than cows receiving a single $PGF_{2\alpha}$ treatment (7D1PGF cows). For cows treated with a second $PGF_{2\alpha}$ treatment, decreasing the duration of the protocol did not increase P/AI (41.4 vs. 43.8% for 7D2PGF vs. 5D2PGF cows). At 60 d after TAI, P/AI did not differ between cows treated with the 1 $PGF_{2\alpha}$ (7D1PGF cows) or 2 $PGF_{2\alpha}$ (7D2PGF + 5D1PGF cows) treatments (32.5 vs. 37.9%, respectively). In addition, reducing the duration of the protocol did not increase P/AI at 60 d after TAI (37.8 vs. 38.5% for 7D2PGF vs. 5D2PGF cows). Pregnancy loss from 32 to 60 d after TAI was not affected by the number of $PGF_{2\alpha}$ treatments (8.5 vs. 10.6%, for 7D1PGF vs. 7D2PGF +

5D2PGF cows) or the duration of the protocol (9.1 vs. 12.1%, for 7D2PGF vs. 5D2PGF cows). The percentage of cows with incomplete luteal regression at the second GnRH treatment tended to differ among treatments and was lowest for 7D2PGF cows, intermediate for 5D2PGF cows, and greatest for 7D1PGF cows (1.9 vs. 6.9 vs. 11.0%, respectively). In conclusion, addition of a second $PGF_{2\alpha}$ treatment tended to decrease the percentage of cows with incomplete luteal regression and increased P/AI 32 d after AI, whereas decreasing the duration of the Ovsynch protocol did not increase P/AI.

Key words: resynchronization, timed artificial insemination, fertility, dairy cow

INTRODUCTION

Ovsynch protocols that allow for timed artificial insemination (TAI; Pursley et al., 1995) use sequential treatments of GnRH and $PGF_{2\alpha}$ in a coordinated fashion and allow cows to be inseminated without the need for detection of estrus (Pursley et al., 1997). Modifications of the Ovsynch protocol to include pre-synchronization with 2 $PGF_{2\alpha}$ treatments administered 10 to 14 d apart (Presynch-Ovsynch, Moreira et al., 2001; El-Zarkouny et al., 2004; Navanukraw et al., 2004; Galvão et al., 2007) or a combination of $PGF_{2\alpha}$ and GnRH (G-6-G, Bello et al., 2006; Double-Ovsynch, Souza et al., 2008) increase P/AI by optimizing the responses to each treatment of the Ovsynch protocol (Vasconcelos et al., 1999; Bello et al., 2006; Ayres et al., 2013). Of particular importance to the present study, recent modifications of the Ovsynch protocol may include a decrease in the interval between the first GnRH treatment and the $PGF_{2\alpha}$ treatment of the Ovsynch protocol (Bridges et al., 2008; Santos et al., 2010) and addition of a second $PGF_{2\alpha}$ treatment administered 24 h after the first (Brusveen et al., 2009; Carvalho et al., 2015a).

Decreasing the interval between the first GnRH and the $PGF_{2\alpha}$ treatments from 7 d (7-d protocol) to 5 d

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(5-d protocol) was first described in a series of experiments using beef cows (Bridges et al., 2008). Although timing of AI after the PGF_{2α} treatment differed for cows in the 7-d protocol and those in the 5-d protocol, more cows become pregnant in the 5-d protocol than in the 7-d protocol (80 vs. 66.7% in Exp. 3, and 65.3 vs. 56.2% in Exp. 4 of Bridges et al., 2008). In 2010, the 5-d protocol was tested in lactating dairy cows and was compared with the 7-d protocol (Santos et al., 2010). In that study, cows in the 5-d protocol received 2 PGF_{2α} treatments, whereas cows in the 7-d protocol received only one PGF_{2α} treatment. Pregnancies/AI (P/AI) were greater for cows in the 5-d protocol than for those in the 7-d protocol (37.9 vs. 30.9%). Similarly, Colazo and Ambrose (2015) compared the 5-d protocol with 2 PGF_{2α} treatments to the 7-d protocol with 1 PGF_{2α} treatment; however, P/AI did not differ between treatments (38.7 vs. 34.1%).

Incomplete luteal regression, measured as progesterone (P₄) ≥ 0.4 ng/mL at the last GnRH treatment, is associated with decreased P/AI (Brusveen et al., 2009; Carvalho et al., 2015a). In a recent study evaluating the relationship between P₄ concentrations at the last GnRH treatment and fertility in 3,148 cows, P/AI were 41% (1,125/2,713) for cows with P₄ < 0.4 ng/mL compared with only 14% (61/435) for cows with P₄ ≥ 0.4 ng/mL (Carvalho et al., 2015b). Modifications of the Ovsynch protocol (7-d protocol) to include a second PGF_{2α} treatment increased the percentage of cows with complete luteal regression from 80% to 98% at both first and resynch TAI, and increased P/AI by 6 to 12 percentage points at first and resynch TAI, respectively (Brusveen et al., 2009; Carvalho et al., 2015a).

Although the 5-d protocol produced more P/AI in the studies mentioned above, it is not clear if the increased fertility resulted from the decreased duration of the Ovsynch protocol or inclusion of a second PGF_{2α} treatment. Therefore, our objective was to evaluate the effects of addition of a second PGF_{2α} treatment and a decrease in the duration of the Ovsynch protocol on P₄ concentrations and P/AI after resynchronization of ovulation in lactating Holstein cows receiving a PRID. Our hypotheses were that addition of a second PGF_{2α} treatment would increase P/AI by decreasing the percentage of cows with incomplete luteal regression, whereas decreasing the duration of the Ovsynch protocol would have no effect on P/AI.

