



Presynchronization using a modified Ovsynch protocol or a single gonadotropin-releasing hormone injection 7 d before an Ovsynch-56 protocol for submission of lactating dairy cows to first timed artificial insemination

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ABSTRACT

Presynchronization strategies, such as Presynch-Ovsynch and Double-Ovsynch, increase fertility to timed artificial insemination (TAI) compared with Ovsynch alone; however, simpler presynchronization strategies could reduce costs and simplify reproductive management. Lactating Holstein cows ($n = 601$) were randomly assigned to 1 of 2 presynchronization treatments before beginning an Ovsynch-56 protocol (GnRH at 70 ± 3 DIM, PGF_{2 α} 7 d later, GnRH 56 h after PGF_{2 α} , and TAI 16 h later at 80 ± 3 DIM) for first TAI. Cows ($n = 306$) in the first treatment (Double-Ovsynch; DO) were presynchronized using a modified Ovsynch protocol (GnRH at 53 ± 3 DIM, 7 d later PGF_{2 α} , and GnRH 3 d later) ending 7 d before the first GnRH injection (G1) of an Ovsynch-56 protocol. Cows ($n = 295$) in the second treatment (GGPG) were presynchronized using a single injection of GnRH 7 d before G1 of an Ovsynch-56 protocol at 63 ± 3 DIM. Blood samples were collected at G1 and the PGF_{2 α} injections of the Ovsynch-56 protocol to determine progesterone (P4) concentrations. Pregnancy diagnosis was performed using ultrasonography 32 d after TAI, and pregnant cows were reexamined 46 and 70 d after TAI. Overall, DO cows had more pregnancies per artificial insemination (P/AI) compared with GGPG cows 32 d after TAI (53 vs. 43%). Overall, P/AI did not differ by parity (primiparous vs. multiparous), and pregnancy loss did not differ between treatments or parities. More DO cows had P4 in a medium range (>0.5 to <4 ng/mL) at G1 of the Ovsynch-56 protocol compared with GGPG cows (82 vs. 50%), and more DO cows had high P4 (>4 ng/mL) at the PGF_{2 α} injection of the Ovsynch-56 protocol compared with GGPG cows (67 vs. 36%). Thus, presynchronization with a modified Ovsynch protocol increased P/AI after TAI

at first AI by increasing synchrony to the Ovsynch-56 protocol compared with presynchronization using a single injection of GnRH.

Key words: presynchronization, Ovsynch, Double-Ovsynch

INTRODUCTION

Despite improvements in nutrition, cow comfort, and genetic selection for reproductive efficiency that have occurred over the past decade, pregnancies per artificial insemination (P/AI) continues to be low ($\sim 30\%$) in high-producing dairy cows inseminated after a detected estrus (Valenza et al., 2012; Fricke et al., 2014). Expression of behavioral estrus is poor in high-producing dairy cows (Lopez et al., 2004) and ranged from 50% after visual observation (Washburn et al., 2002) to 70% when insemination was based on an activity monitoring system for detection of estrus (Valenza et al., 2012), and inadequate and inaccurate detection of estrus continues to be a challenge on dairy farms (Palmer et al., 2010). Timed artificial insemination (TAI) programs, such as the Ovsynch protocol (Pursley et al., 1995), have been developed and widely adopted by commercial dairies (Caraviello et al., 2006). The implementation of synchronization protocols that allow for TAI increases the rate at which cows are inseminated after calving and, in some situations, results in more P/AI compared with cows inseminated to a detected estrus (Pursley et al., 1997; Gumen et al., 2012; Fricke et al., 2014).

Presynchronization strategies that increase fertility by optimizing the hormonal milieu, in which the ovulatory follicle grows, have been developed. Presynchronization strategies have used 2 injections of PGF_{2 α} administered 14 d apart, with the second injection administered 10 to 14 d before initiation of an Ovsynch protocol (i.e., Presynch; Moreira et al., 2001; Navanukraw et al., 2004), a single GnRH injection 7 d before Ovsynch [i.e., GnRH-GnRH-PGF_{2 α} -GnRH (GGPG); Lopes et al., 2013; Bruno et al., 2014], and a combination of GnRH and PGF_{2 α} 6 to 7 d before initiation of an Ovsynch protocol [i.e., G-6-G and Double-Ovsynch (DO); Bello

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et al., 2006; Souza et al., 2008]. Independent of the presynchronization strategy used (Double-Ovsynch, Presynch-Ovsynch, G-6-G), an improvement in fertility was reported when progesterone (**P4**) concentrations were increased at the time of the $\text{PGF}_{2\alpha}$ injection of an Ovsynch protocol (Bello et al., 2006; Bisinotto et al., 2010; Martins et al., 2011; Denicol et al., 2012). Although presynchronization strategies have led to dramatic improvements in P/AI compared with AI after a detected estrus or an Ovsynch protocol alone, they have increased or doubled the number of sequential injections required for a cow to complete the protocol.

Simplification of hormonal protocols for TAI may improve compliance and reduce costs and labor associated with reproductive management. Presynchronization of cows receiving second and greater TAI using a single injection of GnRH administered 7 d before initiation of an Ovsynch-56 protocol (i.e., a GGPG protocol) improved fertility compared with Ovsynch alone (Dewey et al., 2010; Lopes et al., 2013; Bruno et al., 2014). Although a direct comparison of a Double-Ovsynch protocol to a GGPG protocol has not been reported, comparison of presynchronization with a single human chorionic gonadotropin treatment 7 d before Ovsynch (**hGPG**) during a Resynch program yielded similar P/AI compared with cows resynchronized using a Double-Ovsynch protocol (Giordano et al., 2012b). A subsequent study comparing strategies for resynchronization showed no statistical difference between P/AI after an hGPG protocol compared with a GGPG protocol (Giordano et al., 2012b). For submission of cows for first TAI, the Double-Ovsynch protocol has been directly compared with a Presynch-Ovsynch protocol (Souza et al., 2008; Herlihy et al., 2012; Ribeiro et al., 2012); however, a direct comparison between the Double-Ovsynch protocol and a simpler GGPG protocol for submission of cows for first TAI has not been reported.

