Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination

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ABSTRACT

The objective was to determine if using a Double-Ovsynch protocol [DO; Pre-Resynch: GnRH-7 d-PGF_{2α}-3 d-GnRH, 7 d later Breeding-Resynch: GnRH-7 d-PGF_{2α}-56 h-GnRH-16 h-timed artificial insemination (TAI)] to resynchronize ovulation after a previous TAI would increase synchrony and pregnancies per AI (P/AI) compared with an Ovsynch protocol initiated 32 d after TAI (D32; GnRH-7 d-PGF_{2α}-56 h-GnRH-16 h-TAI). Lactating Holstein cows at various days in milk and prior AI services were blocked by parity and randomly assigned to resynchronization treatments. All DO cows received the first GnRH injection of Pre-Resynch 22 d after TAI, and cows (n = 981) diagnosed not pregnant using transrectal ultrasonography 29 d after TAI continued the protocol. Pregnancy status for all D32 cows was evaluated 29 d after TAI so fertility and pregnancy loss could be compared with that of DO cows. All D32 cows received the first GnRH injection of Ovsynch 32 d after TAI, and cows (n = 956) diagnosed not pregnant using transrectal palpation 39 d after TAI continued the protocol. In a subgroup of cows from each treatment, ultrasonography (n = 751) and serum progesterone (P4) concentrations (n = 743) were used to determine the presence of a functional corpus luteum (CL) and ovulation to the first GnRH injection of D32 and Breeding-Resynch of DO (GnRH1), luteal regression after PGF before TAI, and ovulation to the GnRH injection before TAI (GnRH2). Overall, P/AI 29 d after TAI was not affected by parity and was greater for DO compared with D32 cows (39 vs. 30%). Pregnancy loss from 29 to 74 d after TAI was not affected by parity or treatment. The percentage of cows with a functional CL (P4 ≥ 1.0 ng/mL) at GnRH1 was greater for DO than D32 cows (81 vs. 58%), with most DO cows having medium P4 (60%; 1.0 to 3.49 ng/mL), whereas most D32 cows had either low (42%; <1.0

ng/mL) or high (36%; ≥ 3.5 ng/mL) P4 at GnRH1. Ovulation to GnRH1 was similar between treatments but was affected by serum P4 at GnRH. Cows with low P4 (<1.0 ng/mL) had the greatest ovulatory response (59%), followed by cows with medium (≥ 1.0 to 3.49 ng/mL; 38%) and then high (≥ 3.50 ng/mL; 16%) P4 at GnRH1. A greater percentage of DO cows were synchronized compared with D32 cows (72 vs. 51%) primarily due to a greater percentage of D32 than DO cows without a functional CL at the PGF injection before TAI (35 vs. 17%) or without complete CL regression before GnRH2 (17 vs. 7%). We conclude that DO increased fertility of lactating dairy cows during a resynchronization program primarily by increasing synchronization of cows during the Ovsynch protocol before TAI.

Key words: Double-Ovsynch, resynchronization, fertility, dairy cow

INTRODUCTION

Dairy herds have widely adopted programs for synchronization of ovulation and timed artificial insemination (TAI) to improve AI service rates, reduce reliance on detection of estrus, and inseminate anovular cows (Caraviello et al., 2006). More recently, presynchronization protocols, such as 2 injections of prostaglandin F_{2α} (PGF) 14 d apart or the Double-Ovsynch protocol, have improved fertility in lactating cows receiving first TAI compared with Ovsynch alone (Moreira et al., 2001; Navanukraw et al., 2004). When implemented on farms, these advanced presynchronization strategies achieve conception rates of >40% on many farms, a rate of fertility substantially greater than the current average for US Holstein dairy herds (Norman et al., 2009). Nevertheless, many dairy cows fail to conceive at first service, making it critical that dairies implement a strategy to identify and re-inseminate nonpregnant cows. On many farms, an Ovsynch protocol or modifications of Ovsynch are used to resynchronize ovulation for second and subsequent TAI and are commonly referred to as Resynch protocols (Fricke et al., 2003;

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Bartolome et al., 2005; Sterry et al., 2006). A common approach is to initiate Resynch 32 d after TAI, as was done in the original validation studies for Ovsynch (Pursley et al., 1997; Fricke et al., 2003; Sterry et al., 2006). Despite improving the overall service rate and decreasing the interbreeding interval, pregnancies per AI (**P/AI**) for resynchronized services are almost always less than those at first service (Galvao et al., 2007; Silva et al., 2009; Thompson et al., 2010). One reason for the poor fertility to Resynch services is that between 15 to 26% of cows lack a corpus luteum (**CL**) or have low progesterone (**P4**) at initiation of Resynch (Fricke et al., 2003; Sterry et al., 2006; Silva et al., 2009). This is problematic because initiating a Resynch protocol in a low P4 environment reduces fertility compared with that in ovular cows (Fricke et al., 2003; Silva et al., 2007b). Indeed, increased concentrations of circulating P4 during the period of follicle growth before AI has been associated with improved embryo quality (Cerri et al., 2009; Rivera et al., 2010), and fertility in lactating dairy cows (Fonseca et al., 1983; Folman et al., 1990; Bisinotto et al., 2010a).

Several strategies have been used to improve fertility of Resynch services. Silva et al. (2007b) reported an improvement in fertility for cows presynchronized using a single PGF injection 12 d before initiation of Resynch. Although no differences between treatments were observed in the percentage of cows having high versus low P4 at the first GnRH injection of Resynch, an important observation was that cows having high P4 concentration (≥ 1 ng/mL) at the first GnRH injection of Resynch had more P/AI than cows with low P4 regardless of treatment (Silva et al., 2007b). In another study, Dewey et al. (2010) reported an improvement in fertility when cows were presynchronized with GnRH 7 d before initiation of Resynch at 39 ± 3 d after a previous AI. A better understanding of ovarian physiology during Resynch programs using novel presynchronization strategies that improve fertility for first postpartum AI may contribute to the development of practical management programs to improve the overall fertility of second and subsequent AI services.

In the present study, a Double-Ovsynch (**DO**) protocol was compared with an Ovsynch protocol initiated 32 d after TAI (**D32**) for resynchronizing lactating dairy cows that failed to conceive to a prior TAI. The objectives were (1) to determine if P/AI would be greater for cows resynchronized with DO than for cows resynchronized with the D32 Resynch protocol, (2) to determine the percentage of cows that had their estrous cycle synchronized at specific points during the Resynch protocol in cows synchronized with DO or D32 Resynch, and (3) to determine the effect of progesterone concentrations at specific points during the

Resynch protocol on subsequent fertility to the timed AI. Our hypothesis was that fertility of TAI breedings in lactating dairy cows would be improved by DO due to increases in the percentage of cows with a functional CL and therefore increased progesterone concentrations at the first GnRH injection of the breeding Resynch protocol.

MATERIALS AND METHODS

Animals and Management

Lactating Holstein cows from a commercial dairy in south-central Wisconsin (Brodhead, WI) milking approximately 1,800 cows were used in this study. The study was performed from December 2007 to November 2008. Cows were housed in freestall barns and fed a TMR diet once a day with ad libitum access to feed and water. The diet was formulated to meet or exceed NRC requirements (NRC, 2001) for high-producing lactating dairy cows. Throughout the experiment, cows were milked 3 times daily at approximately 8-h intervals, and all cows received subcutaneous injections of bST (Posilac, 500 mg, Monsanto Co., St. Louis, MO) at 14-d intervals beginning at approximately 60 d postpartum. All procedures, including injections, ovarian ultrasonography, pregnancy diagnosis, blood collection, and TAI, were performed while cows were restrained in self-locking head gates at the feedline.