MATERIALS AND METHODS

All animal handling and experimental procedures were approved by the Animal Care and Use Committee of the College of Agricultural and Life Sciences at the University of Wisconsin–Madison.

Cows, Housing, and Feeding

This study was conducted from October 2014 to July 2015 on 3 commercial dairy herds in Portugal. Lactating Holstein cows ($n = 821$) were milked twice daily at approximately 12-h intervals in herd A, and thrice daily at approximately 8-h intervals in herds B and C. Cows were fed twice daily (herds A and B) or once daily (herd C) a TMR consisting of corn silage and alfalfa hay as forage with corn and soybean meal-based concentrate formulated to meet or exceed the minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Cows were housed in freestall barns bedded with mattresses (herds A and C) or sand (herd B) and had ad libitum access to feed and water. Primiparous cows were housed separately from multiparous cows. The rolling herd average and daily milk production were 10,950 kg and 35 kg/cow per day, respectively, with 3.8% fat and 3.3% protein for herd A; 11,160 kg and 38 kg/cow per day with 4.2% fat and 3.3% protein for herd B; and 10,900 kg and 35 kg/cow per day with 3.8% fat and 3.2% protein for herd C, during the experiment.

Treatments and AI

Within each herd, cows with at least one AI service after a nonpregnancy diagnosis were randomly assigned within parity (primiparous vs. multiparous) to 1 of 3 resynchronization protocols (Figure 1): (1) GnRH, d 0; PGF_{2α}, d 7; GnRH, d 9.5 (**7D1PGF**); (2) GnRH, d 0; PGF_{2α}, d 7; PGF_{2α}, d 8; GnRH, d 9.5); (**7D2PGF**); or (3) GnRH, d 2; PGF_{2α}, d 7; PGF_{2α}, d 8; GnRH, d 9.5 (**5D2PGF**). All cows received a progesterone-releasing intravaginal device (PRID Delta) at the first GnRH treatment of the resynchronization protocols that was removed at the first PGF_{2α} treatment. All cows received TAI approximately 16 h after the second GnRH treatment of the protocols. The GnRH (100 µg/dose of gonadorelin diacetate tetrahydrate, Ovarelin), the PGF_{2α} (25 mg/dose of dinoprost tromethamine, Enzaprost-T), and the PRID Delta (1.55 g of progesterone, PRID Delta) were from CEVA Santé Animale (Libourne, France). Cows detected in estrus between study d 7 and 9 were inseminated 12 h later. Multiple AI sires were used within each herd, and cows were inseminated by farm personnel that were blind to treatment.

Ultrasound Evaluation of the Ovaries, Vaginal Discharge, Blood Sampling, and Progesterone Assay

Ovaries of all cows were evaluated on study d 0 by transrectal ultrasonography using a portable scanner

Figure 1. Each week, cows with at least one AI service after a nonpregnancy diagnosis were randomly assigned to treatments within parity (primiparous vs. multiparous) to have ovulation and timed AI (TAI) resynchronized with 1 of 3 protocols: (1) 7D1PGF [$n = 273$; d 0, GnRH and insertion of a progesterone-releasing intravaginal device (PRID Delta; CEVA Santé Animale, Libourne, France); d 7, PGF_{2α} and removal of PRID Delta; d 9.5, GnRH; and d 10, TAI]; (2) 7D2PGF [$n = 277$; d 0, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]; and (3) 5D2PGF [$n = 271$; d 2, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]. B = blood sample; US = ultrasound examination of the ovaries.

(Easi-Scan, BCF Technology Ltd., Livingston, UK) equipped with a 7.5-MHz linear-array transducer. Cows diagnosed not pregnant were eligible for resynchronization and were classified based on the presence or absence [corpus luteum (CL) <10 mm in diameter] of a CL.

Vaginal discharge was evaluated in all cows at PRID removal using a scale of 0 to 2 as described previously (Walsh et al., 2007a,b). Briefly, a score of 0 indicated clear or no debris on the PRID, a score of 1 indicated small flecks of purulent debris on the PRID, and a score ≥ 2 indicated abundant purulent mucus on the PRID.

Blood samples from a subgroup of cows in one herd were collected via puncture of the median caudal blood vessels into 8-mL evacuated serum collection tubes (Vacuette, Greiner Bio-One North America Inc., Monroe, NC). Samples were collected immediately before the first GnRH treatment (G1: 7D1PGF, $n = 101$; 7D2PGF, $n = 106$; 5D2PGF, $n = 102$), before the first treatment with PGF_{2α} (7D1PGF, $n = 102$; 7D2PGF, $n = 102$; 5D2PGF, $n = 102$), and at the last GnRH treatment (G2: 7D1PGF, $n = 102$; 7D2PGF, $n = 102$; 5D2PGF, $n = 100$) of the resynchronization protocols (Figure 1). After collection, blood samples were refrigerated until centrifuged ($1,600 \times g$; 4°C) for 20 min. Serum was harvested and stored at -20°C until being assayed for P4 concentration using a chemiluminescent assay (Immulite; Siemens, Wales, UK). The average sensitivity for the 6 assays was <0.2 ng/mL. The average intraassay CV was 6.3%, and the interassay CV

was 4.9% based on a quality control sample (2.50 ng/mL of P4), which was replicated within each assay.