Therefore, the aim of the current study was to compare presynchronization using a modified Ovsynch protocol (i.e., Double-Ovsynch) or a single GnRH injection administered 7 d before initiation of an Ovsynch-56 protocol for submission of lactating dairy cows for first TAI (i.e., GGPG). We further wanted to determine the effect of these presynchronization strategies on P4 concentrations during and after the Ovsynch-56 portion of the protocols. Based on data from resynchronized cows (Giordano et al., 2012b), our hypothesis was that submission of cows for first TAI using a GGPG protocol would result in similar fertility to cows receiving first TAI after a Double-Ovsynch protocol.

MATERIALS AND METHODS

All animal handling and 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

The current study was conducted at the University of Wisconsin-Madison Emmons Blaine Dairy Cattle Research Center (Arlington, WI) from May 2012 to August 2013. Holstein cows ($n = 601$) were milked twice daily approximately 12 h apart 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 sand and had ad libitum access to feed and water. Primiparous cows were housed in separate pens from multiparous cows. Barns were equipped with fans, but not sprinklers, that were automatically activated when the temperature inside the barns reached 68°F (20°C). As per the management procedures in place at the dairy, multiparous cows received somatotropin (500 mg/dose; Posilac, Monsanto Co., St Louis, MO) every 14 d starting at 67 ± 3 d postpartum, whereas somatotropin was not administered to primiparous cows. The rolling herd average was 13,880 kg and average daily milk production was 41.3 kg/cow per day with 3.6% fat and 3.1% protein for the entire herd during the time period of this experiment.

Treatments and AI

Every week, cows from 53 ± 3 DIM were blocked by parity (primiparous vs. multiparous) and were randomly assigned to have their estrous cycle presynchronized using either a modified Ovsynch protocol or a single GnRH injection administered 7 d before an Ovsynch-56 protocol to receive their first TAI (Figure 1). The 2 presynchronization schemes resulted in a DO protocol (Souza et al., 2008; Herlihy et al., 2012) and a GGPG protocol (Giordano et al., 2012b; Lopes et al., 2013), respectively. Prostaglandin $\text{F}_{2\alpha}$ (25 mg of dinoprost tromethamine; Lutalyse) was from Zoetis (New York, NY). The GnRH (100 μg of Gonadorelin diacetate tetrahydrate; Cystorelin) was from Merial (Duluth, GA). Cows ($n = 306$) in the DO treatment received the first GnRH injection of the Presynch portion of the DO protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, and then began the Ovsynch-56 protocol 7 d later. Cows ($n = 295$) in the GGPG treatment received a GnRH injection at 63 ± 3 DIM, and then began the Ovsynch-56 protocol 7 d later. All cows received the same Ovsynch-56 protocol [first GnRH (**G1**) at $70 \pm$

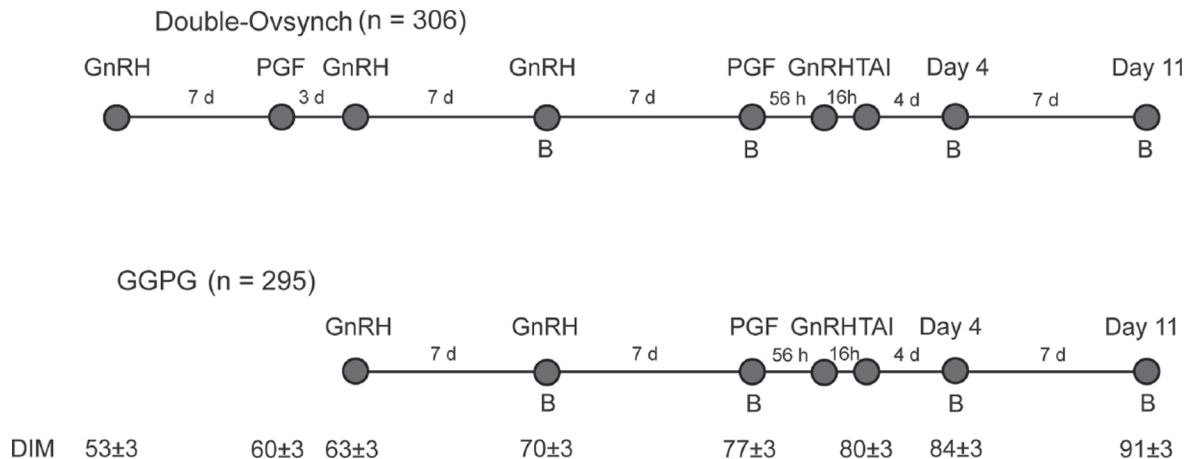


Figure 1. Schematic representation of the synchronization protocols. Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later. The GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later. All cows received a similar Ovsynch-56 protocol: at 70 ± 3 d cows received a GnRH injection, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 56 h after $\text{PGF}_{2\alpha}$, and were given a timed AI (TAI) 16 to 20 h after the last GnRH injection. B = blood sample.

3 DIM, $\text{PGF}_{2\alpha}$ 7 d later, GnRH 56 h after $\text{PGF}_{2\alpha}$, and AI 16 to 20 h later] and received TAI at 80 ± 3 DIM. Three experienced AI technicians that were blinded to treatments performed all inseminations. Multiple sires with high genetic merit and proven fertility were used and were equally balanced between treatments.

Pregnancy Diagnosis

Pregnancy diagnosis was performed 32 d after TAI for all cows in both treatments using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a corpus luteum on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant was reconfirmed at 46 and 70 d after TAI using the same ultrasound machine. Twin pregnancy diagnosis was performed on the day of the third pregnancy diagnosis at 70 d after AI. Cows diagnosed pregnant and then diagnosed not pregnant at the subsequent pregnancy examination were considered to have undergone pregnancy loss.

