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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1532963 since 2017-05-11T16:44:51Z

Published version:

DOI:10.3168/jds.2015-9719

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10 September 2026

UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin F2α treatment.

Journal of dairy science. 98:8741-8752, 2015, DOI: 10.3168/jds.2015-9719

The definitive version is available at:
http://www.journals.elsevier.com/theriogenology/

Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin F2α treatment.

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ABSTRACT

Lactating Holstein cows (n = 897) were randomly assigned to a 2 × 2 factorial arrangement of treatments to compare the main effects of presynchronization with GnRH 6 d before beginning an Ovsynch protocol GnRH) and a second PGF2α treatment 24 h after the first (1 vs. 2 PGF2α) on pregnancies per artificial insemination (P/AI). This resulted in the following 4 treatments: (1) an Ovsynch protocol (GPG, control); (2) presynchronization with GnRH followed by an Ovsynch protocol (GGPG); (3) an Ovsynch protocol with a second PGF2α treatment (GPPG); and (4) presynchronization with GnRH followed by an Ovsynch protocol with a second PGF2α treatment (GGPPG). All cows were submitted for first timed artificial insemination (TAI) using a Presynch Ovsynch protocol, and cows detected in estrus after the second PGF2α treatment of the Presynch portion of the protocol were inseminated and removed from the experiment. Nonpregnant cows were resynchronized using an Ovsynch protocol initiated 32 □} 3 d after artificial insemination.

Blood samples were collected at the first GnRH treatment (G1), at the PGF2α treatment (PGF), and at the last GnRH treatment (G2) of the Ovsynch protocol and were assayed for progesterone (P4) concentrations. Overall, P/AI tended to be greater for cows receiving a second PGF2α treatment compared with cows not receiving the second PGF2α treatment (40 and 37% for GGPPG and GPPG treatments, respectively, vs. 33 and 32% for GGPG and GPG treatments, respectively).

Interestingly, treatment effects on P/AI were detected only for resynchronized cows receiving second and greater TAI, but not for cows receiving first TAI. Fewer cows presynchronized with GnRH had low (<0.5 ng/mL) P4 at G1 than cows not presynchronized (13

vs. 25%), and P4 was greater at PGF2α for cows presynchronized with GnRH (4.4 vs. 4.0 ng/mL). Cows receiving 2 PGF2α treatments had lower P4 at G2 than cows receiving 1 PGF2α treatment

(0.15 vs. 0.35 ng/ mL). Differences in P4 at PGF2α were detected only for resynchronized cows and not for cows submitted for first TAI. We conclude that presynchronization with GnRH 6 d before beginning an Ovsynch protocol tended to increase P4 at the first GnRH treatment of an Ovsynch protocol, and that a second PGF2α treatment 24 h after the first decreased P4 at TAI, thereby increasing P/AI in cows resynchronized for second and greater TAI.

INTRODUCTION

Programs that allow for timed AI (**TAI**) have been developed and widely adopted by commercial dairy farms (Caraviello et al., 2006). These programs use a sequence of GnRH and PGF2α treatments (i.e., Ovsynch) to control follicular development, regression of the corpus luteum (**CL**), and ovulation, thereby allowing for TAI rather than relying on detection of estrus for timing of AI. The implementation of a synchronization protocol that allows for TAI increases the rate at which cows are inseminated (Pursley et al., 1995, 1997). Furthermore, optimization of the hormonal milieu during the Ovsynch protocol increases pregnancies per AI (**P/ AI**) for cows at first TAI (Souza et al., 2008; Carvalho et al., 2014), as well as for cows resynchronized to receive second and greater TAI (Giordano et al., 2012a; Lopes et al., 2013).

Optimization of the hormonal milieu during the Ovsynch protocol has been achieved by presynchronizing cows before initiation of the Ovsynch protocol. Presynchronization strategies have included the following: 2 PGF2α treatments administered 14 d apart 10 to 14 d before initiation of an Ovsynch protocol (i.e., Presynch Ovsynch; Moreira et al., 2001; Navanukraw et al., 2004); a PGF2α treatment followed by a GnRH treatment 2 d later with initiation of an Ovsynch protocol 6 or 7 d after the GnRH treatment (i.e., G6G; Peters and Pursley, 2002; Bello et al., 2006); presynchronization with an Ovsynch protocol 7 d before initiation of an Ovsynch protocol (i.e., Double Ovsynch; Souza et al., 2008; Carvalho et al., 2014); and treatment with GnRH or human chorionic gonadotropin (hCG) 7 d before initiation of an Ovsynch protocol (i.e., GGPG; Giordano et al., 2012a; Lopes et al., 2013; Carvalho et al., 2014). Treatment with GnRH 6 or 7 d before initiation of an Ovsynch protocol is an attractive strategy, particularly for resynchronization of ovulation, because all cows can be treated with GnRH before pregnancy diagnosis without causing iatrogenic pregnancy loss (Fricke et al., 2003; Giordano et al., 2012b).