Pregnancy Diagnosis

Pregnancy diagnosis was performed 33 ± 3 d after AI using a portable scanner (Easi-Scan, BCF Technology Ltd.) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a CL on the ovary ipsilateral to the fluid-filled uterine horn containing an embryo with a visualized heartbeat. Pregnancy status for cows diagnosed pregnant was reconfirmed 60 d after AI using the same ultrasound machine and transducer. Cows diagnosed pregnant and subsequently diagnosed not pregnant at pregnancy reconfirmation were considered to have undergone pregnancy loss.

BCS Evaluation and Milk Yield

Body condition score was evaluated for all cows at G2 using a 5-point scale with 0.25 increments where 1 = thin and 5 = fat (Ferguson et al., 1994). The same individual performed all BCS evaluations throughout the experiment. Based on previous research (Carvalho et al., 2014b), BCS was categorized as either low (≤ 2.50) or high (≥ 2.75) for statistical analysis.

Milk weights for the 7 d preceding AI were recorded daily and stored in the on-farm computer software program (Bovisync, Dairy LLC, WI; Dairyplan, GEA, Germany; or Alprowindow, DeLaval, Sweden). For statistical analysis, the average of the milk weights for the 7 d preceding AI were used, and cows were categorized by parity (primiparous vs. multiparous) according to milk production as being less than or greater than mean milk production.

Statistical Analyses

All statistical analyses were performed using SAS software (version 9.4 for Microsoft Windows; SAS Institute Inc., Cary, NC). Two statistical approaches were used to evaluate the effect of treatment: a more conservative approach, in which the effects of treatment were tested using the treatment $\times$ herd interaction as the error term, and a less conservative approach, in which the effects of treatment were tested using the residual error as the error term. The more conservative approach resulted in a lack of statistical differences due to treatment than the less conservative approach because of a decrease in error degrees of freedom. We therefore chose to report results based on the less conservative approach in which the residual error was used as the error term to test the effects of treatment.

Analysis of binary response data (P/AI and pregnancy loss) was performed by logistic regression using the GLIMMIX procedure of SAS. Fixed effects included in the initial models were treatment, parity, herd, BCS category (≤ 2.50 vs. ≥ 2.75), level of milk production (high vs. low), presence of CL (yes vs. no), timing of AI (AI after estrus vs. TAI), and vaginal discharge score (0, 1, and 2), as well as the interaction between these variables. Cow was the experimental unit. Selection of the fixed effects model that best fit the data for each variable of interest was performed by finding the model with the lowest value for the Akaike information criterion using a backward elimination procedure that removed all variables with a P -value > 0.10 from the model. Treatment, herd, and parity were forced to remain in the final model.

For P/AI 32 and 60 d after TAI, the final model included the fixed effects of treatment, herd, and parity. The same categorical variables and interactions used for P/AI were used to obtain the models for analysis of pregnancy loss. The procedure and criteria used for model selection were similar to those used for P/AI. The final model included fixed effects of treatment, herd, and parity. A contrast statement was used to compare P/AI and pregnancy loss between cows treated with 1 PGF_{2 α} (7D1PGF cows) and those treated with 2 PGF_{2 α} (7D2PGF + 5D2PGF cows), and to only compare P/AI and pregnancy loss between cows based on duration of the Ovsynch protocol (7D2PGF vs. 5D2PGF cows). Data on pregnancy diagnosis and on pregnancy loss from 2 cows (1 from 7D1PGF and 1 from 7D2PGF) were not available because these cows were sold before the second pregnancy diagnosis.

Because presence of CL (yes vs. no) was not significant in the initial model, a second analysis of P/AI was performed including, first, only cows with a CL and then, only cows without a CL when enrolled in the study (d 0). Differences in P/AI for cows receiving AI before scheduled TAI and those receiving TAI were evaluated by logistic regression using the GLIMMIX procedure with a model containing only the fixed effect of timing of AI. In addition, the effect of vaginal discharge score on P/AI was evaluated by logistic regression using the GLIMMIX procedure with a model containing only the fixed effect of vaginal discharge score.

Differences in DIM at AI, milk production, number of previous AI, days from previous AI, vaginal discharge score at PRID removal, and BCS were determined by ANOVA using the MIXED procedure of SAS. The model contained as fixed effects treatment, herd, and parity. Differences in the proportion of cows with low (≤ 2.50) BCS, in the proportion of cows with CL on d 0, and in the percentage of cows detected in estrus before G2 were determined by logistic regression using

the LOGISTIC procedure of SAS. The model contained as fixed effects treatment, herd, parity, and P4 concentration at the PGF_{2 α} treatment (< 1.0 vs. ≥ 1.0 ng/mL, only for percentage of cows detected in estrus before G2).

Differences in P4 concentrations among treatments were determined by ANOVA using the MIXED procedure of SAS. The model contained as fixed effects treatment, parity, and their interaction. Data were examined for normality using the Shapiro-Wilk test. A significant P -value indicated that data were not normally distributed; therefore, data were transformed to ranks. Cows were then distributed into 7 categories using P4 concentration at G1 and the first PGF_{2 α} treatment of the protocols (from 0.0 to ≥ 6.0 ng/mL in 1.0 ng/mL increments). At G2, cows were divided into 4 categories (< 0.20 to ≥ 0.4 ng/mL in 0.1 ng/mL increments). Differences in the proportion of cows within each P4 category were analyzed by logistic regression using the LOGISTIC procedure of SAS. Cows with P4 ≥ 0.4 ng/mL at G2 were considered to have incomplete luteal regression (Brusveen et al., 2009; Giordano et al., 2012c; Carvalho et al., 2015a).