Treatments

Each week, cows at various DIM and having at least one previous AI were blocked by parity (primiparous vs. multiparous) and randomly assigned to either D32 Resynch or DO Resynch treatment for resynchronization of ovulation and TAI for second and subsequent postpartum TAI. During the experiment, all first-service TAI were performed after synchronization of ovulation using a Double-Ovsynch protocol (Souza et al., 2008). All D32 cows received an i.m. injection of GnRH (100 μ g of Fertagyl, 50 μ g/mL of gonadorelin diacetate tetrahydrate, Intervet Animal Health, Millsboro, DE) 32 d after TAI regardless of their pregnancy status, and cows ($n = 956$) diagnosed not pregnant using transrectal palpation 39 d after TAI received an i.m. injection of PGF_{2 α} (500 μ g of Estrumate, 250 μ g/mL of cloprostenol sodium, Schering-Plough Animal Health, Summit, NJ) and continued the protocol to receive an injection of GnRH 56 h after PGF and TAI 16 to 20 h later (Figure 1). All DO cows received the first GnRH injection of the Pre-Resynch portion of the Double-Ovsynch protocol 22 d after TAI regardless of their pregnancy status. At 29 d post-TAI, all DO

Figure 1. Resynchronization protocols for cows assigned randomly to day 32 (D32) or Double-Ovsynch (DO) treatments for second and subsequent postpartum timed AI (TAI) services. Cows received an initial TAI after synchronization of ovulation with DO for first service postpartum. Every week, cohorts of cows were randomly assigned to D32 or DO treatment for resynchronization of ovulation and TAI. In a subgroup of cows, ovarian ultrasonography (US) was performed at the time of the first GnRH (GnRH1) and PGF of D32 and Breeding-Resynch of DO and then at the time of TAI and 7 d later ($n = 439$) to confirm ovulation to the second GnRH (GnRH2) of D32 and Breeding-Resynch of DO. Blood samples (BS) were collected at GnRH1 to determine progesterone concentrations and then at the time of PGF and GnRH2 of D32 and DO to determine luteal regression after PGF administration.

cows were diagnosed for pregnancy using transrectal ultrasonography (US), and cows diagnosed not pregnant ($n = 981$) received PGF and GnRH 72 h later to complete the Pre-Resynch portion of the Double-Ovsynch protocol. Seven days later, cows began the Breeding-Resynch portion of the Double-Ovsynch protocol by receiving GnRH, PGF 7 d later, GnRH 56 h after PGF, and TAI 16 to 20 h later. The first GnRH injection of the Ovsynch protocols resulting in a TAI (i.e., the first GnRH injection of the Breeding-Resynch for DO cows and the first injection of Resynch for the D32 cows) represents a key endpoint in this experiment and will be referred to as **GnRH1** in the text and as G1 in figures for cows in each resynchronization treatment, whereas the last GnRH injection preceding TAI for both resynchronization treatments will be referred to as **GnRH2** in the text and as G2 in the figures (Figure 1). Eighteen days after TAI, all cows were re-randomized to 1 of the 2 resynchronization treatments to begin resynchronization of ovulation and subsequent TAI. Cows were randomized to treatments and inseminated until they conceived or were removed from the herd before the study was concluded.

Pregnancy Diagnosis, Ovarian US, and Ovulatory Responses

Pregnancy diagnosis 29 d after TAI and ovarian US were performed using a portable scanner (Easi-Scan,

BCF Technology Ltd., Livingston, UK) fitted with a 7.5-MHz linear-array transducer. Pregnancy diagnoses 39 and 74 d after TAI were performed using transrectal palpation of the uterine contents by the herd veterinarian. For all cows, pregnancy diagnosis was performed 29 d after TAI regardless of their resynchronization treatment to compare P/AI and pregnancy loss at similar time points between Resynch treatments. All cows diagnosed pregnant 29 d after TAI were rechecked using transrectal palpation 39 and 74 d after TAI. All D32 cows diagnosed not pregnant 39 d after TAI received PGF to continue the protocol as described previously. Conversely, DO cows diagnosed pregnant 29 d after TAI and later diagnosed not pregnant 39 d after TAI were considered to have undergone pregnancy loss and were resynchronized for subsequent breeding using an Ovsynch protocol. Outcomes from these cows were not included in the final data set for analysis of P/AI or ovulatory responses.

In a subgroup of cows from both treatments (D32; $n = 380$ and DO; $n = 371$), transrectal US was performed to record the number and size of structures present on the ovaries for subsequent determination of ovulatory response to GnRH treatment and cyclicity status (Figure 1). Follicle and CL diameters were estimated and recorded using on-screen background gridlines comprising squares with 10-mm sides in the portable scanner. Ultrasonography was performed at GnRH1 for cows in both treatments to determine the presence or absence

of a CL and diameter of follicles present on the ovaries. Seven days later at the PGF injection, ovarian US was performed to determine ovulatory response to GnRH1. Ovulation was defined as the presence of a follicle at GnRH1 and presence of a new or an additional CL in the same location 7 d later at the second US examination. Ovarian US was also performed in a subgroup of cows ($n = 439$) from both treatments at the time of AI and 7 d later to determine ovulatory response to GnRH2.

Blood Sampling and Progesterone Analysis

In a subgroup of cows from each treatment (D32; $n = 375$ and DO; $n = 368$), blood samples (~ 8 mL) were collected via puncture of the median caudal vein or artery into evacuated tubes (Vacutainer, BD, Franklin Lakes, NJ) at GnRH1 for both treatments to determine the percentage of cows with a functional CL (≥ 1.0 ng/mL). In the same subgroup of cows, blood samples were collected at the PGF injection and 56 h later at GnRH2 to determine luteal regression in response to PGF treatment (Figure 1). Samples were transported to the laboratory and centrifuged ($2,000 \times g$, 20 min), and serum was harvested and stored at -20°C until assayed for P4 concentrations using a solid-phase, no-extraction RIA (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). Average sensitivity for the 15 P4 assays was 0.03 ng/mL and intraassay CV was 7.0% whereas interassay CV was 9.6%.

Calculation of Percentage of Cows with Their Estrous Cycle Synchronized

To determine the cumulative effect of individual injections of each Resynch protocol, the percentage of cows with their estrous cycle synchronized was calculated. Cows were considered to have their estrous cycle synchronized when they had high progesterone (≥ 1.0 ng/mL) before PGF administration, low progesterone after PGF administration (i.e., at GnRH2), and responded to GnRH2 by ovulating a follicle.

Statistical Analyses

The experimental design was a complete randomized block design with parity as the blocking factor. Analyses of binary response data (P/AI, pregnancy loss, ovulatory response to GnRH, luteal response to PGF) were performed by logistic regression using the GLIMMIX procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). For P/AI 29, 39, and 74 d after TAI, the initial model contained the following categorical explanatory variables as fixed effects: treatment (D32 vs. DO Re-

synch), parity (primiparous vs. multiparous), AI number (2, 3, 4, and ≥ 5), resynchronization treatment at the time of pregnancy diagnosis (D32 vs. DO Resynch), AI technician (1 vs. 2), and breeding season (cool vs. warm), as well as the interaction between treatment and parity, and between treatment and breeding season. For the classification of inseminations by breeding season, those occurring from January 10 to May 8 and then from September 18 to November 27, 2008, were considered to occur in the cool season, whereas inseminations occurring from May 15 to September 11, 2008, were considered to occur during the warm season of the year.

Given that the experimental unit was the AI breeding after resynchronization with either D32 or DO treatments, all initial models contained both fixed and random effects. Fixed effects included in the initial models were the classification variables treatment, parity, AI number, resynchronization treatment at pregnancy diagnosis, AI technician, breeding season, and their interactions, whereas cow was included as a random effect to account for any potential variation due to cows receiving multiple AI breedings. Based on a covariance parameter estimate test, the random effect of cow was removed from the model so that all final models contained only fixed effects. The covariance parameter test using the ZeroG option for the GLIMMIX procedure is used to evaluate if the matrix containing random effects can be reduced to zero. A covariance parameter test based on the residual pseudo-likelihood is run and a nonsignificant Chi-squared P -value indicates that random effects can be eliminated from the model (SAS Documentation for GLIMMIX, SAS 9.2). After removing random effects, 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 (AIC) using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. Both treatment and parity were forced to remain in each model. Parity was kept in the final models because it was used as a blocking factor for randomization of cows to treatments. Therefore, for P/AI at d 29, 39, and 74 d after AI, the final model contained the fixed effects of treatment, parity, and breeding season.

For analysis of pregnancy loss, the same categorical variables and interactions used for P/AI were applied to obtain the models for pregnancy loss from 29 to 39 d, 39 to 74 d, and 29 to 74 d. Procedures and criteria used for model selection were similar to those used for P/AI. The final model for pregnancy loss from 29 to 39 d included the effect of treatment and parity only, whereas the final model for pregnancy loss from 39 to 74 d and total pregnancy loss from 29 to 74 d included

effects of breeding season and interaction of treatment and breeding season.