Compared with Ovsynch alone, presynchronization with GnRH 7 d before initiation of an Ovsynch protocol increased the number of CL per cow (Dewey et al., 2010) and the proportion of cows with CL at the first GnRH treatment (**G1**) of the Ovsynch protocol (Galvao et al., 2007; Bruno et al., 2013, 2014). In another experiment (Dewey et al., 2010), presynchronization with GnRH increased

the proportion of cows ovulating to the first GnRH treatment, the proportion of cows with high P4 concentrations at the PGF2α treatment, and the number of CL per cow at the PGF2α treatment of the Ovsynch protocol. Despite the observed improvements in these physiological parameters, increases in P/AI are minimal (~4 percentage points; Dewey et al., 2010; Alkar et al., 2011; Giordano et al., 2012a; Mendonca et al., 2012; Bruno et al., 2013; Lopes et al., 2013). The lack of a presynchronization effect on P/AI may occur because presynchronization with GnRH might increase ovulatory response to the first GnRH treatment, thereby increasing the proportion of cows with young (~d 7) CL at the time of the PGF2α treatment of the protocol (Dewey et al., 2010). Thus, lack of complete luteal regression of these young CL after a single PGF2α treatment may limit P/AI. Increased P4 concentration at the last GnRH treatment of the Ovsynch protocol (**G2**) measured as P4 concentrations >0.5 ng/mL is associated with a decrease in P/AI compared with cows having P4 concentrations <0.5 ng/mL (Souza et al., 2007; Brusveen et al., 2009). Furthermore, rates of luteal regression after a single PGF2α treatment during the Ovsynch protocol are not optimal (Brusveen et al., 2009; Giordano et al., 2013). Brusveen et al. (2009) reported that cows receiving 2 PGF2α treatments during an Ovsynch protocol regressed more CL at first TAI (98 vs. 86%; $P = 0.001$) as well at second and greater TAI (93 vs. 82%) than cows receiving 1 PGF2α treatment. Similarly, Giordano et al. (2013) evaluated the effect of increasing the dose of PGF2α administered during an Ovsynch protocol and reported an increase in the proportion of cows with low P4 concentrations at G2 (82 vs. 77%), which tended to increase P/AI 74 d after TAI (42 vs. 38%). Based on these studies, there might be an additive or synergistic effect of presynchronization with GnRH with addition of a second PGF2α treatment during an Ovsynch protocol on P/AI.

The objective of this experiment was to compare the main effects of (1) presynchronization with GnRH (GnRH) 6 d before beginning an Ovsynch protocol, and (2) addition of a second PGF2α treatment 24 h after the first (1 vs. 2 PGF2α) on P4 concentration during the Ovsynch protocol and on P/AI. Our hypotheses were that (1) presynchronization with a GnRH injection 6 d before beginning an Ovsynch protocol would decrease the proportion of cows with low P4 concentrations at G1 and the PGF2α treatments of the Ovsynch protocol, and (2) that addition of a second PGF2α treatment would increase the proportion of cows with low P4 concentrations at G2.

MATERIALS AND METHODS

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

Cows, Housing, and Feeding

This study was conducted from March to December 2013 on a commercial dairy farm (located near Waunakee, WI) milking approximately 1,100 cows. Cows were milked thrice daily at approximately 8-h intervals and were fed once daily a TMR consisting of corn and alfalfa silage 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 mattress and had ad libitum access to feed and water. Primiparous cows were housed separately from multiparous cows. Barns were equipped with fans and sprinklers that were automatically activated when the temperature inside the barns exceeded 20°C (68°F). All cows received bST (500 mg/dose; Posilac, Elanco Animal Health, Greenfield, IN) every 14 d beginning 67,3 d postpartum until dry-off. The rolling herd average during the experiment was 12,450 kg, and average daily milk production was 34.5 kg/cow per day with 4.1% fat and 3.0% protein.

Reproductive Management Protocol

Cows were submitted to a Presynch Ovsynch protocol for first AI as described by Navanukraw et al. (2004). Briefly, cows received 2 PGF2α (25 mg i.m. of dinoprost tromethamine, Lutalyse; Zoetis, Madison, NJ) treatments 14 d apart at 39±3 and 53±3 DIM. Cows detected in estrus based on rubbed tail chalk evaluated daily after the second PGF2α treatment of the presynchronization protocol were inseminated by a professional AI technician, whereas cows not detected in estrus within 14 d of the second PGF2α treatment initiated an Ovsynch56 protocol as described by Brusveen et al. (2008). Briefly, cows received the first GnRH treatment (100 μg i.m. of gonadorelin hydrochloride, Factrel; Zoetis), followed by treatment with PGF2α 7 d later (25 mg i.m. of dinoprost tromethamine) and a second GnRH treatment (G2; 100 μg i.m. of gonadorelin hydrochloride) approximately 56 h after the PGF2α treatment. Cows received TAI approximately 16 to 20 h after the second GnRH treatment. For second and subsequent AI, cows were observed for signs of estrus based on rubbed tail chalk, which was evaluated daily, and cows identified in estrus were inseminated by a professional AI technician. Cows not detected in estrus by 34±3 d after AI were submitted for pregnancy diagnosis, and cows diagnosed not pregnant were enrolled in an Ovsynch56 protocol for resynchronization of ovulation as described for first AI. Cows were continuously observed for signs of estrus based on rubbed tail chalk, which was evaluated daily, and cows identified in estrus were inseminated by a professional AI technician. For cows synchronized for first TAI, only cows not detected in estrus within 8 d after the second PGF2α treatment of the Presynch portion of the Presynch Ovsynch protocol were enrolled in the experiment. For second and greater TAI, cows not detected in estrus within 28±3 d after AI were

enrolled in the experiment. Multiple sires with high genetic merit and proven fertility were used and were randomly balanced among treatments.

Experimental Treatments

Each week, cows were blocked by parity (primiparous vs. multiparous) and service number (first TAI vs. second and greater TAI) as part of the randomization procedure and were randomly assigned to a 2 × 2 factorial arrangement of treatments 6 d before initiation of an Ovsynch protocol (Figure 1) to test the main effects of (1) presynchronization with GnRH (GnRH) 6 d before initiation of an Ovsynch protocol, and (2) addition of a second PGF2α treatment 24 h after the first (1 vs. 2 PGF2α). This resulted in the following 4 treatments: (1) an Ovsynch protocol (**GPG**, control, n =221); (2) presynchronization with GnRH followed by an Ovsynch protocol (**GGPG**, n = 241); (3) an Ovsynch protocol with a second PGF2α treatment (**GPPG**, n=205); and (4) presynchronization with GnRH followed by an Ovsynch protocol with a second PGF treatment (**GGPPG**, n = 230).

