Effect of dose and frequency of prostaglandin F$_{2\alpha}$ treatments during a 7-day Ovsynch protocol with an intravaginal progesterone releasing device on luteal regression and pregnancy outcomes in lactating Holstein cows

M. Hölper,[1] **L. Bretzinger,**[1] **F. Randi,**[2] **W. Heuwieser,**[1]* **and S. Borchardt**[1]
[1]Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universitaet Berlin, Koenigsweg 65, 14163 Berlin, Germany
[2]CEVA Sante animale, 10 avenue de la Ballastière, 33500 Libourne, France

ABSTRACT

Our objective was to evaluate the effect of 3 different Ovsynch protocols on progesterone (P4) and pregnancies per artificial insemination (P/AI), where all cows received a P4 releasing intravaginal device (PRID) from d 0 until d 8. We hypothesized that (1) both modified PGF$_{2\alpha}$ treatments lead to decreased P4 at the second GnRH treatment (G2), resulting in greater P/AI, (2) the treatment effect is influenced by the presence of a corpus luteum (CL) at the beginning of the protocol, and (3) potential vaginal discharge caused by the PRID does not have a negative influence on fertility. Lactating Holstein cows (n = 1,056) were randomly assigned to 1 of 3 treatment groups on a weekly basis (n = 356; control: d 0, 100 µg of GnRH + PRID; d 7, 25 mg of dinoprost; d 8, PRID removal; d 9, 100 µg of GnRH). Cows in the second group (n = 353) received an Ovsynch protocol with a double dose of PGF$_{2\alpha}$ (DoubleDose: d 0, 100 µg of GnRH + PRID; d 7, 50 mg of dinoprost; d 8, PRID removal; d 9, 100 µg of GnRH). Cows in the third group (n = 347) received an Ovsynch protocol with a second PGF$_{2\alpha}$ treatment 24 h after the first one (2PGF: d 0, 100 µg of GnRH + PRID; d 7, 25 of mg dinoprost; d 8, 25 mg of dinoprost and PRID removal; d 9, 100 µg of GnRH). All cows had their ovaries scanned to determine the presence of a CL at the beginning of the Ovsynch protocol. Vaginal discharge score (VS) was evaluated at PRID removal. All cows received timed artificial insemination approximately 16 h after G2. Pregnancy diagnosis was performed via transrectal ultrasonography (d 38 ± 3 after timed artificial insemination) and rechecked on d 80 ± 7 after timed artificial insemination. Blood samples were collected on d 0, 7, and 9 of the protocol to determine P4 concentrations. Treatment affected P4 at G2. Progesterone was lower for 2PGF and DoubleDose cows compared with cows in the control group (control 0.35 ± 0.02 ng/mL; Double-Dose 0.29 ± 0.02 ng/mL; 2PGF 0.30 ± 0.02 ng/mL). Overall, P/AI did not differ among treatments. We found, however, an interaction between treatment and CL at the first GnRH treatment. Cows lacking a CL at the first GnRH treatment in the 2PGF group had greater P/AI (47.9%) compared with the same type of cows in the DoubleDose group (32.7%). We observed an effect of VS on P4 concentration at d 7. We found an increase in P4 with greater VS. Vaginal discharge score at PRID removal tended to have a positive effect on P/AI at d 38 (VS0: 36.5%; VS1: 41.3%; VS2: 49.7%). In conclusion, the addition of a second PGF treatment on d 7 and 8 of a 7-d Ovsynch protocol increased luteal regression and decreased mean P4 at G2. Cows treated with PGF$_{2\alpha}$ 2 times 24 h apart showed greater P/AI, compared with cows treated with an increased dose of PGF$_{2\alpha}$.