Blood Sampling and Progesterone Assay

Blood samples from a subgroup of cows were collected via puncture of the median caudal vein or artery into 8-mL evacuated serum collection tubes (Vacurette, Greiner Bio-One North America Inc., Monroe, NC). Samples were collected immediately before G1 (DO, $n = 255$; GGPG, $n = 244$) and the $\text{PGF}_{2\alpha}$ (DO, $n = 256$;

GGPG, $n = 244$) injections of the Ovsynch-56 protocol, as well as at 4 (DO, $n = 170$; GGPG, $n = 156$) and 11 d (DO, $n = 171$; GGPG, $n = 156$; Figure 1) after TAI. After collection, blood samples were refrigerated for 24 h and centrifuged ($1,600 \times g$; 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 Corp., Los Angeles, CA). The average sensitivity for the 4 assays was 0.035 ng/mL. The average intraassay CV was 4.04% and the interassay CV was 4.32% based on a quality control sample (2.50 ng/mL of P4), which was replicated within each assay.

BCS Evaluation and Milk Yield

Body condition score of a subgroup of cows (DO, $n = 228$; GGPG, $n = 218$) was evaluated on the day of the $\text{PGF}_{2\alpha}$ injection of the Ovsynch-56 protocol (77 ± 3 DIM) using a 5-point scale with 0.25 increments where 1 = thin and 5 = fat (Edmonson et al., 1989). The same individual performed all BCS evaluations throughout the experiment. For statistical analysis, BCS was categorized as either low (≤ 2.50) or high (≥ 2.75).

Milk weights were recorded at each milking and stored in the on-farm computer software program (DairyComp 305, Valley Agricultural Software, Tulare, CA). Average weekly milk weights were extracted from the software program and used to calculate milk production from calving to the first pregnancy diagnosis. For statistical analysis, cows were categorized by parity (primiparous vs. multiparous) according to milk production as being below or above mean milk production.

Temperature and Humidity Recording and Heat Stress

Temperature (°C) and relative humidity (RH; %) data were obtained from the University of Wisconsin Extension Ag Weather website (http://www.soils.wisc.edu/uwex_agwx/). Weather data were collected from May 2012 to August 2013. The weather station is located in Arlington, Wisconsin, approximately 0.5 km from the dairy. For each 24-h period, average daily mean and maximum temperature and RH were recorded and a temperature humidity index (THI) was calculated. Ambient temperature was converted from Celsius to Fahrenheit [temperature in °F = (temperature in °C × 1.8) + 32], and the average and maximum daily THI was calculated as $THI = td - [0.55 - (0.55 \times RH / 100)] (td - 58)$, in which *td* is the temperature (in °F) and RH is expressed as a percentage (NOAA, 1976). The THI was averaged over the 10-d period preceding the day of AI (from G1 to the day of TAI), and was the criterion used to determine the season in which the insemination occurred. Inseminations performed when the average maximum THI was <72 were considered to have occurred during the cool season, whereas inseminations performed when the average maximum THI was ≥72 were considered to have occurred during the warm season. For statistical analysis, cows inseminated when the average of maximum THI was >72 during the 10-d period before AI (from G1 to the day of TAI) were classified as being exposed to heat stress, whereas cows in which the average maximum THI was ≤72 during the 10-d period before AI were classified as not being exposed to heat stress.

Statistical Analyses

The experimental design was a complete randomized block design with parity (primiparous vs. multiparous) as the blocking factor. 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, pregnancy loss, and twins) was performed by logistic regression using the GLIMMIX procedure of SAS. For P/AI at 32, 46, and 70 d after TAI, the initial model contained the following categorical explanatory variables as fixed effects: parity (primiparous vs. multiparous), treatment (DO vs. GGPG), level of milk production (high vs. low), BCS (≤2.5 vs. >2.5), and season (warm vs. cool), as well as the interactions between these variables.

All initial models contained both fixed and random effects. Fixed effects included in the initial models were treatment, parity, level of milk production, BCS,

and season, as well as the interactions between these variables, whereas cow was included as a random effect. 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 was used to evaluate if the matrix containing random effects could be reduced to zero. A covariance parameter test based on the residual pseudolikelihood was run and a nonsignificant chi-squared *P*-value indicated that random effects could be eliminated from the model (SAS Documentation for GLIMMIX, SAS 9.3). 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 using a backward elimination procedure that removed all variables with a *P*-value exceeding 0.10 from the model. Both treatment and parity were forced to remain in each model. Parity was forced into the final model because it was used as a blocking factor for randomization of cows to treatments. Therefore, for P/AI at 32, 46, and 70 d after TAI, the final model contained the fixed effects of treatment and parity. Because season and parity were not significant in the logistic regression model, differences in P/AI between parities within treatments, as well as between seasons within treatments, were obtained using a chi square test.

The same categorical variables and interactions used for P/AI were used to obtain the models for analysis of pregnancy loss (from 32 to 46, 46 to 70, and from 32 to 70 d after TAI) and twin pregnancies. The procedures and criteria used for model selection were similar to those used for P/AI. The final model included the fixed effects of treatment and parity.