Pregnancy Diagnosis

Pregnancy diagnosis was performed 34±3 d after AI using a portable scanner (Easi-Scan, BCF Technology Ltd., Livingston, UK) equipped with a 7.5-MHz lineararray transducer. A positive pregnancy diagnosis was based on visualization of a CL on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant was reconfirmed 60 d after AI using the same ultrasound machine. Cows diagnosed pregnant and subsequently diagnosed not pregnant at pregnancy reconfirmation were considered to have undergone pregnancy loss.

Blood Sampling and Progesterone Assay

Blood samples from a subgroup of cows (n = 851) 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 G1, before treatment with PGF2α, and at G2 of the Ovsynch protocol (Figure 1). After collection, blood samples were refrigerated for 24 h and centrifuged (1,600 × *g*; 4°C) for 20 min. Serum was harvested and stored at −20°C until assayed for P4 concentration using a solid-phase, no-extraction radioimmunoassay (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA). The average sensitivity for the 6 assays was 0.027 ng/mL. The average intraassay CV was 3.34%, and the interassay CV was 5.81% based on a quality control sample (2.50 ng/mL of P4), which was replicated within each assay.

Ultrasound Evaluation of the Ovaries

Ultrasound evaluation of the ovaries was performed in a subgroup of cows (n = 568) at G2 by transrectal ultrasonography using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. The ultrasound image was frozen when follicles were determined visually to be at their maximal size. Mean follicle diameter was calculated by taking the average of 2 perpendicular measurements at the largest cross-sectional area of the follicle [length (L) and width (W)] using the built-in calipers of the ultrasound machine.

Statistical Analyses

The experimental design was a complete randomized block design with parity (primiparous vs. multiparous) and service number (first TAI vs. resynch TAI) as the blocking factors. All statistical analyses were performed using SAS computational software, version 9.3 for Microsoft Windows (SAS Institute Inc., Cary, NC). Analysis of binary response data (P/AI at 32 and 60 d after TAI and pregnancy loss between 32 and 60 d) was performed by logistic regression using the GLIMMIX procedure of SAS. Initial analysis included cows receiving all services, and subsequent analysis was performed including cows receiving first service or cows receiving resynch services. For P/AI at 32 and 60 d after TAI and pregnancy loss between 32 and 60 d, the model contained the following categorical explanatory variables as fixed effects: parity (primiparous vs. multiparous), service number (first TAI vs. resynch TAI), main effect of GnRH (GnRH vs. no GnRH), main effect of PGF2α (1 PGF2α vs. 2 PGF2α), as well as the interaction between the main effect of GnRH and the main effect of PGF2α. The analyses of P/AI at 32 and 60 d after TAI, pregnancy loss between 32 and 60 d for cows receiving first service contained the following categorical explanatory variables as fixed effects: parity (primiparous vs. multiparous), main effect of GnRH (GnRH vs. no GnRH), main effect of PGF2α (1 vs. 2 PGF2α), as well as the interaction between the main effect of GnRH and the main effect of PGF2α. The same categorical variables and interactions were used to analyze outcomes for cows receiving TAI after resynchronization of ovulation.

Treatment differences in P4 concentrations at G1, PGF2α, and at G2 of the Ovsynch protocols were determined by ANOVA using the MIXED procedure of SAS. 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. For P4 concentrations at G1 and PGF2α, the model contained as fixed effects parity (primiparous vs. multiparous), service number (first TAI vs. resynch TAI), and the main effect of GnRH (GnRH vs. no GnRH). For P4 concentration at G2, the model contained as fixed effects parity (primiparous vs. multiparous), service number (first TAI vs. second and greater TAI), the main effect of GnRH (GnRH vs. no GnRH), the main effect of PGF2α (1 vs. 2 PGF2α), as well as the interaction between the main effect of GnRH and the main effect of

PGF2α. Cows were divided into 9 categories using P4 concentrations at G1 (from 0.00 to ≥4.00 ng/mL in 0.5 ng/mL increments) and the PGF2α (from 0.00 to ≥8.00 ng/mL in 1.0 ng/mL increments) treatments of the Ovsynch protocol, whereas cows were distributed into 6 categories using P4 concentrations (from 0.00 to ≥0.50 ng/mL in 0.10 ng/mL increments) at G2. Cows with P4 concentrations <1.0 ng/mL at the PGF2α treatment were removed from the analysis of the effect of P4 concentrations at G2 on fertility to eliminate the effect of lack of synchronization. Differences in the proportion of cows within each P4 category were analyzed by logistic regression using the LOGISTIC procedure of SAS. Differences in ovulatory follicle size were determined by ANOVA using the MIXED procedure of SAS. Data were examined for normality using the Shapiro-Wilk test. A nonsignificant *P*-value indicated that data were normally distributed. The model contained as fixed effects parity (primiparous vs. multiparous), service number (first TAI vs. resynch TAI), the main effect of GnRH (GnRH vs. no GnRH), the main effect of PGF2α (1 vs. 2 PGF2α), P4 concentrations as categorical variable (<1.0 or ≥1.0 ng/mL), as well as the interaction between the main effect of GnRH and the main effect of PGF2α. The model for the analyses of ovulatory follicle size for cows with P4 concentration at G1 <1.0 or ≥1.0 ng/mL contained as fixed effects parity (primiparous vs. multiparous), service number (first TAI vs. resynch TAI), the main effect of PGF2α (1 vs. 2 PGF2α), P4 concentrations as categorical variable (<1.0 or ≥1.0 ng/mL), and the interaction between main effect of PGF2α and P4 concentrations as categorical variable (<1.0 or ≥1.0 ng/mL). The proportion of cows with high P4 (≥0.4 ng/mL) concentrations at G2 among those cows with P4 concentrations at G1 <1.0 or ≥1.0 ng/mL, and P/AI between cows receiving 1 versus 2 PGF2α treatments for cows with P4 concentrations <1.0 ng/mL at G1 was evaluated by logistic regression using the GLIMMIX procedure of SAS. The model contained as fixed effects parity (primiparous vs. multiparous), service number (first TAI vs. resynch TAI), the main effect of PGF2α (1 vs. 2 PGF2α), P4 concentrations as categorical variable (<1.0 or ≥1.0 ng/mL), and the interaction between main effect of PGF2α and P4 concentrations as categorical variable (<1.0 or ≥1.0 ng/mL). The effect of P4 at G1, PGF2α, and G2 on P/AI was analyzed by logistic regression using the LOGISTIC procedure of SAS with a model that contained only the categorical variable P4 concentration as a fixed effect to eliminate the effect of lack of synchronization, for evaluation of the effect of P4 at the PGF2α injection of the Ovsynch protocol on P/AI among cows with high P4 concentrations, cows with P4 concentrations <1.0 ng/mL (n = 165) were removed from the analysis. A significant difference between the levels of a classification variable was declared 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±standard errors of the mean obtained using the MEANS procedure of SAS.