Data collected from the subgroup of cows sampled to determine circulating P4 concentrations at the GnRH and PGF injections, ovulatory response to GnRH treatment, and luteal regression after PGF treatment were used to generate multiple logistic regression models to assess the interactions between various physiologic variables and their effect on fertility. Specific models were generated to determine the percentage of cows having high versus low P4 concentrations at GnRH1 for both resynchronization protocols and its effect on ovulatory response to GnRH treatment and P/AI at 29 d, ovulatory response to GnRH1 and GnRH2 of both resynchronization treatments and its effect on P/AI at 29 d, percentage of cows with high versus low P4 concentrations at the time of PGF and its effect on P/AI at 29 d, and luteal regression after PGF treatment. Each model contained a specific set of categorical explanatory variables that may affect the response variable analyzed. The categorical variables included in the models were treatment, parity, AI number, circulating P4 concentration at GnRH1 (either low vs. high or low vs. medium vs. high), PGF (low vs. high), and GnRH2 (low vs. high) for both resynchronization treatments, ovulatory response to the GnRH injection (yes vs. no), as well as the interaction between these variables. Model selection was performed following a similar procedure and criteria used for P/AI. Treatment differences in circulating P4 concentration at GnRH1 and PGF before TAI were determined by ANOVA using PROC MIXED in SAS 9.2. The model contained as fixed effects treatment, parity, and their interaction, whereas cow was used as a random effect in the model.

To further assess the effect of P4 concentration on P/AI, cows were separated into 8 groups using serum P4 concentration (0.00 to ≥ 3.50 ng/mL in 0.5 ng/mL increments) at the time of the first GnRH of D32 and Breeding-Resynch of DO and then at the time of PGF before TAI. Differences in the percentage of cows within each P4 concentration group were assessed by logistic regression using PROC LOGISTIC of SAS 9.2. The effect of P4 on P/AI was assessed by logistic regression using PROC GLIMMIX of SAS 9.2 with a model that only contained the variable P4 concentration as fixed effect. The same model was used to determine differences between the 8 P4 groups in percentage of cows with low P4 concentration at the time of the PGF injection.

Finally, data from a subgroup of cows with available information for circulating P4 concentration at GnRH1, PGF, and GnRH2 of both resynchronization treatments as well as ovulatory response to both GnRH injections were used to evaluate and compare the percentage of

cows that had their estrous cycle synchronized and P/AI after completion of both treatments. Both percentage of cows and P/AI were calculated and compared for the different intermediate classification groups created sequentially as follows: cows with low versus high P4 at the time of the PGF injection, low versus high P4 at GnRH2, ovulatory response to GnRH2, and whether or not cows synchronized to the protocols (synchronized vs. nonsynchronized). Statistical differences for the percentage of cows and P/AI for the different groups were analyzed by logistic regression either using PROC GLIMMIX or PROC LOGISTIC of SAS 9.2.

A significant difference between the levels of a classification variable was considered when $P < 0.05$, whereas differences between $P \geq 0.05$ and $P \leq 0.10$ were considered a statistical tendency. Further, the least significant difference (LSD) post hoc mean separation test was used to determine differences between least squares means (LSM). Data included in the text are presented as arithmetic means obtained using PROC FREQ or PROC MEANS of SAS, whereas LSM estimates and standard error of the mean ($\pm$ SEM) are also reported in the tables.

RESULTS

Effect of Treatment on P/AI and Pregnancy Loss

At 29 d after TAI, DO cows had more ($P < 0.01$) P/AI than D32 cows (Table 1). Overall, P/AI at 29 d after TAI were similar for primiparous and multiparous cows (33.9 vs. 35.0%, respectively), and were greater ($P = 0.04$) during the cool compared with the warm season (36.5 vs. 31.6%). At 39 d after TAI, DO cows had more ($P < 0.01$) P/AI than D32 cows (Table 1). Overall, P/AI at 39 d after TAI were similar for primiparous and multiparous cows (30.8 vs. 31.7%, respectively) and only tended ($P = 0.06$) to differ by season (33.0 vs. 28.7%, for cool vs. warm seasons, respectively). At 74 d after TAI, P/AI was greater ($P < 0.01$) for DO than for D32 cows (Table 1). At 74 d after TAI, no differences were detected for primiparous compared with multiparous cows (28.8 vs. 28.7%, respectively), and P/AI for cows receiving AI during the cool season tended ($P = 0.10$) to differ from that of cows receiving AI during the warm season (30.3 vs. 26.5%, for cool vs. warm seasons, respectively).

Pregnancy loss from 29 to 39 d after TAI was not affected by resynchronization treatment (Table 1) or parity (9.0 vs. 8.8% for primiparous and multiparous, respectively). Overall pregnancy loss from 29 to 39 d, 39 to 74 d, and total loss from 29 to 74 d after TAI for both D32 and DO cows was 8.9% ($n = 59/662$), 7.1% ($n = 42/595$), and 15.4% ($n = 101/654$), respectively.

Table 1. Pregnancies per AI (P/AI) at 29, 39, and 74 d after timed AI, and pregnancy loss from 29 to 39, 39 to 74, and 29 to 74 d after timed AI for cows resynchronized using Ovsynch initiated 32 d after a previous timed AI (D32) or Double-Ovsynch (DO)

Item ¹	Treatment		P-value
	D32	DO	
P/AI 29 d	30.0 (287/956) 29.7 ± 1.0	38.7 (380/981) 38.2 ± 2.0	<0.01
P/AI 39 d	27.0 (258/955) 26.7 ± 1.0	35.3 (345/977) 34.8 ± 2.0	<0.01
P/AI 74 d	24.5 (233/952) 24.2 ± 2.0	32.9 (320/972) 32.5 ± 1.5	<0.01
Loss 29 to 39 d	9.8 (28/286) 9.8 ± 1.8	8.2 (31/376) 8.2 ± 1.4	0.62
Loss 39 to 74 d	8.6 (22/255) 6.8 ± 2.0	5.9 (20/340) 6.1 ± 1.0	0.76
Loss 29 to 74 d	17.7 (50/283) 16.3 ± 2.0	13.8 (51/371) 14.0 ± 2.0	0.44

¹Each value includes % (no./no.) and LSM ± SEM below.

From 39 to 74 d after TAI, pregnancy loss was not affected by treatment (Table 1), parity (6.0 vs. 8.1% for primiparous and multiparous respectively), or breeding season (7.8 vs. 5.8% for cool vs. warm seasons, respectively). Unexpectedly, a treatment by breeding season interaction ($P = 0.03$) was found in which D32 cows inseminated during the cool season had more pregnancy loss (11.7%) than D32 cows inseminated during the warm season (3.9%) or DO cows inseminated during the cool season (5.0%), but not more pregnancy loss than DO cows inseminated during the warm season (7.4%). Finally, when accounting for the total pregnancy loss occurring from 29 to 74 d after TAI, no effect of treatment (Table 1), parity (14.6 vs. 16.4% for primiparous vs. multiparous cows, respectively), or breeding season (16.3 vs. 13.9% for cool vs. warm seasons, respectively) was observed, but a tendency ($P = 0.08$) was found for a treatment by season interaction.

Physiologic Role of P4 Concentration and Ovulatory Response to GnRH During Resynchronization Protocols

Mean P4 concentration at GnRH1 for D32 cows and DO cows did not differ between treatments (2.6 vs. 2.4 ng/mL for D32 vs. DO cows, respectively) or parity (2.4 vs. 2.5 ng/mL for primiparous vs. multiparous cows, respectively); however, the distribution of cows by P4 concentrations at GnRH1 of the breeding Resynch differed substantially between treatments (Figure 2). A greater ($P < 0.01$) percentage of DO cows had high (≥ 1.0 ng/mL) P4 concentration (81.0 vs. 58.3% for DO vs. D32 cows, respectively) at GnRH1 compared with D32 cows. In addition, a greater percentage of DO cows had medium P4 concentrations (1.0 to 3.49 ng/mL; 60.1 vs. 22.4% for DO vs. D32 cows, respectively),

whereas a greater percentage of D32 cows had high P4 concentrations (≥ 3.50 ng/mL; 35.7 vs. 20.9% for D32 vs. DO cows, respectively).