A significant difference between the levels of a classification variable was considered when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a statistical tendency. Data are presented as means $\pm$ SEM, obtained using the MEANS procedure of SAS.

RESULTS

Milk Production and BCS

Average milk production during the 7 d before AI did not differ ($P = 0.80$) among treatments (Table 1); however, primiparous cows produced less ($P < 0.01$) milk than multiparous cows (30.7 vs. 37.2 kg/d, respectively). Average BCS did not differ ($P = 0.74$) among treatments (Table 1); however, primiparous cows had a greater ($P < 0.01$) BCS than multiparous cows (2.92 vs. 2.79, respectively). The proportion of cows with low (≤ 2.50) BCS did not differ ($P = 0.77$) among treatments (Table 1). Fewer ($P < 0.01$) primiparous cows had low (≤ 2.50) BCS than multiparous cows [18.3% (45/246) vs. 32.9% (189/575), respectively]. Other characteristics of cows enrolled in the experiment are reported in Table 1.

Progesterone Concentrations

At G1, mean P4 concentrations did not differ ($P = 0.71$) among treatments (3.1 ± 0.2 vs. 2.8 ± 0.2 vs. 2.9 ± 0.3 ng/mL, for 7D1PGF, 7D2PGF, and 5D2PGF

Table 1. Characteristics of Holstein cows enrolled in the experiment

Item	Treatment ¹			<i>P</i> -value
	7D1PGF	7D2PGF	5D2PGF	
Number of cows	273	277	271	—
DIM at AI	184.5 ± 5.1	184.4 ± 5.9	182.3 ± 4.9	0.97
Milk production (kg/d)	35.2 ± 0.5	35.3 ± 0.5	35.7 ± 0.5	0.80
Number of previous AI	2.8 ± 0.1	3.0 ± 0.2	3.2 ± 0.1	0.25
Days from previous AI	36.8 ± 1.1	36.3 ± 1.0	34.9 ± 0.9	0.39
BCS	2.83 ± 0.02	2.84 ± 0.02	2.82 ± 0.02	0.74
Cows with BCS ≤ 2.50 [% (no./no)]	27.5 (75/273)	27.8 (77/277)	30.3 (82/271)	0.77
Cows with corpus luteum on study d 0 [% (no./no)]	74.0 (202/273)	69.7 (193/277)	71.2 (193/271)	0.52

¹Each week, cows with at least one AI service after a nonpregnancy diagnosis at 33 ± 3 d after previous AI were randomly assigned to treatments within parity (primiparous vs. multiparous) to have ovulation and timed AI (TAI) resynchronized with 1 of 3 protocols: (1) 7D1PGF [n = 273; d 0, GnRH and insertion of a progesterone-releasing intravaginal device (PRID Delta; CEVA Santé Animale, Libourne, France); d 7, PGF_{2α} and removal of PRID Delta; d 9.5, GnRH; and d 10, TAI]; (2) 7D2PGF [n = 277; d 0, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]; and (3) 5D2PGF [n = 271; d 2, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI].

cows, respectively) or parities ($P = 0.45$; 2.8 ± 0.3 vs. 2.9 ± 0.2 ng/mL, for primiparous vs. multiparous cows, respectively). Furthermore, the distribution of cows based on P4 concentrations at G1 did not differ among treatments (Figure 2A). Overall, 30% of cows had P4 concentrations <1.0 ng/mL, which did not differ ($P = 0.73$) among treatments (Figure 2A) or parities [$P = 0.68$; 34% (27/80) vs. 31% (71/226), for primiparous vs. multiparous cows, respectively].

At the first PGF_{2α} treatment, mean P4 concentrations did not differ ($P = 0.66$) among treatments (3.8 ± 0.2 vs. 4.0 ± 0.2 vs. 3.5 ± 0.3 ng/mL, for 7D1PGF, 7D2PGF, and 5D2PGF cows, respectively); however, primiparous cows had lower ($P = 0.05$) mean P4 concentrations than multiparous cows (3.3 ± 0.2 vs. 3.9 ± 0.2 ng/mL, respectively). When cows were stratified based on P4 concentrations at the first PGF_{2α} treatment, the proportion of cows with P4 ≥ 6 ng/mL tended to differ ($P = 0.06$) among treatments and more 7D1PGF and 7D2PGF cows had P4 concentrations ≥ 6 ng/mL than 5D2PGF cows (Figure 2B). The proportion of cows with P4 concentrations <1.0 ng/mL did not differ ($P = 0.28$) among treatments (Figure 2A) or parities [7.4% (6/81) vs. 5.9% (13/222), for primiparous vs. multiparous cows, respectively].

At G2, mean P4 concentrations did not differ ($P = 0.26$) among treatments (0.24 ± 0.01 vs. 0.22 ± 0.01 vs. 0.24 ± 0.01 ng/mL, for 7D1PGF, 7D2PGF, and 5D2PGF cows, respectively) or parities ($P = 0.66$; 0.24 ± 0.01 vs. 0.23 ± 0.01 ng/mL, for primiparous vs. multiparous cows, respectively). When cows were stratified based on P4 concentrations at G2, the proportion of cows with P4 ≥ 0.4 ng/mL tended ($P = 0.06$) to differ among treatments and was less for 7D2PGF cows, intermediate for 5D2PGF cows, and greater for 7D1PGF cows (Figure 2C). The proportion of cows with P4 ≥ 0.4

ng/mL did not differ ($P = 0.28$) between parities [9.4% (8/85) vs. 5.5% (12/218), for primiparous vs. multiparous cows, respectively].