RESULTS

Pregnancies per AI and Pregnancy Loss

At first TAI, there was no effect (P = 0.59) of presynchronization with GnRH 6 d before initiation of the Ovsynch protocol on P/AI 32 d after TAI (Table 1). Similarly, we found no effect (P = 0.53) of a second PGF2α treatment on P/AI, and there was no interaction (P = 0.90) between the main effects of presynchronization with GnRH and addition of a second PGF2α treatment (Table 1). Primiparous cows had more (P =0.02) P/AI than multiparous cows [47% (68/144) vs. 35% (72/207), respectively]. At 60 d after first TAI, we detected no effect (P =0.32) of presynchronization with GnRH on P/AI (Table1), addition of a second PGF2α treatment did not affect (P = 0.62) P/AI, and there was no interaction (P = 0.70) between the main effects of presynchronization with GnRH and addition of a second PGF2α treatment (Table 1). Primiparous cows had more (P < 0.01) P/AI than multiparous cows [45% (64/143) vs. 28% (57/205)]. Neither presynchronization with GnRH (P= 0.32) nor addition of a second PGF2α treatment (P =0.74; Table 1) affected pregnancy loss between 32 and 60 d after TAI; however, primiparous cows had fewer (P = 0.02) pregnancy losses than multiparous cows [5% (3/67) vs. 19% (13/70), respectively]. For cows resynchronized to receive second and greater TAI, presynchronization with GnRH did not affect (P= 0.24) P/AI 32 d after TAI (Table 1). Cows receiving 2 PGF2α treatments, however, had more (P < 0.01) P/AI than control cows (Table 1). Nevertheless, P/AI after TAI for resynchronized cows did not differ (P = 0.44) between primiparous and multiparous cows [35% (72/208) vs. 31% (106/338), respectively]. Presynchronization with GnRH did not affect (P = 0.90) P/AI 60 d after TAI (Table 1) for resynchronized cows; however, cows receiving 2 PGF2α treatments had more (P = 0.04) P/AI than cows receiving 1 PGF2α treatment (Table 1). At 60 d after TAI, P/AI did not differ (P = 0.30) between primiparous and multiparous cows [30% (63/207) vs. 26% (88/336), respectively]. Pregnancy loss between 32 and 60 d after TAI did not differ (P = 0.97) between cows presynchronized with GnRH and cows not presynchronized with GnRH (P = 0.97) or between cows receiving 1 versus 2 PGF2α treatments (Table 1). Pregnancy loss did not differ (P = 0.38) between primiparous and multiparous cows [11% (8/71) vs. 15% (16/104), respectively]. 8746 CARVALHO ET AL. Journal of Dairy Science Vol. 98 No. 12, 2015 Overall, P/AI at 32 d (P = 0.03), and at 60 d (P = 0.04) after TAI was greater for cows receiving first TAI than for resynchronized cows receiving second and greater TAI [40% (140/351) vs. 33% (178/546) at 32 d; and 35% (211/348) vs. 28% (151/543) at 60 d]. Nevertheless, pregnancy loss between 32 and 60 d after TAI did not differ (P = 0.77) between cows receiving first TAI and for resynchronized cows receiving second and greater TAI [12% (16/137) vs. 14% (24/175)].

Progesterone Concentration During the Protocol and Follicle Size at the last GnRH