Figure 3 shows the effect of P4 concentrations at GnRH1 on the percentage of cows with P4 <1.0 ng/mL at PGF ($P < 0.01$; panel B) and on the P/AI at 29 d after TAI ($P < 0.01$; panel A). Cows with either lower (<1.0 ng/mL) or higher (≥ 3.50 ng/mL) P4 concentrations at GnRH1 were more likely to have P4 concentrations <1.0 ng/mL at PGF compared with cows with medium P4 concentrations at GnRH1. The P/AI followed a similar trend, with cows with either lower or higher P4 concentrations at GnRH1 having the lowest P/AI at TAI. Cows with medium P4 concentrations at GnRH1 (1.0 to 3.49 ng/mL) had greater ($P < 0.01$) P/AI (38.8%) than cows with low P4 (<1.0 ng/mL, P/AI = 21.6%) or high P4 (≥ 3.5 ng/mL, P/AI = 27.5%) at GnRH1.

Ovulatory response to GnRH1 for D32 and DO cows did not differ between treatments (38.7 vs. 37.2% for D32 vs. DO cows, respectively) or parity (38.4 vs. 37.6% for primiparous vs. multiparous cows, respectively) but was affected ($P < 0.01$) by P4 concentration at GnRH1 (cows without functional CL = 59.3%, cows with functional CL = 28.8%). Furthermore, when cows were grouped by P4 concentration at GnRH1 in low (<1.0 ng/mL of P4), medium (≥ 1.0 to 3.49 ng/mL of P4), and high (≥ 3.50 ng/mL of P4) P4 groups, P4 concentration affected ($P < 0.01$) ovulatory response (Figure 4A). Cows with low P4 had the greatest ovulatory response (59.0%), followed by cows with medium (38.1%) and finally high (15.6%) P4 concentration at GnRH1. Interestingly, P/AI was also affected ($P < 0.01$) by P4 concentration group at GnRH1, regardless of ovulatory response. Cows in the middle P4 concentration group had the greatest P/AI (38.8%), whereas

Figure 2. Distribution of cows according to progesterone (P4) concentration at the first GnRH injection (GnRH1) of the D32 protocol and the Breeding-Resynch of Double-Ovsynch Resynch protocol. Concentration groups vary by 0.5 ng/mL increments. *P*-values indicate treatment differences in the percentage of cows within a P4 concentration group.

cows in low and high P4 had the lowest P/AI (21.6 and 27.5%, respectively; Figure 4B).

We also analyzed the combined effect of P4 concentration and ovulatory response to GnRH1 on P/AI for all cows (Figure 4B). Interestingly, an overall effect was observed on P/AI at 29 d of P4 concentration (38.8, 27.5, 21.6% for medium, high, and low P4, respectively; $P < 0.01$), and a tendency for an effect of ovulatory response to GnRH1 (28.8 vs. 33.0% for cows that did not ovulate vs. cows that ovulated, respectively; $P = 0.06$). Furthermore, a tendency ($P = 0.06$) was observed for the effect of P4 and ovulation to GnRH1 on P/AI. Pregnancies per AI for cows that ovulated or did not ovulate to GnRH1 differed only for cows with low P4 at GnRH1 (Figure 4B).

Mean circulating P4 concentration at the PGF injection was greater ($P < 0.01$) for DO than for D32 cows (3.3 vs. 2.4 ng/mL), whereas primiparous cows tended ($P = 0.06$) to have greater P4 concentration than multiparous cows (3.0 vs. 2.8 ng/mL). The distribution of cows by their circulating P4 at the PGF injection and their respective P/AI is shown in Figure 5. The percentage of cows with high P4 at the PGF injection before TAI was affected by treatment (81.8 vs. 66.0% for DO vs. D32 cows, respectively; $P < 0.01$) and ovula-

tory response to GnRH1 (85.0 vs. 67.1% for ovulation vs. no ovulation, respectively; $P < 0.01$) and tended to be affected by the treatment and ovulatory response interaction ($P = 0.07$; Table 2). Conversely, parity did not affect ($P = 0.84$) the percentage of cows with high P4 at PGF (73.9 vs. 74.0% for primiparous vs. multiparous cows, respectively). When assessing the effect of concentration of P4 at the PGF injection before TAI on P/AI, neither treatment ($P = 0.61$) nor parity ($P = 0.26$) affected P/AI 29 d after TAI. Conversely, cows in the high P4 group had more ($P < 0.01$) P/AI than cows in the low P4 group (37.3 vs. 10.3%).

Luteal Regression After PGF_{2α} Treatment and Ovulatory Response to the GnRH Injection Before TAI

To determine the cutoff point for circulating serum P4 concentration that was used to indicate complete CL regression in this experiment, the relationship between the concentration of P4 56 h after PGF treatment and subsequent P/AI 29 d after TAI was evaluated using cows from both treatments (Figure 6). A value of 0.4 ng/mL was selected based on the concentration of P4 at which P/AI was reduced by more than 50% compared

Figure 3. Pregnancies per AI (P/AI; A) and percentage of cows with low progesterone (P4; <1.0 ng/mL; B) at PGF treatment before timed AI for cows grouped based on their circulating concentrations of P4 at the time of the first GnRH (GnRH1) of D32 and Breeding-Resynch of Double-Ovsynch Resynch protocols. ^{a-c}Different letters indicate a significant difference ($P < 0.05$).

with cows in progesterone concentration groups having the greatest P/AI. This P4 cutoff value is identical to that determined in a previous experiment using the same methodology (Brusveen et al., 2009).

Luteal regression (≥ 1 ng/mL at PGF treatment and <0.4 ng/mL 56 h after PGF treatment) did not differ between D32 ($n = 246$) and DO cows ($n = 294$; 83.7 and 89.1%, respectively) but was greater ($P = 0.03$) for primiparous than for multiparous cows (89.7 vs. 83.9%) and was greater ($P < 0.01$) for cows that did not ovulate compared with cows that did ovulate to GnRH1 of both treatments (93.0 vs. 79.4%, respectively).

Figure 4. Ovulatory response at the time of the first GnRH of D32 and Breeding-Resynch of Double-Ovsynch Resynch protocols (GnRH1) for cows grouped based on their circulating concentrations of progesterone (P4) at the time of GnRH1 (A). For ovulatory response cows were grouped based on their circulating P4 concentration into low (<1.0 ng/mL of P4), medium (≥ 1.0 to 3.49 ng/mL of P4), and high (≥ 3.50 ng/mL of P4) concentration of P4 at GnRH1. Panel B represents pregnancies per AI (P/AI) for cows allocated into 6 groups based on their circulating P4 concentration and ovulatory response: low P4 (<1.0 ng/mL of P4) ovulating or not, medium P4 (≥ 1.0 to 3.49 ng/mL of P4) ovulating or not, and high P4 (≥ 3.50 ng/mL of P4) ovulating or not to GnRH1. ^{a-c}Different lowercase letters indicate a significant difference ($P < 0.05$) in P/AI for cows ovulating or not within the 3 different P4 groups. ^{A-C}Different uppercase letters indicate a significant difference ($P < 0.05$) in P/AI for cows in different P4 concentration (low, medium, and high) groups.

Overall, ovulatory response to GnRH2 was 86.6% ($n = 439$). A small percentage of cows (1.8%, $n = 8/447$) underwent premature ovulation (from PGF treatment to TAI) and were excluded from further analysis of

Figure 5. Distribution of cows according to progesterone (P4) concentration at the PGF injection before timed AI of the D32 protocol (D32) and the Breeding-Resynch of Double-Ovsynch Resynch (DO) protocol (A) and pregnancies per AI (P/AI) for cows in the different P4 concentration groups at the time of PGF before TAI in cows from both treatment groups (D32 and DO; B). Concentration groups vary by 0.5 ng/mL increments. *P*-values indicate treatment differences in the percentage of cows within a P4 concentration group. ^{a-c}Different letters indicate a significant difference ($P < 0.05$).

ovulatory response to GnRH2. Cows with low P4 ($n = 388$; < 0.4 ng/mL) at GnRH2 tended ($P = 0.06$) to have a greater ovulatory response than cows with high ($n = 51$) P4 (87.9 vs. 76.5%, for high vs. low P4, respectively). Although the ovulatory response between cows with high versus low P4 at GnRH2 only tended to differ, P/AI 29 d after TAI was greater ($P < 0.01$) for cows with low than for cows with high P4 at GnRH2 (32.5 vs. 7.8%). Furthermore, when including only those cows with low P4 (< 0.4 ng/mL) at GnRH2 in

the analysis, DO cows ($n = 206$) tended ($P = 0.10$) to have a greater ovulatory response than D32 cows ($n = 182$) at GnRH2 (90.3 vs. 85.2%). No effect of parity ($P = 0.90$) on ovulatory response to GnRH2 was detected (87.8 vs. 87.9%, for primiparous vs. multiparous cows, respectively).