Treatment differences in circulating P4 concentrations at G1 and the PGF_{2α} injection of the Ovsynch-56 protocol, as well as at 4 and 11 d after TAI, were determined by ANOVA using the MIXED procedure of SAS. The model contained as fixed effects treatment, parity, and their interaction, whereas cow was used as a random effect in the model. Data were examined for normality using the Shapiro-Wilk test. A significant *P*-value indicated that data were not normally distributed; therefore data were transformed to ranks. Cows were distributed into 9 categories using P4 concentration at G1 and the PGF_{2α} injection of the Ovsynch-56 protocol (from 0.00 to ≥4.00 ng/mL in 0.5-ng/mL increments). At 11 d after TAI, cows were divided into 7 categories (0.00 to ≥7.00 ng/mL in 1.00-ng/mL increments). Differences in the proportion of cows within each P4 category were analyzed by logistic regression using the LOGISTIC procedure of SAS. The effect of P4 on P/AI

was assessed by logistic regression using the LOGISTIC procedure of SAS with a model that only contained the variable P4 concentration as fixed effect. For evaluation of the effect of P4 at the PGF_{2α} injection of the Ovsynch-56 protocol on P/AI, cows with P4 concentrations <1.00 ng/mL (*n* = 58) were removed from the analysis, whereas cows with P4 concentrations >10.0 ng/mL were classified as having P4 concentrations of 10 ng/mL. The effect of milk production during the week before AI on P/AI was also evaluated by logistic regression using the LOGISTIC procedure of SAS with a model that contained the fixed effects of milk production on the week before AI (continuous), parity (primiparous vs. multiparous), and their interaction.

Differences in milk production and BCS were determined by ANOVA using the MIXED procedure of SAS. The model contained as fixed effects treatment, week postpartum (for evaluation of milk production only), parity, and their interaction, whereas cow was used as a random effect in the model.

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

RESULTS

Milk Production and BCS

The average milk yield from calving to the first pregnancy diagnosis after first TAI did not differ ($P = 0.70$) between DO and GGPG cows (47.1 vs. 46.7 kg/d, respectively). In addition, milk production did not differ ($P = 0.71$) between DO and GGPG cows (50.1 vs. 49.7 kg/d, respectively) during the week before TAI. Milk production from calving to the first pregnancy diagnosis after first TAI was greater ($P < 0.001$) for multiparous cows compared with primiparous cows (53.3 vs. 36.0 kg/d, respectively). For the subgroup of cows (*n* = 446) with BCS evaluated at the PGF_{2α} injection of the Ovsynch-56 protocol, BCS did not differ ($P = 0.68$) between DO and GGPG cows (3.08 vs. 3.09, respectively); however, primiparous cows had a greater ($P < 0.001$) BCS compared with multiparous cows (3.21 vs. 3.04, respectively).

P/AI, Pregnancy Loss, and Twins

At 32 d after TAI, DO cows had more ($P = 0.01$) P/AI than GGPG cows (Table 1). Nevertheless, P/AI at 32 d after TAI did not differ ($P = 0.16$) between primiparous and multiparous cows [51 (115/224) vs. 46% (172/377), respectively]. Furthermore, P/AI did

not differ ($P = 0.35$) between treatments for primiparous cows, whereas multiparous GGPG cows had fewer ($P = 0.02$) P/AI compared with multiparous DO cows (Table 2). Pregnancies per AI at 32 d after TAI did not differ ($P = 0.15$) between cows receiving AI during the cool and warm seasons [50 (204/412) vs. 44% (83/189), respectively]; however, P/AI at 32 d after TAI did not differ ($P = 0.88$) for DO cows receiving TAI during the cool and warm season, whereas GGPG cows tended to have fewer ($P = 0.07$) P/AI after TAI during the warm compared with the cool season (Table 2). Overall, P/AI did not differ ($P = 0.16$) between cows with high versus low BCS near TAI [48 (201/418) vs. 36% (10/28), respectively].

At 46 d after TAI, DO cows had more ($P = 0.03$) P/AI than GGPG cows (Table 1). Overall, P/AI at 46 d after TAI did not differ ($P = 0.17$) between primiparous and multiparous cows [50 (112/224) vs. 44% (167/377), respectively]. At 70 d after TAI, DO cows had more ($P = 0.05$) P/AI than GGPG cows (Table 1). Nevertheless, P/AI at 70 d after TAI did not differ ($P = 0.13$) between primiparous and multiparous cows [49 (109/224) vs. 42% (160/377), respectively].

Overall pregnancy loss for all cows from 32 to 46, 46 to 70, and total loss from 32 to 70 d after TAI was 2.8, 3.6, and 6.3, respectively. Pregnancy loss from 32 to 46 d after TAI did not differ between treatments (Table 1), parities ($P = 0.92$; 2.6 vs. 2.9%, for primiparous vs. multiparous cows, respectively), or season ($P = 0.73$; 2.9 vs. 2.5%, for TAI during the cool vs. warm season, respectively). Similarly, pregnancy loss from 46 to 70 d after TAI did not differ between treatments (Table 1) or parities ($P = 0.52$; 2.7 vs. 4.2% for primiparous vs. multiparous cows, respectively). Overall pregnancy loss from 32 to 70 d after TAI did not differ between treatments (Table 1) or parities ($P = 0.58$; 5.2 vs. 7.0% for primiparous vs. multiparous cows, respectively).

Percentage of cows diagnosed with twins at 70 d after TAI did not differ ($P = 0.55$) between DO vs. GGPG cows (4.9 vs. 6.2%, for, respectively); however, multiparous cows tended ($P = 0.06$) to have more twin pregnancies compared with primiparous cows (7.8 vs. 1.9%, respectively). Overall, the percentage of cows diagnosed with twins at 70 d after TAI was 5.4%.

P4 Concentrations

At G1 of the Ovsynch-56 protocol, mean P4 concentrations did not differ ($P = 0.71$) between DO and GGPG cows (2.61 vs. 3.11 ng/mL, respectively), and did not differ ($P = 0.16$) between parities (3.02 vs. 2.79 ng/mL, for primiparous vs. multiparous cows, respectively). When cows were divided into 9 categories

Table 1. Effect of treatment on pregnancies per artificial insemination (P/AI) after first timed AI and pregnancy loss in lactating Holstein cows

Item	Treatment ¹		P-value
	Double-Ovsynch	GGPG	
P/AI, % (n/n)			
32 d	52.6 (161/306)	42.7 (126/295)	0.01
46 d	50.7 (155/306)	42.0 (124/295)	0.03
70 d	48.7 (149/306)	40.7 (120/295)	0.05
Pregnancy loss, % (n/n)			
32 to 46 d	3.7 (6/161)	1.6 (2/126)	0.29
46 to 70 d	3.9 (6/155)	3.2 (4/124)	0.80
32 to 70 d	7.5 (12/161)	4.8 (6/126)	0.37

¹Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of PGF_{2α} 7 d later and GnRH 72 h after PGF_{2α}, then began an Ovsynch-56 protocol 7 d later. The GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later.

based on P4 concentrations at G1 (Figure 2), more ($P < 0.01$) DO cows had medium P4 concentrations (0.50 to 3.99 ng/mL) compared with GGPG cows (82 vs. 50%). In addition, fewer DO cows had low (<0.5 ng/mL) P4 concentrations (3 vs. 17%; $P < 0.01$) or high (≥ 4.00 ng/mL) P4 concentrations (15 vs. 33%; $P < 0.01$) compared with GGPG cows.