At G1 of the Ovsynch protocol, P4 concentrations tended to be greater (P = 0.09) for cows presynchronized with GnRH than for cows not presynchronized with GnRH (3.09 vs. 2.95 ng/mL, respectively). In addition, primiparous cows had greater (P = 0.01) P4 concentrations than multiparous cows (3.34 vs. 2.85 ng/mL). Nevertheless, P4 concentration did not differ (P = 0.51) between cows receiving first TAI and resynchronized cows receiving second and greater TAI (3.10 vs. 2.90 ng/mL, respectively), and there was no interaction (P = 0.45) between presynchronization with GnRH and AI service number. When cows were divided into 9 categories based on P4 concentration at G1 (Figure 2), a greater (P < 0.01) proportion of cows that were presynchronized with GnRH had medium (0.5 to 3.99 ng/mL) P4 concentrations compared with cows that were not presynchronized (55 vs. 43%, respectively). Furthermore, more (P < 0.01) cows that were not presynchronized had low P4 (<0.5 ng/mL) concentrations compared with cows that were presynchronized with GnRH (25 vs. 13%, respectively; Figure 2). At the PGF2α treatment of the Ovsynch protocol, P4 concentrations were greater (P = 0.04) for cows presynchronized with GnRH than for cows not presynchronized with GnRH (4.42 vs. 3.99 ng/mL, respectively). In addition, primiparous cows had greater (P = 0.03) P4 concentrations than multiparous cows (4.57 vs. 3.99 ng/mL). Nevertheless, P4 concentrations did not differ (P = 0.14) between cows receiving first TAI and cows **Table 1.** Effect of treatment on pregnancies per AI [P/AI; % (no./no.)] after timed AI (TAI) and pregnancy loss for lactating Holstein cows Item Treatment1 P-value GPG GGPG GPPG GGPPG GnRH PGF2α GnRH×PGF2α Service Parity P/AI at 32 d First TAI 43 (34/79) 40 (38/95) 40 (34/86) 37 (34/91) 0.59 0.53 0.90 — 0.02 ≥2 TAI 26 (37/142) 28 (41/146) 35 (41/119) 42 (59/139) 0.24 <0.01 0.54 — 0.44 P-value <0.01 0.05 0.46 0.44 Overall 32 (71/221) 33 (79/241) 37 (75/205) 40 (93/230) 0.53 0.07 0.59 0.03 0.04 P/AI at 60 d First TAI 37 (29/78) 35 (33/95) 37 (32/86) 30 (27/89) 0.32 0.62 0.70 — <0.01 ≥2 TAI 26 (37/142) 22 (32/146) 29 (34/118) 35 (48/137) 0.90 0.04 0.19 — 0.30 P-value 0.09 0.03 0.19 0.46 Overall 30 (66/220) 27 (65/241) 32 (66/204) 33 (75/226) 0.65 0.19 0.48 0.04 <0.01 Pregnancy loss First TAI 12 (4/33) 13 (5/38) 6 (2/34) 16 (5/32) 0.32 0.74 0.33 — 0.02 ≥2 TAI 0 (0/37) 22 (9/41) 15 (6/40) 16 (9/57) 0.97 0.97 0.97 — 0.38 P-value 0.04 0.31 0.28 0.98 Overall 6 (4/70) 18 (14/79) 11 (8/74) 16 (14/89) 0.02 0.49 0.30 0.77 0.02 1Six days before initiation of an Ovsynch protocol, cows were randomly assigned to receive (1) an Ovsynch protocol starting 6 d later (GPG); (2) treatment with GnRH and start the Ovsynch protocol 6 d later (GGPG); (3) an Ovsynch protocol 6 d later and a second PGF2α treatment 24 h after the first (GPPG); or (4) treatment with GnRH 6 d before initiation of the Ovsynch protocol and a second treatment with PGF2α 24 h after the first (GGPPG). Statistical significance for the main effect of presynchronization with GnRH, addition of a second PGF2α treatment, their interaction, as well as service number (first TAI vs. ≥2 TAI) and

parity (primiparous vs. multiparous) are shown. **Figure 2.** Main effect of presynchronization with GnRH 6 d before initiation of an Ovsynch protocol on distribution of cows based on progesterone concentration at the first GnRH treatment of the Ovsynch protocol (G1). *P*-values denote treatment differences in the proportion of cows within a progesterone concentration category. Journal of Dairy Science Vol. 98 No. 12, 2015 MODIFICATIONS OF OVSYNCH TO IMPROVE FERTILITY 8747 receiving second and greater TAI (4.37 vs. 3.98 ng/mL, respectively), and there was no interaction ($P = 0.72$) between presynchronization with GnRH and AI service number. When cows were divided into 9 categories based on P4 concentrations at PGF2α (Figure 3), the distribution of cows based on P4 concentration at the PGF2α did not differ based on whether or not cows were presynchronized with GnRH (Figure 3) At G2 of the Ovsynch protocol, P4 concentrations were greater ($P < 0.01$) for cows receiving 1 PGF2α treatment than for cows receiving 2 PGF2α treatments (0.35 vs. 0.15 ng/mL, respectively). Furthermore, primiparous cows had greater ($P < 0.01$) P4 concentrations than multiparous cows (0.30 vs. 0.23 ng/ mL). Nevertheless, P4 concentrations did not differ ($P = 0.82$) between cows receiving first TAI and cows receiving second and greater TAI (0.21 vs. 0.28 ng/ mL, respectively), and there was no interaction ($P = 0.50$) between the main effects of presynchronization with GnRH and addition of a second PGF2α treatment. When cows were divided into 6 categories based on P4 concentrations at G2 (Figure 4), a greater proportion of cows receiving a second PGF2α treatment had low P4 (<0.4 ng/mL) concentrations, whereas more ($P < 0.01$) cows receiving 1 PGF2α treatment had high P4 (≥0.4 ng/mL) concentrations [5% (18/401) vs. 16% (69/425), respectively]. For cows receiving 2 PGF2α treatments, the proportion of cows with high P4 (≥0.4 ng/mL) concentrations did not differ ($P = 0.70$) between cows receiving first TAI and cows receiving second and greater TAI [5% (8/146) vs. 4% (9/223), respectively]. For cows receiving 1 PGF2α treatment, however, the proportion of cows with high (≥0.4 ng/mL) P4 concentrations was less ($P = 0.05$) for cows receiving first TAI than for cows receiving second and greater TAI [13% (19/152) vs. 19% (45/241), respectively]. At the last GnRH treatment of the Ovsynch protocol, average follicle size was greater ($P < 0.01$) for cows receiving first service than for cows receiving resynch services (16.4 vs. 16.0 mm, respectively). In addition, average follicle size was greater ($P = 0.02$) for multiparous than for primiparous cows (16.4 vs. 15.7 mm, respectively). Furthermore, cows presynchronized with GnRH had smaller ($P < 0.01$) ovulatory follicles than cows that were not presynchronized (16.0 vs. 16.4 mm, respectively), and cows receiving 2 PGF2α treatments had larger ($P < 0.01$) ovulatory follicles than cows receiving 1 PGF2α treatment (16.3 vs. 16.0 mm, respectively). There was, however, no interaction ($P = 0.76$) between presynchronization with GnRH and addition of a second PGF2α treatment.