Percentage of Cows with Their Estrous Cycle Synchronized

The percentage of cows that had their estrous cycle synchronized by treatment and P/AI of synchronized and nonsynchronized cows was calculated for a subset of cows ($n = 433$) that had complete information including P4 concentration at GnRH1, PGF, and GnRH2, as well as the ovulatory response to both GnRH1 and GnRH2 (Table 3). A greater ($P < 0.01$) percentage of D32 cows (34.8%) had P4 < 1.0 ng/mL at the PGF injection than in DO cows (17.0%); however, both groups of cows with P4 < 1.0 ng/mL at the PGF injection had low P/AI (Table 3). After elimination of cows with P4 < 1.0 ng/mL, a greater ($P < 0.01$) percentage of D32 cows did not completely regress their CL by the time of GnRH2 compared with DO cows (16.8 vs. 6.5%); however, both groups of cows that did not have complete CL regression had low P/AI (Table 3). Cows that had P4 ≥ 1.0 ng/mL at PGF and low P4 at GnRH2 were further analyzed for ovulation to GnRH2. Treatment did not affect ovulation to GnRH2; however, cows in both treatments that did not ovulate to GnRH2 did not conceive (Table 3). Based on the criteria used to classify cows (≥ 1 ng/mL at PGF, < 0.4 ng/mL of P4 at GnRH2, and ovulation to GnRH2), a greater percentage ($P < 0.01$) of DO cows were synchronized (71.8%) compared with D32 cows (50.5%; Table 3). Cows in both treatments that were not synchronized had low P/AI. Cows that were synchronized had greater P/AI than nonsynchronized cows; however, P/AI for synchronized cows was similar for D32 and DO cows (Table 3).

The percentage of cows that had their estrous cycle synchronized was reassessed for the same subgroup of cows; however, in this analysis cows from both treatments were pooled and grouped based on concentration of P4 and ovulatory response to GnRH1 (Table 4). Seven treated cows were eliminated from the data set because of missing data for P4 concentration and ovulation, leaving 426 inseminations for this analysis. Cows with high P4 that did not ovulate to GnRH1 represented the greatest percentage of cows (51.6%). The percentage of cows that had their estrous cycles synchronized was affected by P4 concentration ($P < 0.01$) and ovulation ($P < 0.01$; Table 4). Overall, a greater ($P < 0.01$) percentage of cows with high P4 (69.7%) had their estrous cycles synchronized than with

Table 2. Percentage of cows with high versus low (1 ng/mL cutoff) progesterone (P4) at PGF_{2α} treatment before timed AI by ovulatory response to GnRH1 for cows resynchronized using Ovsynch initiated 32 d after a previous timed AI (D32) or Double-Ovsynch (DO)

Item	Treatment				P-value		
	D32		DO		Treatment	Ovulation	Treatment × ovulation
	No ovulation	Ovulation	No ovulation	Ovulation			
High P4, % (no./no.)	55.3 (126/228)	83.2 (119/143)	78.8 (182/231)	86.9 (119/137)	<0.01	<0.01	0.07
High P4 (LSM ± SEM)	55.6 ± 3.3 ^c	83.1 ± 3.1 ^{ab}	78.6 ± 2.7 ^b	87.5 ± 2.8 ^a			

^{a-c}LSM with different superscripts differ ($P < 0.05$) among groups.

¹GnRH1 = the first GnRH injection of the D32 protocol and the first GnRH injection of the Breeding-Resynch portion of the Double-Ovsynch protocol.

low P4 (41.3%) at GnRH1 with no effect of ovulation to GnRH1 on the percentage of cows that had their estrous cycles synchronized in cows with high P4. Cows with low P4 at GnRH1 that did not ovulate to GnRH1 represented the lowest percentage of cows (25.5%) with intermediate values for cows with low P4 at GnRH1 that did ovulate to GnRH1 (53.5%; Table 4). The primary reason for lack of synchronization in cows with low P4 at GnRH1 that did not ovulate to GnRH1 was low P4 at PGF (67.3% of these cows). In contrast, the primary reason for lack of synchronization in cows with low P4 at GnRH1 that ovulated to GnRH1 was lack of complete CL regression (35.6% of these cows). Overall, P/AI for synchronized cows was similar for all groups and did not differ by their original P4 status or ovulation group (Table 4).

DISCUSSION

The results of this study support our original hypothesis that resynchronization with Double-Ovsynch would increase fertility compared with the typical D32 Resynch protocol. Considering the large number of cows included in this experiment (~1,900 TAI breedings), the improvement in P/AI observed at 29 d (~8.5%) for DO cows is substantial for resynchronized breedings. In a previous study (Souza et al., 2008), presynchronization with Ovsynch (i.e., Double-Ovsynch) increased P/AI compared with Presynch (PGF-14 d-PGF-11 d-Ovsynch); however, only first-service TAI services were included. The improvement in fertility observed in the present experiment agrees with reports in which presynchronization before Resynch by administering PGF 12 d before or GnRH 7 d before initiation of a Resynch protocol increased P/AI (Silva et al., 2007b; Dewey et al., 2010).

The improvement in fertility due to presynchronization of Resynch breedings observed in the present experiment and in results from Silva et al. (2007b) and Dewey et al. (2010) are in contrast to another study in which presynchronization of ovulation before second Resynch TAI was attempted by insertion of a controlled internal drug-releasing device for the 7 d before initiation of an Ovsynch protocol 25 d after a previous TAI (Thompson et al., 2010). Although P/AI at 32 d after TAI in the presynchronized cows was increased for resynchronized services after the Ovsynch protocol, they did not differ from that of the control cows that were not presynchronized. In that study, however, fewer cows had a CL at the time of the first GnRH injection of the resynchronization protocol and cows had larger follicles at this time. The lack of improvement in P/AI for the presynchronized group may have resulted from a failure to increase the percentage of cows with a functional CL and therefore high P4 at initiation of the resynchronization protocol, potentially reducing the beneficial effect of the increased ovulatory response to the first GnRH injection of the protocol on P/AI.

Figure 6. Effect of progesterone (P4) concentrations at second GnRH treatment (GnRH2) on pregnancies per AI (P/AI) 29 d after timed AI for cows from both resynchronization treatments. Number within each bar indicates the number of cows per group. ^{a-c}Bars with different letters differ ($P < 0.05$).

Table 3. Percentage of cows that had their estrous cycle synchronized after resynchronization using Ovsynch initiated 32 d after a previous timed AI (D32) or Double-Ovsynch (DO)

Item	Treatment, % (no./no.)		<i>P</i> -value
	D32	DO	
P4 at time of PGF ¹			
Cows with low P4	34.8 (73/210)	17.0 (38/223)	<0.01
P/AI ² for cows with low P4	9.6 (7/73)	5.3 (2/38)	0.44
P/AI for cows with high P4	35.8 (49/137)	37.8 (70/185)	0.70
P4 at time of GnRH ² ³			
Cows with high P4	16.8 (23/137)	6.5 (12/185)	<0.01
P/AI for cows with high P4	13.0 (3/23)	8.3 (1/12)	0.68
P/AI for cows with low P4	40.4 (46/114)	39.9 (69/173)	0.94
Ovulation to the GnRH ²			
Cows with no Ovu	7.0 (8/114)	7.5 (13/173)	0.87
P/AI for cows with no Ovu ⁴	0.0 (0/8)	0.0 (0/13)	1.00
P/AI for cows with Ovu	43.4 (46/106)	43.1 (69/160)	0.97
Estrous cycle synchronization			
Synchronized cows	50.5 (106/210)	71.8 (160/223)	<0.01
P/AI not synchronized	9.6 (10/104)	4.8 (3/63)	0.27
P/AI synchronized	43.4 (46/106)	43.1 (69/160)	0.97

¹Cutoff for low versus high progesterone (P4) was 1.0 ng/mL.²Pregnancies per AI.³GnRH² = the second GnRH injection of the D32 protocol and the second GnRH injection of the Breeding-Resynch portion of the Double-Ovsynch protocol. Cutoff for low versus high P4 was 0.4 ng/mL. Cows were considered synchronized when they had high progesterone before PGF administration, low progesterone after PGF administration (i.e., at GnRH²), and responded to GnRH² by ovulating a follicle.⁴No Ovu = no ovulation.