P/AI, Pregnancy Loss, and Estrus Expression Before the Last GnRH

At 32 d after AI, P/AI did not differ ($P = 0.14$) among treatments (Table 2); however, primiparous cows had more ($P = 0.03$) P/AI than multiparous cows [46.1% (112/243) vs. 37.8% (210/556)]. Pregnancies per AI did not differ ($P = 0.64$) among herds. Although cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF) had more ($P = 0.05$) P/AI than cows receiving 1 PGF_{2α} treatment (7D1PGF; Table 2), P/AI did not differ ($P = 0.56$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 2).

At 60 d after AI, P/AI did not differ ($P = 0.27$) among treatments (Table 2); however, primiparous cows had more ($P < 0.01$) P/AI than multiparous cows [43.4% (105/242) vs. 33.0% (183/555)]. Pregnancies per AI did not differ ($P = 0.89$) among herds. At 60 d after AI, P/AI did not differ ($P = 0.11$) between cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) and cows receiving 1 PGF_{2α} treatment (7D1PGF cows; Table 2). In addition, P/AI did not differ ($P = 0.77$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 2).

Pregnancy loss between 32 and 60 d after AI did not differ ($P = 0.64$) among treatments (Table 2); however, primiparous cows had fewer ($P = 0.05$) pregnancy losses than multiparous cows [5.4% (6/111) vs. 12.4% (26/209), respectively]. Pregnancy loss did not differ ($P = 0.33$) among herds. Furthermore, pregnancy loss did not differ ($P = 0.64$) between cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) and cows

Figure 2. Distribution of cows based on progesterone (P4) concentration at G1 (upper panel), at PGF (middle panel), and at G2 (lower panel). Each week, cows with at least one AI service after a nonpregnancy diagnosis were randomly assigned to treatments within parity (primiparous vs. multiparous) to have ovulation and timed AI (TAI) resynchronized with 1 of 3 protocols: (1) 7D1PGF [$n = 273$; d 0, GnRH and insertion of a progesterone-releasing intravaginal device (PRID Delta; CEVA Santé Animale, Libourne, France); d 7, PGF_{2α} and removal of PRID Delta; d 9.5, GnRH; and d 10, TAI]; (2) 7D2PGF [$n = 277$; d 0, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]; and (3) 5D2PGF [$n = 271$; d 2, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]. Within a progesterone category, bars with different letters denote statistical differences ($P < 0.05$).

receiving 1 PGF_{2α} treatment (7D1PGF cows), or ($P = 0.43$) between 7D2PGF and 5D2PGF cows (Table 2).

The percentage of cows detected in estrus before G2 did not differ ($P = 0.31$) among treatments [15.9% (43/271) vs. 12.5% (34/272) vs. 6.7% (18/267), for 7D1PGF, 7D2PGF, and 5D2PGF cows, respectively]; however, more ($P < 0.01$) cows with P4 < 1.0 ng/mL at the first PGF_{2α} treatment were detected in estrus before G2 than cows with P4 ≥ 1.0 ng/mL [47.4% (9/19) vs. 11.3% (32/282), respectively]. At 32 d after AI, P/AI did not differ ($P = 0.84$) between cows receiving TAI and those receiving AI after a detected estrus [40.4% (285/705) vs. 39.4 (37/94), respectively].

Effect of Presence of CL

Overall, 72% (588/821) of cows had a CL at study d 0, and the proportion of cows with a CL at study d 0 did not differ ($P = 0.52$) among treatments (Table 1). Furthermore, the proportion of cows with a CL on study d 0 did not differ ($P = 0.58$) between parities [70.3% (173/246) vs. 72.2% (415/575), for primiparous vs. multiparous cows, respectively]. For cows with a CL, P/AI 32 d after AI did not differ ($P = 0.51$) among treatments, or ($P = 0.35$) between cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) and cows receiving 1 PGF_{2α} treatment (7D1PGF cows; Table 3). Decreasing the duration of the protocol did not affect ($P = 0.49$) P/AI for cows with a CL (7D2PGF vs. 5D2PGF cows; Table 3). Similarly, at 60 d after AI, P/AI did not differ ($P = 0.71$) among treatments or ($P = 0.43$) between cows receiving 2 PGF_{2α} (7D2PGF and 5D2PGF cows) and cows receiving 1 PGF_{2α} treatment (7D1PGF cows; Table 3). In addition, P/AI did not differ ($P = 0.82$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 3). Furthermore, pregnancy loss did not differ ($P = 0.42$) among treatments for cows with a CL at study d 0, between ($P = 0.87$) cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) and cows receiving 1 PGF_{2α} treatment (7D1PGF cows), or ($P = 0.21$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 3).