At the PGF_{2α} injection of the Ovsynch-56 protocol, mean P4 concentrations were greater ($P < 0.001$) for DO cows compared with GGPG cows (5.35 vs. 3.55 ng/mL, respectively), whereas mean P4 concentrations were similar ($P = 0.75$) between primiparous and multiparous cows (4.47 vs. 4.48 ng/mL, respectively). When cows were divided into 9 categories based on P4 concentrations at the PGF_{2α} injection of the Ovsynch-56 protocol (Figure 3), more ($P < 0.01$) DO cows had high P4 (>4.0 ng/mL) compared with GGPG cows (67 vs. 36%), whereas more ($P < 0.01$) GGPG cows had lower P4 concentrations (0 to 1.99 ng/mL) than DO cows (39.8 vs. 7%). Furthermore, more GGPG cows had P4 concentrations from 0 to 1.0 and 1.0 to 4.0 ng/mL, whereas more DO cows had P4 concentrations from 4 to 7 and >7 ng/mL (Table 3). For these ranges of P4 concentrations, P/AI were similar between treatments

for cows with P4 concentrations of 0 to 1.0, 1.0 to 4.0, and >7 ng/mL, whereas P/AI were less for GGPG cows with P4 concentrations between 4 and 7 ng/mL (Table 3). Furthermore, when cows from both treatments were combined to evaluate the effect of P4 concentration on P/AI (Table 3), P/AI differed ($P < 0.01$) among P4 categories and was least for cows with P4 concentrations of 0 to 1.0 ng/mL and greatest for cows with P4 concentrations from 1.0 to 4.0, 4.0 to 7, and >7 ng/mL (27.6, 47.3, 46.0, and 60.2%, respectively).

At 4 d after TAI, mean P4 concentrations did not differ ($P = 0.17$) between DO vs. GGPG cows (0.89 vs. 1.09 ng/mL, respectively); however, mean P4 concentrations were greater ($P = 0.01$) for primiparous compared with multiparous cows (1.13 vs. 0.93 ng/mL, respectively). In addition, the distribution of cows based on P4 concentrations 4 d after TAI was similar between treatments (Figure 4). When the effect of P4 concentrations 4 d after TAI was evaluated, cows with extremely low [$P4 = <0.3$ ng/mL; 13.0% (3/23)] or high [$P4 = \geq 2.0$ ng/mL; 10.3% (3/29)] P4 had fewer ($P < 0.001$) P/AI compared with cows with medium P4 concentrations [$0.3 \geq P4 < 2.0$ ng/mL; 50.7% (139/274)].

Table 2. Effect of parity and season on pregnancies per AI (P/AI) at 32 d after first timed AI for lactating Holstein cows synchronized using a Double-Ovsynch protocol or a GGPG protocol¹

Item, P/AI, % (n/n)	Double-Ovsynch	GGPG	P-value
Parity			
Primiparous	54.5 (61/112)	48.2 (54/112)	0.35
Multiparous	51.5 (100/194)	39.3 (72/183)	0.02
P-value	0.62	0.14	
Season			
Warm	52.0 (52/100)	34.8 (31/89)	0.02
Cool	52.9 (109/206)	46.1 (95/206)	0.17
P-value	0.88	0.07	

¹Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of PGF_{2α} 7 d later and GnRH 72 h after PGF_{2α}, then began an Ovsynch-56 protocol 7 d later. The GGPG cows received a GnRH injection at 63 ± 3 DIM, and then began the Ovsynch-56 protocol 7 d later.

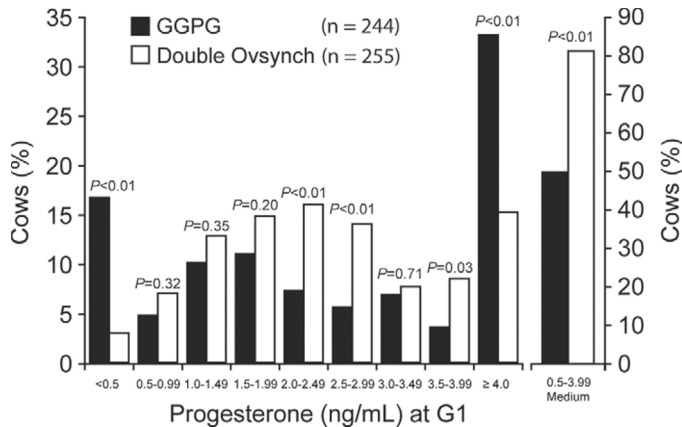


Figure 2. Distribution of cows based on progesterone concentrations at the first GnRH injection (G1) of an Ovsynch-56 protocol for cows presynchronized using a single GnRH injection (GGPG; black bars) or a modified Ovsynch protocol (Double-Ovsynch; white bars) for first timed AI. Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later, whereas GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later. All cows received a similar Ovsynch-56 protocol: at 70 ± 3 d cows received a GnRH injection, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 56 h after $\text{PGF}_{2\alpha}$, and were given a timed AI 16 to 20 h after the last GnRH injection.