Unlike the results from Souza et al. (2008) that showed a treatment by parity interaction on P/AI for cows synchronized with Double-Ovsynch, the lack of parity effect and treatment by parity interaction on P/AI in the present study indicates that resynchronization using Double-Ovsynch increased P/AI for all parities. Souza et al. (2008) suggested that having more primiparous cows that were anovular at initiation of Ovsynch, as previously reported by others (Chebel et al., 2006; Silva et al., 2007a), may explain the major improvement in P/AI in primiparous but not multiparous cows after first-service AI. Other than the difference in AI service number (i.e., first vs. resynchronized TAI), it is not clear why our results using Double-Ovsynch do not agree with those of Souza et al. (2008) regarding parity differences. The lack of parity effect or treatment by parity interaction on P/AI in the present study contrasts not only with the data from Souza et al. (2008) but also with that of other resynchronization experiments (Sterry et al., 2006, 2007) that reported parity effects on P/AI. Our data are, however, in agreement with others that reported no differences in P/AI by parity for second and subsequent AI services (Galvao et al., 2007; Brusveen et al., 2008; Silva et al., 2009). The detrimental effect of increased ambient temperatures and humidity on reproductive efficiency of lactating dairy cows inseminated after estrus or TAI is well documented (Aréchiga et al., 1998; de la Sota et

al., 1998; Fricke et al., 2003; Chebel et al., 2004) with multiple physiological mechanisms involved (Hansen and Aréchiga, 1999). Therefore, the decrease in P/AI observed during the warm season of the year in the present study was not surprising.

The total pregnancy loss from 29 to 74 d after TAI was 15.4%. This value is in agreement with previous studies performing early pregnancy diagnosis by transrectal ultrasound (Fricke et al., 1998; Vasconcelos et al., 1999; Chebel et al., 2004; Silva et al., 2009). Moreover, the time elapsed from the first pregnancy exam to the last reconfirmation of pregnancy is substantial (45 d), covering most of the period in which pregnancy loss may occur in lactating dairy cows (Santos et al., 2004). Implicit to the experimental design, cows that conceived to a previous TAI in both treatments received an injection of GnRH 32 or 22 d after TAI, respectively. The lack of a treatment effect on P/AI at 29 d after TAI suggests that treatment with GnRH 22 d after a previous TAI neither favored the establishment of pregnancy nor increased the rate of pregnancy loss. This observation agrees with studies reporting that treating cows of unknown pregnancy status with GnRH does not affect pregnancy loss (Fricke et al., 2003; Chebel et al., 2004; Stevenson and Martel, 2009; Buttrey et al., 2010). Furthermore, it was not surprising that we failed to detect treatment or parity differences in the rate of pregnancy loss because most studies comparing TAI

Table 4. Percentage of cows that had their estrous cycle synchronized for cows grouped by progesterone (P4) concentration and ovulatory response to GnRH1¹

Item	Low progesterone		High progesterone		P-value	
	No ovulation, % (no./no.)	Ovulation, % (no./no.)	No ovulation, % (no./no.)	Ovulation, % (no./no.)	P4	P4 × ovulation
Cows at GnRH1 ¹						
Overall P/AI ²	12.9 (55/426)	16.7 (71/426)	51.6 (220/426)	18.8 (80/426)	—	—
Cows with low P4 at PGF ³	12.7 (7/55)	25.4 (18/71)	32.7 (72/220)	36.3 (29/80)	<0.01	0.22
Cows with high P4 at GnRH2 ⁴	67.3 (37/55)	16.9 (12/71)	24.6 (54/220)	7.5 (6/80)	<0.01	0.14
Cows with no ovulation to GnRH2	16.7 (3/18)	35.6 (21/59)	3.0 (5/166)	8.2 (6/74)	<0.01	0.98
Estrous cycle synchronization	6.7 (1/15)	0.0 (0/38)	8.1 (13/161)	10.3 (7/68)	0.97	0.97
P/AI synchronized cows	25.5 (14/55)	53.5 (38/71)	67.3 (148/220)	76.3 (61/80)	<0.01	0.12
	42.9 (6/14)	39.5 (15/38)	44.6 (66/148)	42.6 (26/61)	0.78	0.93

¹GnRH1 = the first GnRH injection of the D32 protocol and the first GnRH injection of the Breeding-Resynch portion of the Double-Ovsynch protocol.
²Pregnancies per AI.
³PGF = cutoff for low versus high P4 was 1.0 ng/mL.
⁴GnRH2 = the second GnRH injection of the D32 protocol and the second GnRH injection of the Breeding-Resynch portion of the Double-Ovsynch protocol. Cutoff for low versus high P4 was 0.4 ng/mL.

programs after resynchronization have shown similar results (Sterry et al., 2006; Silva et al., 2009; Thompson et al., 2010).

Analysis of physiologic parameters also support our hypothesis that resynchronization of lactating dairy cows using a Double-Ovsynch protocol would increase the percentage of cows having a functional CL at GnRH1 compared with D32 cows. The effect of treatment on the distribution of cows according to their P4 concentration at GnRH1 is a reflection of the stage of the estrous cycle of the cows at this time point during each protocol. The D32 cows were concentrated at both extremes of the distribution, with 42% of the cows having P4 concentration <1.0 ng/mL, 36% of the cows with serum P4 concentration ≥3.5 ng/mL, and the remainder of the cows in intermediate P4 concentration groups. Assuming that all cows present a normal estrous cycle and ovulation occurs every 22.9 ± 0.7 d (Sartori et al., 2004), most cows should have a P4 concentration ≥1.0 ng/mL 32 d after TAI. In contrast, we observed that 42% of D32 cows did not have a functional CL at GnRH1, suggesting that a significant percentage of these cows became anovular between the time of the previous TAI and GnRH1 (as suggested by Silva et al., 2007a), underwent early luteal regression after ovulating around 21 d after TAI, or were not properly synchronized by the previous synchronization protocol. The percentage of cows without a functional CL in the present study was slightly greater than that reported in previous experiments for cows 32 to 34 d after TAI, which was in the range of 17 to 28% (Sterry et al., 2006; Silva et al., 2009; Bisinotto et al., 2010a; Thompson et al., 2010), but comparable to another in which 30 to 35% of cows had low P4 concentration (<1.0 ng/mL) at the time of pregnancy diagnosis between 27 to 29 d after a previous AI (Stevenson et al., 2003). Within the group of cows having P4 concentration ≥1.0 ng/mL, 36% had P4 concentration ≥3.5 ng/mL 32 d after TAI, suggesting that these cows either came into estrus and ovulated very early after TAI and were at a late stage of their estrous cycle (i.e., late diestrus) or, despite failing to conceive, did not undergo luteal regression after TAI and their CL was still actively producing P4.

In contrast, the distribution of DO cows by P4 group differed considerably from that of D32 cows. Interestingly, 20% of DO cows had circulating P4 concentrations <1.0 ng/mL, which represents a substantial reduction in the percentage of cows in the lowest P4 group compared with D32 cows. In addition, fewer DO cows were in the high P4 group, leaving most of the DO cows distributed among the intermediate concentration groups. Considering that DO cows completed the final GnRH injection of an Ovsynch protocol 7 d before GnRH1, it was surprising that 20% had low P4 at this

time. In general, the distribution of DO cows reflected the stage of the estrous cycle (i.e., early diestrus) in which most cows are found approximately 6 to 8 d after ovulation that occurred either spontaneously after the PGF injection or induced after the second GnRH injection of the Pre-Resynch portion of the Double-Ovsynch protocol.