Effect of P4 Concentration at G1 on Luteal Regression and Follicle Size

For evaluation of luteal regression according to P4 concentration at G1 (<1.0 ng/mL vs. ≥1.0 ng/mL), only cows (n = 744) with blood samples at all 3 treatments of the Ovsynch protocol were included in the analysis. Furthermore, to eliminate the effect of early luteal regression, cows (n = 144) with P4 concentrations <1.0 ng/mL at the PGF2α treatment of the Ovsynch protocol were removed from the analysis. **Figure 3.** Main effect of presynchronization with GnRH 6 d before initiation of an Ovsynch protocol on distribution of cows based on progesterone concentration at the PGF2α treatment of the Ovsynch protocol (PGF). *P*-values denote treatment differences in the proportion of cows within a progesterone concentration category. **Figure 4.** Main effect of 1 versus 2 PGF treatments during an Ovsynch protocol on distribution of cows based on progesterone concentration at the last GnRH treatment (G2) of the Ovsynch protocol. *P*-values denote treatment differences in the proportion of cows within a progesterone concentration category. 8748 CARVALHO ET AL. Journal of Dairy Science Vol. 98 No. 12, 2015 For cows with P4 concentrations <1.0 ng/mL at G1, a greater ($P < 0.01$) proportion of cows receiving 1 PGF2α treatment had incomplete luteal regression (≥0.4 ng/ mL) compared with cows receiving 2 PGF2α treatments (Table 2). Similarly, for cows with P4 concentrations ≥1.0 ng/mL at G1, a greater ($P < 0.01$) proportion of cows receiving 1 PGF2α treatment had incomplete luteal regression (P4 ≥0.4 ng/mL) compared with cows receiving 2 PGF2α treatments (Table 2). Cows with P4 concentrations <1.0 ng/mL at G1 had larger ($P < 0.01$) ovulatory follicles than cows with P4 concentrations ≥1.0 ng/mL (17.0 vs. 15.8). For cows with P4 concentration <1.0 ng/mL, size of the ovulatory follicle did not differ ($P = 0.80$) between cows receiving 1 versus 2 PGF2α treatments (Table 2). For cows with P4 concentration ≥1.0 ng/mL, size of the ovulatory follicle tended to be greater ($P = 0.09$) for cows receiving 2 PGF2α treatments than cows receiving 1 PGF2α treatment (Table 2).

Effect of P4 Concentration and Follicle Size on Pregnancies per AI

Figure 5 summarizes the effect of P4 concentrations at G1, PGF, and G2 of the Ovsynch protocol on P/AI. At G1, P/AI did not differ ($P = 0.64$) among P4 categories (Figure 5A). To further evaluate the relationship between P4 concentration at G1 and P/AI, cows with information on P4 concentration at G1 of the Ovsynch protocol and receiving 1 PGF2α treatment were compared with cows receiving 2 PGF2α treatments. For cows with P4 concentrations <1.0 ng/mL at G1, cows receiving 2 PGF2α treatments had greater ($P = 0.03$) P/AI than cows receiving 1 PGF2α treatment (Table 2; Figure 6). When cows with incomplete luteal regression (P4 ≥0.4 ng/mL at G2) were removed from the analysis, P/AI did not differ ($P = 0.35$) between cows receiving 1 versus 2 PGF2α treatments [43% (30/70) vs. 47% (48/102), respectively; Figure 6]. For cows with