At 11 d after TAI, mean P4 concentrations did not differ ($P = 0.19$) between DO versus GGPG cows (3.85 vs. 4.07 ng/mL, respectively); however, mean P4 concentrations were greater ($P = 0.006$) for primiparous compared with multiparous cows (4.52 vs. 3.74 ng/mL, respectively). The distribution of cows according to P4 concentrations 11 d after TAI did not differ between DO versus GGPG cows (Figure 5). Cows with P4 concentrations <2 ng/mL had fewer ($P < 0.001$) P/AI compared with cows with P4 concentrations >2 ng/mL

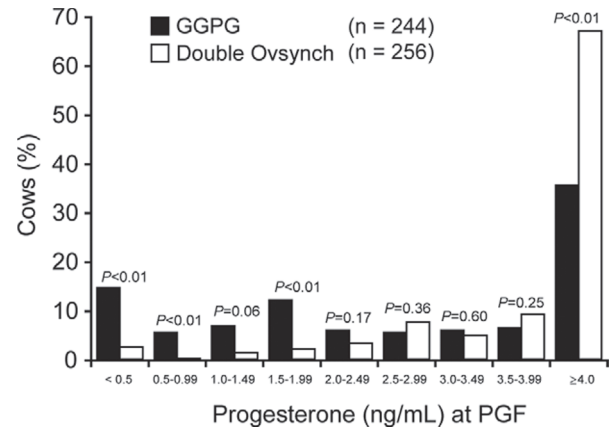


Figure 3. Distribution of cows based on progesterone concentrations at the $\text{PGF}_{2\alpha}$ injection of an Ovsynch-56 protocol for cows presynchronized using a single GnRH injection (GGPG; black bars) or a modified Ovsynch protocol (Double-Ovsynch; white bars) for first timed AI. Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later, whereas GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later. All cows received a similar Ovsynch-56 protocol: at 70 ± 3 d cows received a GnRH injection, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 56 h after $\text{PGF}_{2\alpha}$, and were given a timed AI 16 to 20 h after the last GnRH injection.

[12 (5/43) vs. 50% (141/284)]; however, P/AI did not differ ($P = 0.75$) among cows with P4 concentrations of 2 to 3, 3 to 4, and >4 ng/mL [53.3 (32/60) vs. 50.7 (35/69) vs. 47.7% (74/155), respectively].

DISCUSSION

Cows submitted for first TAI after a Double-Ovsynch protocol had more P/AI compared with cows submitted for first TAI using a Presynch-Ovsynch protocol in

Table 3. Effect of treatment¹ on distribution of cows according to progesterone concentrations at the $\text{PGF}_{2\alpha}$ injection of the Ovsynch-56 protocol and pregnancies per artificial insemination (P/AI) after first timed AI

Item	Progesterone (ng/mL) at $\text{PGF}_{2\alpha}$			
	0.0 to 1.0	>1.0 to 4.0	>4.0 to 7.0	>7.0
Proportion of cows, % (n/n)				
Double-Ovsynch	3.1 (8/256)	30.9 (79/256)	43.0 (110/256)	23.0 (59/256)
GGPG	20.5 (50/244)	43.9 (107/244)	21.7 (53/244)	13.9 (34/244)
P-value	<0.01	<0.01	<0.01	0.01
P/AI, % (n/n)				
Double-Ovsynch	25.0 (2/8)	48.1 (38/79)	53.6 (59/110)	59.3 (35/59)
GGPG	28.0 (14/50)	46.7 (50/107)	30.2 (16/53)	61.8 (21/34)
P-value	0.86	0.85	<0.01	0.82
Overall, % (n/n)	27.6 (16/58) ^{b,B}	47.3 (88/186) ^{a,A}	46.0 (75/163) ^{ab,A}	60.2 (56/93) ^{a,A}

^{a,b}Within the same row, means with different lowercase superscripts differ at $P < 0.05$.

^{A,B}Within the same row, means with different uppercase superscripts differ at $P < 0.10$.

¹Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later. The GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later.

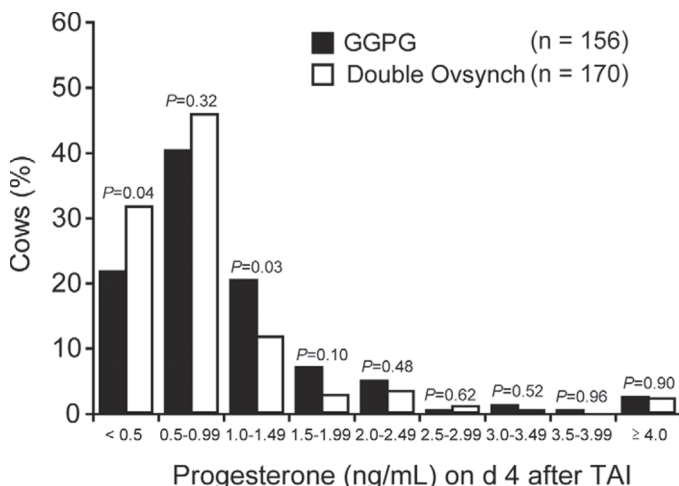


Figure 4. Distribution of cows based on progesterone concentrations 4 d after timed AI (TAI) for cows presynchronized using a single GnRH injection (GGPG; black bars) or a modified Ovsynch protocol (Double-Ovsynch; white bars) for first TAI. Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later, whereas GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later. All cows received a similar Ovsynch-56 protocol: at 70 ± 3 d cows received a GnRH injection, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 56 h after $\text{PGF}_{2\alpha}$, and were given a TAI 16 to 20 h after the last GnRH injection.