The effect of presynchronization for DO cows may have been beneficial because of the significant percentage of cows that had medium P4 serum concentrations (1.0 to 3.49 ng/mL) rather than low or high concentrations at GnRH1. Indeed, cows in the intermediate P4 groups had better fertility than cows at either extreme of the distribution. It was not surprising to find that cows with low P4 at the initiation of Ovsynch had reduced P/AI. Similarly, Bisinotto et al. (2010a) reported lower P/AI for cows with low P4 at the start of Ovsynch for first-service postpartum AI after Presynch (2 PGF_{2α} injections 14 d apart). We did not, however, expect to observe the dramatic reduction in P/AI for cows at the high end of the distribution for P4 concentration at GnRH1. Nevertheless, these cows had reduced P/AI that were comparable to P/AI for cows in the lowest P4 concentration group. This observation may be explained by the unexpectedly high rate of luteal regression before PGF treatment. It is possible that these cows were in the latter stages of the estrous cycle and subsequently underwent luteal regression during the 7-d period from GnRH1 to PGF treatment. Cows in the middle P4 concentration groups, however, were in early to mid diestrus and very few underwent luteal regression from GnRH1 to PGF treatment, resulting in good synchrony and subsequently the greatest fertility. Clearly, the P4 concentration at GnRH1 affected the P4 status of these cows at the PGF injection. Similar to previous reports (Bello et al., 2006; Chebel et al., 2006), cows with low P4 at the PGF injection, regardless of treatment, had low fertility to TAI (<10%). This group of cows accounted for more than 50% of the nonsynchronized cows in both treatments. Obviously, most cows with low P4 at GnRH1 that did not ovulate to GnRH1 had low P4 at the PGF injection. However, the largest group of cows in this study, cows with high P4 at GnRH1 that did not ovulate to GnRH1, also had a surprisingly high percentage of cows with low P4 at the PGF injection. A shorter interval between the GnRH and PGF injections, as used in the 5-d Ovsynch protocol (Bisinotto et al., 2010b; Santos et al., 2010), may allow less time for premature luteal regression in this group of cows.

The extremely low ovulatory response to GnRH1 and the lack of difference between D32 and DO cows in ovulatory response to GnRH1 was unexpected. We anticipated a high ovulatory response in DO cows due to

presynchronization of the follicular wave with this protocol. It is possible, however, that the better synchrony achieved by DO cows was neutralized by the strong effect of circulating P4 concentration to inhibit ovulation. In fact, the ovulatory response to GnRH1 for cows with high (≥ 1.0 ng/mL cutoff) circulating P4 was much less than for cows having low P4. The strong association between endogenous P4 and ovulatory response after administration of exogenous GnRH observed in the present study is relevant because ovulation to the first GnRH injection of the Ovsynch protocol has generally been found to benefit fertility of dairy cows (Vasconcelos et al., 1999; Chebel et al., 2006; Stevenson et al., 2007). Surprisingly in our study, the beneficial effect of ovulation to GnRH1 was observed only for cows with low P4 at GnRH1, and no beneficial effect of ovulation to GnRH1 was found in cows with medium or high P4. The observed suppressive effect of P4 on ovulation agrees with a study that reported a decreased ovulatory response after GnRH treatment between 23 to 35 d after a previous AI in lactating dairy cows having ≥ 1 ng/mL of P4 (Buttrey et al., 2010) and another in which the same trend was observed for lactating dairy cows and heifers (Stevenson et al., 2007). The suppressive effect of P4 on the ovulatory response to GnRH is concerning because a major goal of presynchronizing the estrous cycle before initiation of a resynchronization protocol is to increase the percentage of cows having a functional CL, thereby resulting in high circulating P4 concentration.

Interestingly, the major reason for improved fertility in DO cows was the greater synchrony after DO than D32, because P/AI for synchronized cows was similar for both treatments. The improved percentage of DO cows that had their estrous cycles synchronized seems to be related to the intermediate concentrations of P4 at GnRH1, with a reduced percentage of cows with either low or high P4 at GnRH1. Nevertheless, a lower percentage of DO cows than expected had their estrous cycles synchronized (71.8%), but it was still superior to the 50.5% of D32 cows that had their estrous cycles synchronized. Clearly, lack of synchronization continues to be a problem with these resynchronization protocols.

Cows with low P4 concentrations at GnRH1 and ovulation to GnRH1 had an improved percentage of cows with their estrous cycle synchronized although not to the extent of cows with high P4. It is clear that the major factor responsible for the decrease in the percentage of cows that had their estrous cycle synchronized for this group of cows was the lack of luteal regression (~36% of cows with high P4 at GnRH before TAI). In contrast, for that group of cows with high serum P4 that ovulated after the GnRH injection, luteal regression was high and comparable to that of cows with no

ovulation after GnRH. It appears that the presence of the older CL allowed more efficient luteal regression of the younger CL induced by ovulation at GnRH1.

In addition to the observed relationship between circulating P4 and the percentage of cows that had their estrous cycles synchronized on fertility, another potential contribution to the increased fertility of cows with high P4 may be due to the circulating P4 concentrations during the period of follicle growth before AI. Studies have reported an association between circulating P4 before AI and improved fertility in lactating dairy cows (Fonseca et al., 1983; Folman et al., 1990). This relationship between P4 concentration and fertility may be explained by the suppressive effect of P4 on gonadotropin secretion, specifically on reducing LH pulse amplitude and frequency (Rahe et al., 1980). For example, using persistent dominant follicles as a model, exposure of growing follicles to adequate concentrations of LH could be critical for proper oocyte maturation because exposure to a high pulsatile pattern of LH during a prolonged period may result in oocyte maturation before the preovulatory LH surge (Revah and Butler, 1996; Mihm et al., 1999). Although the physiological conditions characteristic of the persistent follicle model may be extreme to illustrate the potential mechanisms involved in the decreased fertility observed for cows with low P4 before initiating a resynchronization protocol, we speculate that similar physiological conditions may exist, although to a lesser extent than that occurring in cows inseminated after a resynchronization protocol. One caveat of our analysis of P4 concentrations on fertility in the present study is that it is confounded by the Resynch protocols themselves. Further studies are needed to fully clarify the role of progesterone in fertility in lactating dairy cows.

CONCLUSIONS

Synchronization of ovulation for second and subsequent AI services with a Double-Ovsynch protocol improved the fertility of lactating dairy cows compared with an Ovsynch protocol initiated 32 d after TAI. A greater percentage of DO cows had intermediate circulating concentrations of P4 at the time of the first GnRH injection of the Breeding-Resynch of the DO protocol compared with the first GnRH injection of Ovsynch for D32 cows, which resulted in a greater percentage of cows that had their estrous cycle synchronized for DO cows. This study highlights the critical need for careful evaluation of the percentage of cows that have their estrous cycle synchronized during future research aimed at improving timed AI protocols. The development of less complex protocols with shorter interbreed-

ing intervals may be possible by focusing on improving synchronization during the protocol.

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REFERENCES

- Aréchiga, C. F., C. R. Staples, L. R. McDowell, and P. J. Hansen. 1998. Effects of timed insemination and supplemental beta-carotene on reproduction and milk yield of dairy cows under heat stress. *J. Dairy Sci.* 81:390–402.
- Bartolome, J. A., F. T. Silvestre, S. Kamimura, A. C. Arteche, P. Melendez, D. Kelbert, J. McHale, K. Swift, L. F. Archbald, and W. W. Thatcher. 2005. Resynchronization of ovulation and timed insemination in lactating dairy cows. I: Use of the Ovsynch and Heatsynch protocols after non-pregnancy diagnosis by ultrasonography. *Theriogenology* 63:1617–1627.
- Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. *J. Dairy Sci.* 89:3413–3424.
- Bisinotto, R. S., R. C. Chebel, and J. E. Santos. 2010a. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. *J. Dairy Sci.* 93:3578–3587.
- Bisinotto, R. S., E. S. Ribeiro, L. T. Martins, R. S. Marsola, L. F. Greco, M. G. Favoreto, C. A. Risco, W. W. Thatcher, and J. E. Santos. 2010b. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. *J. Dairy Sci.* 93:5798–5808.
- Brusveen, D. J., A. P. Cunha, C. D. Silva, P. M. Cunha, R. A. Sterry, E. P. Silva, J. N. Guenther, and M. C. Wiltbank. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. *J. Dairy Sci.* 91:1044–1052.
- Brusveen, D. J., A. H. Souza, and M. C. Wiltbank. 2009. Effects of additional prostaglandin F_{2α} and estradiol-17β during Ovsynch in lactating dairy cows. *J. Dairy Sci.* 92:1412–1422.
- Buttrey, B. S., M. G. Burns, and J. S. Stevenson. 2010. Ovulation and pregnancy outcomes in response to human chorionic gonadotropin before resynchronized ovulation in dairy cattle. *Theriogenology* 73:449–459.
- Caraviello, D. Z., K. A. Weigel, P. M. Fricke, M. C. Wiltbank, M. J. Florent, N. B. Cook, K. V. Nordlund, N. R. Zwald, and C. L. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. *J. Dairy Sci.* 89:4723–4735.
- Cerri, R. L., H. M. Rutigliano, R. C. Chebel, and J. E. Santos. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. *Reproduction* 137:813–823.
- Chebel, R. C., J. E. Santos, R. L. Cerri, H. M. Rutigliano, and R. G. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. *J. Dairy Sci.* 89:4205–4219.
- Chebel, R. C., J. E. Santos, J. P. Reynolds, R. L. Cerri, S. O. Juchem, and M. Overton. 2004. Factors affecting conception rate after ar-