For cows without a CL on study d 0, P/AI 32 d after AI did not differ ($P = 0.11$) among treatments; however, cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) had more ($P = 0.03$) P/AI than cows receiving 1 PGF_{2α} treatment (7D1PGF cows; Table 3). Furthermore, P/AI did not differ ($P = 0.98$) based on the duration of the protocol between 7D2PGF and 5D2PGF cows (Table 3). At 60 d after AI, P/AI did not differ ($P = 0.23$) among treatments; however, cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) tended ($P = 0.08$) to have more P/

Table 2. Effect of treatment on pregnancies per AI (P/AI) and pregnancy loss in lactating Holstein cows

Item	Treatment (Trt) ¹			P-value ²		
	7D1PGF	7D2PGF	5D2PGF	Trt	C1	C2
P/AI at 32 d [% (no./no)]	35.7 (95/266)	41.4 (111/268)	43.8 (116/265)	0.14	0.05	0.56
P/AI at 60 d [% (no./no)]	32.5 (86/265)	37.5 (100/267)	38.5 (102/265)	0.27	0.11	0.77
Pregnancy loss [% (no./no)]	8.5 (8/94)	9.1 (10/110)	12.1 (14/116)	0.64	0.64	0.43

¹Each week, cows with at least one AI service after a nonpregnancy diagnosis at 33 ± 3 d after previous AI were randomly assigned to treatments within parity (primiparous vs. multiparous) to have ovulation and timed AI (TAI) resynchronized with 1 of 3 protocols: (1) 7D1PGF [$n = 273$; d 0, GnRH and insertion of a progesterone-releasing intravaginal device (PRID Delta; CEVA Santé Animale, Libourne, France); d 7, PGF_{2α} and removal of PRID Delta; d 9.5, GnRH; and d 10, TAI]; (2) 7D2PGF [$n = 277$; d 0, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]; and (3) 5D2PGF [$n = 271$; d 2, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI].

²C1 = preplanned contrast between 1 PGF_{2α} (7D1PGF) and 2 PGF_{2α} (7D2PGF + 5D2PGF); C2 = preplanned contrast between 7D2PGF and 5D2PGF.

AI than cows receiving 1 PGF_{2α} treatment (7D1PGF cows; Table 3). Furthermore, P/AI did not differ ($P = 0.88$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 3). Pregnancy loss between 32 and 60 d after AI did not differ ($P = 0.71$) among treatments, between ($P = 0.49$) cows receiving 2 PGF_{2α} treatments (7D2PGF and 5D2PGF cows) and those receiving 1 PGF_{2α} treatment (7D1PGF cows), or ($P = 0.67$) based on duration of the protocol between 7D2PGF and 5D2PGF cows (Table 3).

Effect of Vaginal Discharge at Removal of the PRID

Vaginal discharge score at removal of the PRID did not differ ($P = 0.98$) among treatments (1.2 ± 0.4 vs. 1.2 ± 0.4 vs. 1.2 ± 0.4 , for 7D1PGF, 7D2PGF, and 5D2PGF cows, respectively) or parities ($P = 0.45$; 1.3 ± 0.4 vs. 1.2 ± 0.3 , for primiparous vs. multiparous cows, respectively). Overall, 11% (92/820), 54% (443/820),

and 35% (285/820) of cows had a vaginal discharge score of 0, 1, and >1 , respectively. At 32 d after AI, P/AI did not differ ($P = 0.60$) among cows based on vaginal discharge score [37.9% (33/87), 39.3% (170/433), and 42.7% (119/279), for cows with a vaginal discharge score of 0, 1, and >1 , respectively].

DISCUSSION

The present study provides additional information on the effects of addition of a second PGF_{2α} treatment to and decreasing the duration of an Ovsynch protocol that included a PRID when used for resynchronization of ovulation on fertility to TAI. The repeatability of the effects of addition of a second PGF_{2α} treatment among herds should be interpreted cautiously because of a lack of statistical differences when the more conservative statistical approach was used. Thus, for the 3 herds investigated in the present experiment, addition of a

Table 3. Effect of treatment based on presence or absence of a corpus luteum (CL) at study d 0 on pregnancies per AI (P/AI) and pregnancy loss in lactating Holstein cows

CL at study d 0	Treatment (Trt) ¹			P-value ²		
	7D1PGF	7D2PGF	5D2PGF	Trt	C1	C2
Yes						
P/AI at 32 d [% (no./no)]	37.8 (74/196)	39.8 (76/191)	43.4 (82/189)	0.51	0.35	0.49
P/AI at 60 d [% (no./no)]	33.4 (66/195)	36.3 (69/190)	37.6 (71/189)	0.71	0.43	0.82
Pregnancy loss [% (no./no)]	9.6 (7/73)	8.0 (6/75)	13.4 (11/82)	0.42	0.87	0.21
No						
P/AI at 32 d [% (no./no)]	30.0 (21/70)	45.5 (35/77)	44.7 (34/76)	0.11	0.03	0.98
P/AI at 60 d [% (no./no)]	28.6 (20/70)	40.3 (31/77)	40.8 (31/76)	0.23	0.08	0.88
Pregnancy loss [% (no./no)]	4.8 (1/21)	11.4 (4/35)	8.8 (3/34)	0.71	0.49	0.67

¹Each week, cows with at least one AI service after a nonpregnancy diagnosis at 33 ± 3 d after previous AI were randomly assigned to treatments within parity (primiparous vs. multiparous) to have ovulation and timed AI (TAI) resynchronized with 1 of 3 protocols: (1) 7D1PGF [$n = 273$; d 0, GnRH and insertion of a progesterone-releasing intravaginal device (PRID Delta; CEVA Santé Animale, Libourne, France); d 7, PGF_{2α} and removal of PRID Delta; d 9.5, GnRH; and d 10, TAI]; (2) 7D2PGF [$n = 277$; d 0, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI]; and (3) 5D2PGF [$n = 271$; d 2, GnRH and insertion of PRID Delta; d 7, PGF_{2α} and removal of PRID Delta; d 8, PGF_{2α}; d 9.5, GnRH; and d 10, TAI].