confinement-based dairies using a 7-d Ovsynch protocol (Souza et al., 2008; Herlihy et al., 2012), but not for cows managed in grazing-based dairies using a 5-d Cosynch protocol (Ribeiro et al., 2012). Despite having similar P/AI, cows synchronized with a Double-Ovsynch protocol had fewer pregnancy losses compared with cows synchronized with a Presynch-Ovsynch protocol (Ribeiro et al., 2012). In the present experiment, our objective was to compare synchronization of ovulation and TAI using a Double-Ovsynch protocol with a simpler protocol requiring fewer injections (a GGPG protocol) at first TAI in high-producing dairy cows managed in a confinement-based system. Based on data using hGPG in resynchronized cows (Giordano et al., 2012b), our hypotheses was that cows submitted for first TAI using a GGPG protocol would have similar fertility to cows receiving first TAI after a Double-Ovsynch protocol. Contrary to our hypothesis, DO cows had more P/AI at 32, 46, and 70 d after TAI compared with GGPG cows. The underlying physiology that produces these dramatic differences in fertility appears to be a better synchrony of the estrous cycle for DO compared with GGPG cows, as reflected by changes in circulating P4 at G1 and the $\text{PGF}_{2\alpha}$ injections of the Ovsynch-56 protocol.

The distribution of cows based on P4 concentrations at initiation of the Ovsynch-56 protocol was illustra-

tive. Presynchronization using a modified Ovsynch protocol resulted in more cows with midrange P4 concentrations at G1 of the Ovsynch-56 protocol compared with presynchronization using a single GnRH injection. Midrange P4 concentrations are an indication that more DO cows were on d 7 of the estrous cycle (early diestrus) when the Ovsynch-56 protocol was initiated compared with GGPG cows. By contrast, more GGPG cows were in the early and late stages of the estrous cycle when the Ovsynch-56 protocol was initiated compared with DO cows. Cows in the early stages of the estrous cycle when an Ovsynch-56 protocol is initiated have low P4 concentrations during development of the ovulatory follicle. This low-P4 environment results in overstimulation of the dominant follicle or oocyte by exposure to a greater frequency of LH pulses (Revah and Butler, 1996). This overstimulated oocyte is less likely to produce a good quality embryo 7 d after AI (Rivera et al., 2011; Wiltbank et al., 2011). Even if a good quality embryo develops, establishment of pregnancy is not guaranteed. In fact, cows with low P4 concentrations during development of the ovulatory follicle are more predisposed to prematurely develop pathways leading to $\text{PGF}_{2\alpha}$ secretion by the endometrium causing short luteal phases (Cerri et al., 2011), thereby preventing the establishment of pregnancy.

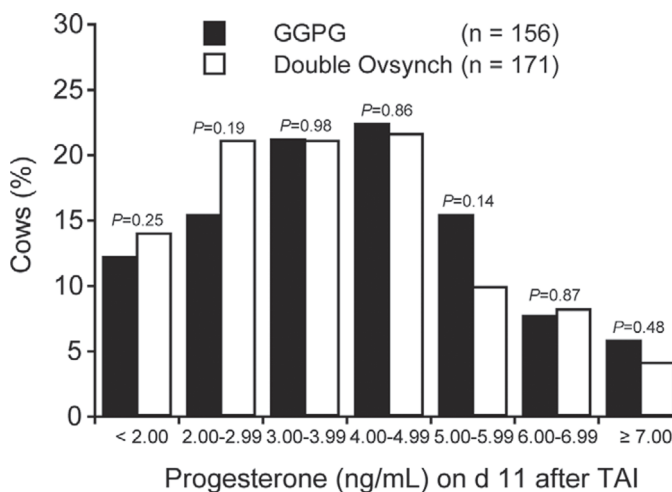


Figure 5. Distribution of cows based on progesterone concentrations 11 d after timed AI (TAI) for cows presynchronized using a single GnRH injection (GGPG; black bars) or a modified Ovsynch protocol (Double-Ovsynch; white bars) for first TAI. Double-Ovsynch cows received the first GnRH injection of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 72 h after $\text{PGF}_{2\alpha}$, then began an Ovsynch-56 protocol 7 d later, whereas GGPG cows received a GnRH injection at 63 ± 3 DIM and then began the Ovsynch-56 protocol 7 d later. All cows received a similar Ovsynch-56 protocol: at 70 ± 3 d cows received a GnRH injection, followed by an injection of $\text{PGF}_{2\alpha}$ 7 d later and GnRH 56 h after $\text{PGF}_{2\alpha}$, and were given a TAI 16 to 20 h after the last GnRH injection.

Cows that are in the late stages of the estrous cycle when an Ovsynch-56 protocol is initiated are less likely to be properly synchronized, resulting in fewer P/AI (Vasconcelos et al., 1999). The reduced synchrony of cows initiating an Ovsynch protocol during late diestrus might be partly attributed to a low ovulatory response to the first GnRH injection of the protocol. Elevated P4 concentrations at the time of the first GnRH injection decrease the amplitude of the GnRH-induced LH surge (Giordano et al., 2012a). Cows in late diestrus that failed to ovulate to the first GnRH injection of the protocol are more likely to have spontaneous luteolysis before the PGF_{2α} injection (Vasconcelos et al., 1999). This leads to an LH surge before the last GnRH injection and ovulation before TAI thereby decreasing the chances of conception (Vasconcelos et al., 1999; Moreira et al., 2000).

In addition, P4 concentrations at the PGF_{2α} injection further support the idea that more DO cows were in early diestrus when the Ovsynch-56 protocol was initiated. Independent of treatment, cows with low P4 (<1.0 ng/mL) at the PGF_{2α} injection had fewer P/AI compared with cows with high P4 (>1.0 ng/mL) concentrations, and more GGPG cows had low P4 concentrations at the PGF_{2α} injection compared with DO cows. Cows without a CL or with low P4 concentrations at the PGF_{2α} injection of the Ovsynch protocol have a greatly reduced P/AI compared with cows with high P4 at the PGF_{2α} injection (Bisinotto et al., 2010; Denicol et al., 2012; Lopes et al., 2013). For example, during a Resynch experiment (Giordano et al., 2012c), 25.6% of cows had low P4 at the PGF_{2α} injection (111/433), and these cows had much lower fertility (8.1%) compared with cows with high P4 (37.0%). In the present study, the reduction in P/AI for cows with low P4 concentrations at PGF_{2α} was also substantial (~20 percentage points), with most cows in this category (86%) being GGPG cows. The reduced fertility in this group of cows might be caused by a lack of synchrony due to premature luteolysis leading to an LH surge and ovulation before TAI as discussed earlier.