P4 concentrations ≥1.0 ng/mL at G1, P/AI did not differ (P = 0.46) between cows receiving 1 versus 2 PGF2α treatments (Table 2). At the PGF2α treatment of the Ovsynch protocol, cows with low P4 (<1.0 ng/mL) concentrations had fewer (P < 0.01) P/AI than cows with high P4 (≥1.0 ng/mL) concentrations (Figure 5B); however, P/AI did not differ (P = 0.74) among cows in P4 categories with high P4 (≥1.0 ng/mL) concentrations (Figure 5B). At G2 of the Ovsynch protocol, P/AI differed (P = 0.002) among P4 categories with the greatest decrease in P/AI occurring when P4 concentrations were ≥0.4 ng/mL (Figure 5C). Cows were divided into 3 categories based on the diameter of the ovulatory follicle at the last GnRH **Table 2.** Effect of 1 versus 2 PGF2α treatments during an Ovsynch protocol on luteal regression, follicle size, and pregnancies per AI [P/AI; % (no./no.)] for cows with low versus high progesterone (P4) concentrations at the first GnRH treatment of an Ovsynch protocol (G1) Item Treatment1 1 × PGF2α 2 × PGF2α P-value Cows with complete luteal regression Low P4 (<1.0 ng/mL) at G1 70 (53/76) 96 (71/74) <0.01 High P4 (≥1.0 ng/mL) at G1 89 (209/236) 98 (210/214) <0.01 P-value <0.01 0.20 Overall 83 (262/312) 98 (281/288) <0.01 Follicle size at G2 (mm) Low P4 (<1.0 ng/mL) at G1 17.0 □} 0.4 16.9 □} 0.3 0.80 High P4 (≥1.0 ng/mL) at G1 15.6 □} 0.2 16.1 □} 0.2 0.09 P-value <0.01 0.04 Overall 16.0 □} 0.2 16.3 □} 0.2 0.51 P/AI 32 d, all cows Low P4 (<1.0 ng/mL) at G1 33 (35/107) 46 (51/110) 0.03 High P4 (≥1.0 ng/mL) at G1 33 (103/312) 37 (106/289) 0.46 P-value 0.93 0.04 Overall 33 (138/419) 39 (157/399) 0.03 P/AI at 32 d, cows with P4 ≥1.0 at PGF2α Low P4 (<1.0 ng/mL) at G1 38 (28/73) 61 (44/72) <0.01 High P4 (≥1.0 ng/mL) at G1 37 (85/227) 41 (87/212) 0.53 P-value 0.93 <0.01 Overall 37 (113/300) 46 (131/284) <0.01 1Six days before initiation of an Ovsynch protocol, cows were randomly assigned to receive (1) an Ovsynch protocol starting 6 d later; (2) treatment with GnRH and start the Ovsynch protocol 6 d later; (3) an Ovsynch protocol 6 d later with a second PGF2α treatment 24 h after the first; or (4) treatment with GnRH 6 d before initiation of an Ovsynch protocol and a second PGF2α treatment 24 h after the first. Results for the main effect of 1 versus 2 PGF2α treatments are shown. Journal of Dairy Science Vol. 98 No. 12, 2015 MODIFICATIONS OF OVSYNCH TO IMPROVE FERTILITY 8749 treatment of the Ovsynch protocol (≤12, 12 to 21, and ≥22 mm). For these ranges of follicle diameters, P/AI did not differ (P = 0.16) and were 27% (15/56), 38% (177/465), and 28% (8/29), respectively. In a subsequent analysis, cows were divided in 2 categories based on the diameter of the ovulatory follicle being either small or large (≤12 mm vs. ≥22 mm) versus cows having medium-sized follicles (12 to 21 mm). For this analysis, cows with small or large follicles had fewer (P = 0.05) P/AI than cows with medium-sized follicles at G2 of the Ovsynch protocol [27% (23/85) vs. 38% (177/465), respectively].

DISCUSSION

Progesterone is a key hormone that affects fertility in many species, including cattle (Wiltbank et al., 2011b, 2014). Of particular importance, P4 concentrations during the Ovsynch protocol can be used as a predictor of fertility. For instance, mid-range P4 concentrations when an Ovsynch protocol is initiated are associated with increased P/AI (Wiltbank et al., 2011b, 2014). In addition, supplementation with exogenous P4 to cows lacking a CL at initiation of the Ovsynch protocol increases P/AI similar to that of cows with a CL at first and later TAI services (Stevenson et al., 2006; Chebel et al., 2010; Bilby et al., 2013; Bisinotto et al., 2013, 2015). At the PGF2α treatment of the Ovsynch protocol, increased P4 concentrations are also associated with increased P/AI, whereas cows with low P4 concentrations at the PGF2α treatment of the protocol have decreased P/AI (Giordano et al., 2012b, 2013; Carvalho et al., 2014). If greater P4 concentrations at G1 and the PGF2α treatments of the Ovsynch protocol increase fertility, assuring complete luteal regression after the **Figure 5.** Pregnancies per AI based on progesterone concentration at the first GnRH treatment (upper panel), the PGF2α treatment (middle panel), and last GnRH treatment (lower panel) of the Ovsynch protocols. Bars with different letters (a and b) differ ($P < 0.05$). Numbers in parentheses denote the number of observations for each progesterone category. **Figure 6.** Main effect of 1 versus 2 PGF2α treatments during an Ovsynch protocol on pregnancies per AI for cows with low (<1.0 ng/ mL) progesterone concentrations at the first GnRH treatment of the Ovsynch protocol for all cows and for cows with complete luteal regression (progesterone ≤0.4 ng/mL at the last GnRH treatment of the Ovsynch protocol). *P*-values denote treatment differences in pregnancies per AI. Numbers in parentheses denote the number of observations. 8750 CARVALHO ET AL. Journal of Dairy Science Vol. 98 No. 12, 2015 PGF2α treatment resulting in low P4 concentrations at G2 of the Ovsynch protocol have been associated with increased P/AI (Souza et al., 2007; Giordano et al., 2013; Lopes et al., 2013). In the present study, we evaluated P4 concentrations and P/AI of cows submitted to a more complex scheme for synchronization of ovulation and TAI, which included the main effects of presynchronization with GnRH 6 d before initiation of the Ovsynch protocol and addition of a second PGF2α treatment 24 h after the first within the Ovsynch protocol. Our first hypothesis was that presynchronization with GnRH 6 d before initiation of an Ovsynch protocol would decrease the proportion of cows with low P4 concentrations at G1 and the PGF2α treatments of the protocol. This hypothesis was based on studies in which treatment with GnRH 7 d before initiation of an Ovsynch protocol induced ovulation of a follicle, resulting in the formation of a CL that would be present and functional during the Ovsynch protocol (Dewey et al., 2010; Giordano et al., 2012a; Bruno et al., 2013, 2014; Lopes et al., 2013). Several studies have highlighted the importance of mid-range P4 concentrations at initiation of the Ovsynch protocol to optimize embryo quality and P/AI after TAI