²C1 = preplanned contrast between 1 PGF_{2α} (7D1PGF) and 2 PGF_{2α} (7D2PGF + 5D2PGF); C2 = preplanned contrast between 7D2PGF and 5D2PGF.

second PGF_{2α} treatment increased P/AI at 32 d after AI, whereas decreasing duration of the protocol did not affect P/AI.

It is well established that cows receiving a single PGF_{2α} treatment on d 5 of the estrous cycle fail to undergo complete luteal regression (Howard and Britt, 1990; Nascimento et al., 2014). In fact, cows receiving a 5-d Ovsynch protocol require 2 sequential PGF_{2α} treatments (one on d 5 and the other on d 6) to maximize fertility (Ribeiro et al., 2012) because ~50% of cows have incomplete luteal regression after a single PGF_{2α} treatment on d 5 (Santos et al., 2010). Experiments evaluating the effect of decreasing the duration of the Ovsynch protocol have reported an increase (Santos et al., 2010) or no difference (Colazo and Ambrose, 2011, 2015) in P/AI after synchronization of ovulation and TAI when comparing a 5-d with a 7-d protocol. The experiment reporting an increase in P/AI used 2 PGF_{2α} treatments in the 5-d protocol and 1 PGF_{2α} treatment in the 7-d protocol, whereas the experiment reporting no difference in P/AI used 1 PGF_{2α} treatment in both protocols. Santos et al. (2010) conducted an analysis to control for a difference in the rate of luteal regression between cows receiving 1 versus 2 PGF_{2α} treatments by analyzing only cows with P4 <1 ng/mL on the day of AI, and P/AI was again greater for the 5-d than the 7-d protocol (39 vs. 34%). The authors attributed this treatment effect to a reduced period of follicle dominance for cows in the 5-d Ovsynch protocol. Based on data from several studies (Souza et al., 2007; Giordano et al., 2012c; Carvalho et al., 2015a), however, the P4 cut-off value of 1 ng/mL used by Santos et al. (2010) was too high because P/AI were substantially decreased when P4 concentrations exceeded 0.4 ng/mL at G2 (Brusveen et al., 2009; Giordano et al., 2012c; Carvalho et al., 2015a).

The importance of optimizing P4 concentrations during the Ovsynch protocol has been the subject of recent research (Wiltbank et al., 2011; Carvalho et al., 2014a). Several studies have evaluated the importance of complete luteal regression during the Ovsynch protocol on fertility (Brusveen et al., 2009; Carvalho et al., 2015a). For example, Brusveen et al. (2009) observed an overall increase in P/AI of 6 percentage points when a second PGF_{2α} treatment was added to a double-Ovsynch protocol for first TAI. A greater increase in P/AI (12 percentage points) has been reported for cows receiving 2 PGF_{2α} treatments during resynchronization protocols (Carvalho et al., 2015a). Cows with P4 concentrations >0.4 ng/mL at G2 had fewer P/AI than cows with <0.4 ng/mL at G2 (Brusveen et al., 2009; Carvalho et al., 2015a). Interestingly, addition of a second PGF_{2α} treatment increased the rate of luteal regression in cows with P4 <1.0 ng/mL (from 70 to 96%) and ≥1.0

ng/mL (from 89 to 98%) at G1; however, a significant increase in P/AI was observed in cows with P4 <1.0 ng/mL at G1 (46 vs. 33%), whereas only a numeric increase (+4 percentage points; 37 vs. 33%) in P/AI was observed for cows with P4 ≥1.0 ng/mL at G1 (Carvalho et al., 2015a). When cows considered to have incomplete luteal regression were removed from the analyses, P/AI for cows with P4 <1.0 ng/mL at G1 did not differ between cows receiving 1 compared with 2 PGF_{2α} treatments, demonstrating the importance of complete luteal regression on fertility to TAI (Carvalho et al., 2015a).

Cows with low P4 concentrations at G1 have a greater amplitude of the GnRH-induced LH surge (Giordano et al., 2012a; Lima et al., 2013; Pulley et al., 2015), resulting in a high ovulatory response to G1 (Ayres et al., 2013; Giordano et al., 2013; Carvalho et al., 2015c), which increases the percentage of cows with a young CL at the time of PGF_{2α} treatment during the protocol. It is well documented that luteal regression rate after a single PGF_{2α} treatment ranges from 80 to 90% in cows synchronized using a 7-d protocol (Brusveen et al., 2009; Martins et al., 2011; Giordano et al., 2012c), and that the rate of luteal regression is greater for primiparous than for multiparous cows (Wiltbank et al., 2015). Incomplete luteal regression is particularly problematic for cows with a young CL at the PGF_{2α} treatment (Howard and Britt, 1990), wherein rates of luteal regression may be as low as 70% (Carvalho et al., 2015a). The lack of regression of the early CL (Nascimento et al., 2014) is an intriguing biological phenomenon that is not yet fully understood; however, the inability to completely activate intracellular signal transduction pathways has been postulated and supported by several studies (Tsai and Wiltbank, 1998; Mondal et al., 2011; Diaz et al., 2013). Experiments evaluating the effects of P4 concentrations or presence of a CL at G1 have reported lower fertility for cows with P4 <1.0 ng/mL or for cows without a CL at initiation of an Ovsynch protocol (Denicol et al., 2012; Bisinotto et al., 2015a). Reasons for the reduced fertility of cows with P4 <1.0 ng/mL at G1 have been attributed to an increased LH pulse frequency leading to overstimulation of the oocyte (Revah and Butler, 1996; Wiltbank et al., 2006; Rivera et al., 2011). In the present experiment, all cows were supplemented with exogenous P4 during the protocols, and we observed a 50% relative increase in P/AI in cows without a CL at G1 when these cows received 2 PGF_{2α} treatments (7D2PGF + 5D2PGF cows vs. 7D1PGF cows; Table 3). Similarly, in the study by Carvalho et al. (2015a), a 39% relative increase in P/AI was observed for cows with P4 <1 ng/mL at G1 that received 2 rather than 1 PGF_{2α} treatment. Based on the results of the present