An interesting observation from the present study was the reduced P/AI for GGPG (30.2%) compared with DO (53.6%) cows with P4 concentrations from 4 to 7 ng/mL at the PGF_{2α} injection. Reasons for this difference are not clear; however, they might be attributed to differences in synchronization of the follicular wave. Double-Ovsynch cows were likely on d 7 on the estrous cycle and better responded to the first GnRH injection by ovulating a dominant follicle. By contrast, GGPG cows might have been in a later stage of the estrous cycle at the first GnRH injection and therefore failed to ovulate a dominant follicle leading to asynchrony of the follicular wave. We did not evaluate these endpoints

in the present study, and further studies are needed to clarify reasons for the reduced P/AI in GGPG cows with P4 concentration of 4 to 7 ng/mL at the time of the PGF_{2α} injection.

Although circulating P4 after AI is essential for establishment and maintenance of pregnancy (Wiltbank et al., 2014), the relationship between post-AI P4 and fertility is not conclusive. Supplementation with exogenous P4 during the early luteal phase after AI increased the length of embryos (Carter et al., 2008; Clemente et al., 2009) possibly due to changes in gene expression in the uterus induced by P4 supplementation (Clemente et al., 2009). Manipulative studies have used various methods to increase P4 after AI with only a marginal effect on P/AI (Wiltbank et al., 2014). For example, increasing P4 after AI by treating cows with human chorionic gonadotropin 5 d after estrus to induce accessory CL increased P/AI by only 3 percentage points compared with control cows (Nascimento et al., 2013). Although the relationship between post-AI P4 and P/AI is debatable (Wiltbank et al., 2014), a recent study reported that fertility increased as P4 concentrations increased after AI in cows synchronized using a Double-Ovsynch protocol, but not for cows synchronized with a Presynch-Ovsynch protocol (Herlihy et al., 2012). In the present study, a reduction in P/AI in cows with lower (<0.3 ng/mL) and higher (≥2.0 mg/mL) P4 at 4 d after AI was observed. The extremes in P4 (very low or very high at 4 d after TAI) are likely indicative of asynchrony to the protocol rather than a direct effect of P4 on embryonic growth. At 11 d after TAI, cows with low P4 (<2.0 ng/mL) had greatly reduced fertility (11.6%) compared with cows with P4 exceeding 2 ng/mL (53%) probably indicating lack of synchrony to the protocol similar to observations in another study (Herlihy et al., 2012). In contrast to another study (Herlihy et al., 2012), when we removed these asynchronous cows (13.1% of cows) from the analysis, we observed no effect on P/AI with increasing P4 at either 4 or 11 d after TAI. Thus, our results indicate that post-AI P4 concentrations are useful for indicating lack of synchrony to the timed AI protocol but do not support the idea that increasing P4 after AI improves P/AI at least after submission of cows to a TAI protocol.

Interestingly, multiparous cows synchronized using a DO protocol had exceptional P/AI (52%) that was similar to that of primiparous cows (55%). By contrast, multiparous cows synchronized using a GGPG protocol had approximately 9 percentage points fewer P/AI than primiparous cows; however, this difference did not reach statistical significance and may represent a Type II error in the statistical analysis. Some studies using the Double-Ovsynch or GGPG protocols observed fewer P/AI for multiparous cows compared with primiparous

cows (Souza et al., 2008; Giordano et al., 2012b; Lopes et al., 2013), whereas others have reported no difference in P/AI between primiparous and multiparous cows synchronized using a Double-Ovsynch protocol (Brusveen et al., 2009; Giordano et al., 2012c, 2013). Reasons for the high P/AI in multiparous cows synchronized using a Double-Ovsynch protocol in the present study are not clear at this time; however, this difference contributed to the increased P/AI for DO cows compared with GGPG cows.

Heat stress causes a well-documented reduction in fertility in lactating dairy cows (Rutigliano et al., 2008; Giordano et al., 2012b; Astiz and Fargas, 2013; Pereira et al., 2013). The decrease in P/AI observed for cows receiving AI during heat stress may be related to decreases in fertilization rate of the oocytes and a lower proportion of zygotes reaching the blastocyst stage of development (Al-Katanani et al., 2002; Sartori et al., 2002; de S. Torres-Júnior et al., 2008; Hackbart et al., 2010). In the present experiment, we observed no decrease in P/AI during periods of heat stress for cows synchronized with a Double-Ovsynch protocol, whereas GGPG cows tended to have fewer P/AI during the warm season compared with the cool season (Table 2). Average maximum THI exceeded 72 from May to September during the present experiment, and this criterion was used to define cows exposed to heat stress; however, average mean THI exceeded 72 only from June to August. The interaction between treatment and season (cool vs. warm) was not a part of our initial hypothesis, and further studies should be designed to evaluate whether a Double-Ovsynch protocol can mitigate the effects of heat stress or if the cows in the present study were not exposed to sufficiently high temperatures to affect P/AI.

CONCLUSIONS

Cows presynchronized for first TAI using a modified Ovsynch protocol (i.e., a Double-Ovsynch protocol) had more P/AI than cows presynchronized using a single injection of GnRH 7 d before initiation of an Ovsynch protocol (i.e., a GGPG protocol). This difference in fertility was due to an increased synchrony during the Ovsynch-56 protocol based on P4 concentrations collected at G1 and the PGF_{2α} injections of the Ovsynch-56 protocol. Thus, although a Double-Ovsynch protocol requires more injections, it resulted in more P/AI compared with a GGPG protocol for first TAI.

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