(Rivera et al., 2011; Wiltbank et al., 2011a; Carvalho et al., 2014). Despite an increase in the proportion of cows with mid-range P4 concentrations at G1, we did not observe an increase in P/AI for cows presynchronized with GnRH. This may have resulted from a failure to decrease the proportion of cows with low P4 concentrations at the PGF2α treatment of the Ovsynch protocol and the lack of an effect of P4 on fertility at G1. Similarly, in a study by Bruno et al. (2014), presynchronization with GnRH did not increase the proportion of cows with high P4 (≥1.0 ng/mL) concentrations or the proportion of cows with a CL at the PGF2α treatment of the protocol. These results may be a consequence of a greater proportion of cows not presynchronized receiving AI after being detected in estrus during the protocol (Bruno et al., 2014), thereby eliminating any potential differences in the proportion of cows without a CL at the PGF2α treatment. In contrast, Lopes et al. (2013) observed a decrease in the proportion of cows with low P4 (<1.0 ng/mL) concentrations (20 vs. 32%) at the PGF2α treatment and an increase in P/AI (34 vs. 39%) for cows presynchronized with GnRH 7 d before initiation of an Ovsynch protocol. Cows with spontaneous luteolysis before the PGF2α treatment of the protocol likely had an LH surge before G2 and ovulated before TAI, thereby decreasing their chances of conception (Vasconcelos et al., 1999; Moreira et al., 2000). In fact, cows without a CL or with low P4 concentrations at the PGF2α treatment of the Ovsynch protocol have dramatically fewer P/AI than cows with high P4 at the PGF2α treatment (Denicol et al., 2012; Giordano et al., 2012b; Lopes et al., 2013). In a recent experiment in which cows were synchronized to receive their first TAI, cows with low P4 concentrations (<1.0 ng/mL) at the PGF2α treatment had dramatically fewer P/AI (27 vs. 50%) than cows with P4 concentrations ≥1.0 ng/mL (Carvalho et al., 2014). Similarly, for cows receiving TAI after resynchronization of ovulation, a similar decrease in P/AI was observed for cows without a CL (29.7 vs. 47.7%; Bisinotto et al., 2010b) or cows with low P4 concentration at the PGF2α treatment of the protocol (8.1 vs. 37.0%; Giordano et al., 2012b). A similar decrease in P/AI for cows with low P4 concentrations at the PGF2α treatment was observed in the present experiment (19 vs. 41%). Our second hypothesis was that addition of a second PGF2α treatment 24 h after the first would increase the proportion of cows with complete luteal regression, thereby increasing P/AI. This hypothesis was based on the observation that 10 to 20% of lactating dairy cows submitted to an Ovsynch protocol fail to undergo complete luteal regression, resulting in poor fertility to TAI (Brusveen et al., 2009; Giordano et al., 2013). In the present experiment, addition of a second PGF2α treatment increased the rate of luteal regression, with the greatest improvement being observed for cows with low P4 concentrations at G1. The observation that cows with low P4 concentrations at G1 had similar P/AI to cows with mid-range P4 concentrations contrasts with results reported by others (Bisinotto et al., 2010a, 2013; Chebel et al., 2010). These results are

attributed, in part, to the greater fertility of the cows with low P4 concentrations at G1 that received a second PGF2α treatment. Cows with low P4 at G1 that ovulate in response to GnRH have a young CL at the time of PGF2α treatment (Nascimento et al., 2014), and young CL do not completely regress in response to a single treatment with exogenous PGF2α (Nascimento et al., 2014). The decreased fertility of cows with low P4 concentrations at G1 reported in some studies has been attributed to an overstimulation of the follicle or oocyte by a greater frequency of LH pulses (Revah and Butler, 1996; Wiltbank et al., 2006), resulting in poor embryo development (Rivera et al., 2011; Wiltbank et al., 2011a). Nevertheless, the decreased fertility of cows with low P4 concentrations at G1 may also be explained by incomplete luteal regression after a single PGF2α treatment during the Ovsynch protocol (Table 2). The increase in P/AI observed in the present study occurred due to an increase in P/AI for resynchronized cows at second and greater TAI, whereas no effect of treatment on P/AI was observed for cows at first TAI. Journal of Dairy Science Vol. 98 No. 12, 2015 MODIFICATIONS OF OVSYNCH TO IMPROVE FERTILITY 8751 Nevertheless, optimization of P4 concentrations was observed for both cows at first TAI and for cows at second and greater TAI. Cows submitted for first TAI were presynchronized with 2 PGF2α treatments administered 14 d apart (Moreira et al., 2001; Navanukraw et al., 2004). This presynchronization strategy may have reduced response to the GnRH treatment used to presynchronize cows before initiating the Ovsynch protocol. In agreement with the lack of increase in P/ AI for cows at first TAI, others who have used a similar strategy for submission of cows for first TAI have reported no increase in P/AI for cows presynchronized with GnRH 7 d before initiation of an Ovsynch protocol (Galvao et al., 2007; Bruno et al., 2013). In conclusion, presynchronization with GnRH 6 d before initiation of an Ovsynch protocol and addition of a second PGF2α treatment 24 h after the first within the Ovsynch protocol optimized P4 concentrations at each treatment of the Ovsynch protocol, and P/AI were increased for cows resynchronized to receive second or greater TAI when treated with a second PGF2α treatment.

ACKNOWLEDGMENTS

We thank the staff at White Gold Dairy for their contribution of time, cows, and facilities to conduct this experiment. The authors extend their gratitude to M. Luchterhand, J. Mulcahy, V. G. Santos, M. C. Amundson, G. M. Baez, and J. N. Guenther from the department of Dairy Science at the University of Wisconsin- Madison for their assistance with data collection and laboratory analyses. We also thank Zoetis (Madison, NJ) for their donation of Lutalyse and Factrel to conduct this project. This research was supported by the USDA National Institute of Food and Agriculture (Washington, DC), Hatch project 231440 to P. M. Fricke.

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