- tificial insemination and pregnancy loss in lactating dairy cows. *Anim. Reprod. Sci.* 84:239–255.
- de la Sota, R. L., J. M. Burke, C. A. Risco, F. Moreira, M. A. DeLorenzo, and W. W. Thatcher. 1998. Evaluation of timed insemination during summer heat stress in lactating dairy cattle. *Theriogenology* 49:761–770.
- Dewey, S. T., L. G. Mendonca, G. Lopes Jr., F. A. Rivera, F. Guagnini, R. C. Chebel, and T. R. Bilby. 2010. Resynchronization strategies to improve fertility in lactating dairy cows utilizing a presynchronization injection of GnRH or supplemental progesterone: I. Pregnancy rates and ovarian responses. *J. Dairy Sci.* 93:4086–4095.
- Folman, Y., M. Kaim, Z. Herz, and M. Rosenberg. 1990. Comparison of methods for the synchronization of estrous cycles in dairy cows. 2. Effects of progesterone and parity on conception. *J. Dairy Sci.* 73:2817–2825.
- Fonseca, F. A., J. H. Britt, B. T. McDaniel, J. C. Wilk, and A. H. Rakes. 1983. Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J. Dairy Sci.* 66:1128–1147.
- Fricke, P. M., D. Z. Caraviello, K. A. Weigel, and M. L. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. *J. Dairy Sci.* 86:3941–3950.
- Fricke, P. M., J. N. Guenther, and M. C. Wiltbank. 1998. Efficacy of decreasing the dose of GnRH used in a protocol for synchronization of ovulation and timed AI in lactating dairy cows. *Theriogenology* 50:1275–1284.
- Galvao, K. N., J. E. Santos, R. L. Cerri, R. C. Chebel, H. M. Rutigliano, R. G. Bruno, and R. C. Bicalho. 2007. Evaluation of methods of resynchronization for insemination in cows of unknown pregnancy status. *J. Dairy Sci.* 90:4240–4252.
- Hansen, P. J., and C. F. Aréchiga. 1999. Strategies for managing reproduction in the heat-stressed dairy cow. *J. Anim. Sci.* 77(Suppl. 2):36–50.
- Mihm, M., N. Curran, P. Hyttel, P. G. Knight, M. P. Boland, and J. F. Roche. 1999. Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *J. Reprod. Fertil.* 116:293–304.
- Moreira, F., C. Orlandi, C. A. Risco, R. Mattos, F. Lopes, and W. W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *J. Dairy Sci.* 84:1646–1659.
- Navanukraw, C., D. A. Redmer, L. P. Reynolds, J. D. Kirsch, A. T. Grazul-Bilska, and P. M. Fricke. 2004. A modified presynchronization protocol improves fertility to timed artificial insemination in lactating dairy cows. *J. Dairy Sci.* 87:1551–1557.
- Norman, H. D., J. R. Wright, S. M. Hubbard, R. H. Miller, and J. L. Hutchison. 2009. Reproductive status of Holstein and Jersey cows in the United States. *J. Dairy Sci.* 92:3517–3528.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Sci., Washington, DC.
- Pursley, J. R., M. R. Kosorok, and M. C. Wiltbank. 1997. Reproductive management of lactating dairy cows using synchronization of ovulation. *J. Dairy Sci.* 80:301–306.
- Rahe, C. H., R. E. Owens, J. L. Fleeger, H. J. Newton, and P. G. Harms. 1980. Pattern of plasma luteinizing hormone in the cyclic cow: Dependence upon the period of the cycle. *Endocrinology* 107:498–503.
- Revah, I., and W. R. Butler. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. *J. Reprod. Fertil.* 106:39–47.
- Rivera, F. A., L. G. Mendonca, G. Lopes, J. E. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderon, and R. C. Chebel. 2010. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post-transfer in lactating dairy cows. *Reproduction* 141:333–342.
- Santos, J. E., C. D. Narciso, F. Rivera, W. W. Thatcher, and R. C. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. *J. Dairy Sci.* 93:2976–2988.
- Santos, J. E., W. W. Thatcher, R. C. Chebel, R. L. Cerri, and K. N. Galvao. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Anim. Reprod. Sci.* 82–83:513–535.
- Sartori, R., J. M. Haughian, R. D. Shaver, G. J. Rosa, and M. C. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J. Dairy Sci.* 87:905–920.
- Silva, E., R. A. Sterry, and P. M. Fricke. 2007a. Assessment of a practical method for identifying anovular dairy cows synchronized for first postpartum timed artificial insemination. *J. Dairy Sci.* 90:3255–3262.
- Silva, E., R. A. Sterry, D. Kolb, N. Mathialagan, M. F. McGrath, J. M. Ballam, and P. M. Fricke. 2009. Effect of interval to resynchronization of ovulation on fertility of lactating Holstein cows when using transrectal ultrasonography or a pregnancy-associated glycoprotein enzyme-linked immunosorbent assay to diagnose pregnancy status. *J. Dairy Sci.* 92:3643–3650.
- Silva, E., R. A. Sterry, D. Kolb, M. C. Wiltbank, and P. M. Fricke. 2007b. Effect of pretreatment with prostaglandin F_{2α} before resynchronization of ovulation on fertility of lactating dairy cows. *J. Dairy Sci.* 90:5509–5517.
- Souza, A. H., H. Ayres, R. M. Ferreira, and M. C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. *Theriogenology* 70:208–215.
- Sterry, R. A., P. W. Jardon, and P. M. Fricke. 2007. Effect of timing of Cosynch on fertility of lactating Holstein cows after first postpartum and Resynch timed-AI services. *Theriogenology* 67:1211–1216.
- Sterry, R. A., M. L. Welle, and P. M. Fricke. 2006. Effect of interval from timed artificial insemination to initiation of resynchronization of ovulation on fertility of lactating dairy cows. *J. Dairy Sci.* 89:2099–2109.
- Stevenson, J. S., J. A. Cartmill, B. A. Hensley, and S. Z. El-Zarkouny. 2003. Conception rates of dairy cows following early not-pregnant diagnosis by ultrasonography and subsequent treatments with shortened Ovsynch protocol. *Theriogenology* 60:475–483.
- Stevenson, J. S., and C. A. Martel. 2009. Resynchronized ovulation in lactating dairy cattle of unknown pregnancy: Occurrence and timing of gonadotropin-releasing hormone. *Prof. Anim. Sci.* 25:605–609.
- Stevenson, J. S., M. A. Portaluppi, and D. E. Tenhouse. 2007. Factors influencing upfront single-and multiple-ovulation incidence, progesterone, and luteolysis before a timed insemination resynchronization protocol. *J. Dairy Sci.* 90:5542–5551.
- Thompson, I. M., R. L. Cerri, I. H. Kim, J. A. Green, J. E. Santos, and W. W. Thatcher. 2010. Effects of resynchronization programs on pregnancy per artificial insemination, progesterone, and pregnancy-associated glycoproteins in plasma of lactating dairy cows. *J. Dairy Sci.* 93:4006–4018.
- Vasconcelos, J. L., R. W. Silcox, G. J. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology* 52:1067–1078.