study and those by Carvalho et al. (2015a), the low fertility of cows with P4 <1.0 ng/mL or without a CL at G1 resulted from incomplete luteal regression for cows receiving a single PGF_{2α} treatment.

Experiments evaluating the effect of P4 concentrations or presence of CL at PGF_{2α} treatment have reported consistent results. For example, for cows synchronized for first TAI using a double-Ovsynch protocol, cows with P4 <1.0 ng/mL at PGF_{2α} treatment had reduced fertility compared with cows with P4 ≥1.0 ng/mL (27 vs. 50%, Carvalho et al., 2014a). A similar decrease in fertility for cows with P4 <1.0 ng/mL has been observed for cows submitted to TAI after a re-synchronization protocol (8 vs. 37%, Giordano et al., 2012c). In addition, cows without a CL at the PGF_{2α} treatment during resynchronization with a 5-d protocol have reduced fertility compared with cows with a CL (30 vs. 48%, Bisinotto et al., 2010). The decreased fertility in those cows may be due to early luteal regression before the PGF_{2α} treatment and ovulation before TAI (Vasconcelos et al., 1999). In this regard, the percentage of cows with P4 concentrations <1.0 ng/mL at the PGF_{2α} treatment did not differ between cows receiving the 7-d protocol and those receiving the 5-d protocol (Figure 2); however, more cows with early luteal regression were detected in estrus before G2, as expected. Longer intervals from onset of estrus to AI can compromise embryo development (Saacke et al., 2000; Dalton et al., 2001; Roelofs et al., 2006) and fertility (Dransfield et al., 1998; Pursley et al., 1998; Brusveen et al., 2008). If cows detected in estrus before G2 were submitted for TAI at the scheduled time, fertility could have been compromised due to incorrect timing of AI relative to ovulation. The use of intravaginal P4 inserts has been reported to improve the level of synchronization (Bisinotto et al., 2015b), which could explain the similar fertility of cows receiving AI after a detected estrus and those receiving TAI. It is worth noting that this level of fertility does not represent the fertility of all cows in these herds but only the fertility of cows receiving AI after detection of estrus that were enrolled in the experiment. In fact, cows in these herds detected in estrus but not enrolled in the experiment had P/AI of 32% during the experiment.

Several studies have reported differences in P/AI and pregnancy loss between primiparous and multiparous cows (Galvão et al., 2007; Giordano et al., 2012b; Carvalho et al., 2015a), whereas others have reported no effect of parity (Giordano et al., 2012b; Carvalho et al., 2014a, 2015c). Synchronization to each treatment of the protocol is a key element for fertility (Giordano et al., 2012c; Lopes et al., 2013), and the rate of luteal regression is greater for primiparous than for multiparous

cows submitted to an Ovsynch protocol (Wiltbank et al., 2015). Interestingly, some studies reporting no difference in P/AI between parities reported that synchronization rate was similar between parities (Carvalho et al., 2014a, 2015c), whereas those that reported fewer P/AI in multiparous cows also reported a lower synchronization rate for multiparous cows (Giordano et al., 2013; Carvalho et al., 2015a). Although uterine size was not measured in the present study, the greater uterine size near AI in multiparous cows may also be a contributor for the reduced fertility in multiparous cows (Baez et al., 2015).

The use of intravaginal P4 inserts in protocols for synchronization of ovulation and TAI increases both synchronization rate and P/AI and decreases pregnancy loss (Chebel et al., 2006; Bilby et al., 2013; Bisinotto et al., 2013, 2015a); however, many farmers and veterinarians are reluctant to use intravaginal P4 inserts due to concerns with vaginal discharge caused by the insert. Results of the present study and those by others (Chenault et al., 2003; Walsh et al., 2007a,b) demonstrate that the vaginal discharge caused by the use of P4 inserts does not significantly affect fertility.

CONCLUSIONS

Adding a second PGF_{2α} treatment increased P/AI 32 d after AI by 7 percentage points, whereas decreasing the duration of the protocol did not increase P/AI in lactating Holstein cows submitted to resynchronization protocols with PRID. Furthermore, addition of a second PGF_{2α} treatment increased P/AI in cows without a CL at G1 but not in cows with a CL at G1. The percentage of cows with incomplete luteal regression tended to be lower for cows receiving a second PGF_{2α} treatment than for cows receiving a single PGF_{2α} treatment. Finally, vaginal discharge score at removal of the PRID did not affect fertility.

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