Key words: prostaglandin, luteal regression, timed artificial insemination, Ovsynch, dairy cow

INTRODUCTION

Protocols for timed AI (**TAI**) have been widely adopted in the dairy industry since their development in the late 1990s (Pursley et al., 1995; Caraviello et al., 2006). The Ovsynch protocol consists of a consecutive treatment with GnRH and PGF$_{2\alpha}$ in a certain time interval, allowing AI that is not dependent on estrus detection (Pursley et al., 1997) due to synchronization of ovulation. Initiating an Ovsynch protocol at a random stage of the estrus cycle may lead to suboptimum fertility because a proportion of cows will not ovulate after the first GnRH treatment (**G1**), and hence have a low concentration of circulating progesterone (**P4**) during the follicle growth phase. This results in reduced oocyte quality, a higher risk for pregnancy loss, and double ovulation (Revah and Butler, 1996; Martins et al., 2018; Carvalho et al., 2019). Intravaginal P4 devices can be used to counteract this effect, providing high P4 levels by mucosal absorption and therefore improving repro-

ductive performance (Bisinotto et al., 2014, 2015a). As a reaction to the devices, a proportion of cows show vaginal discharge (Walsh et al., 2008; Santos et al., 2016b). Also, strategies for presynchronization, such as Presynch-Ovsynch (Moreira et al., 2001; Galvão et al., 2007), Double-Ovsynch (Souza et al., 2008; Herlihy et al., 2012), and G6G (Peters and Pursley, 2002; Bello et al., 2006), have been developed to ensure that cows are in an optimal stage of the estrus cycle to initiate the breeding Ovsynch protocol. This leads to more cows ovulating after G1 and results in a newly formed corpus luteum (**CL**) at d 7 of the Ovsynch protocol. Despite the beneficial effect of providing higher P4 levels during the follicle growth phase (Bisinotto et al., 2010; Wiltbank et al., 2014), the young CL does not necessarily regress completely after a single treatment with PGF$_{2\alpha}$, supposing that a mature CL is not simultaneously present at that time (Nascimento et al., 2014; Carvalho et al., 2018). Although the exact mechanism remains unclear, a co-existing mature CL seems to induce luteal regression after a single PGF$_{2\alpha}$ treatment in the young CL as well (Stevenson, 2016). Incomplete luteal regression, ranging from 0.3 to 0.5 ng/mL (Brusveen et al., 2009; Fricke et al., 2016; Carvalho et al., 2018), at the time of the second GnRH treatment (**G2**) can cause a dramatic decrease in pregnancies per AI (**P/AI**; Giordano et al., 2012b; Wiltbank et al., 2014; Borchardt et al., 2018; Carvalho et al., 2018). Modifications of dose and timing of PGF$_{2\alpha}$ treatments in an Ovsynch protocol have been tested to increase reproductive performance by decreasing the percentage of cows without complete luteal regression at the end of the protocol. One of the strategies was implementing an additional PGF$_{2\alpha}$ treatment 24 h after the first treatment at d 7 of the Ovsynch protocol, increasing the percentage of cows with complete luteal regression and subsequently P/AI (Brusveen et al., 2009; Carvalho et al., 2015; Wiltbank et al., 2015; Santos et al., 2016b; Rheinberger et al., 2020).

Alternatively, a greater dose of PGF$_{2\alpha}$ at the scheduled time (Giordano et al., 2013) was applied, which increased luteal regression in multiparous cows in a 7-d Ovsynch and tended to increase P/AI, but failed to reproduce similar results in a 5-d Ovsynch protocol, compared with cows receiving 2 PGF$_{2\alpha}$ treatments 24 h apart (Ribeiro et al., 2012; Stevenson et al., 2018). Despite several studies examining 1 of the 2 methods, there are only a few studies comparing these different modifications directly and with a control group, regarding reproductive performance and P4 concentrations (Barletta et al., 2018; Minela et al., 2021; Tippenhauer et al., 2021).

The objective of this study was to compare the effect of 2 PGF$_{2\alpha}$ treatments 24 h apart (25 mg of dinoprost)

and the treatment of a double PGF$_{2\alpha}$ dose (50 mg of dinoprost) with a control group (25 mg of dinoprost at d 7) during a 7-d Ovsynch protocol with inclusion of a P4 releasing intravaginal device (**PRID**) on luteal regression and P/AI in lactating Holstein cows. We set out to test 3 hypotheses: (1) the risk of complete luteal regression and P/AI would increase in the modified Ovsynch protocols compared with the traditional 7-d Ovsynch protocol, (2) the treatment effect is influenced by the presence of a CL at the beginning of the protocol, and (3) eventual vaginal discharge due to intravaginal insertions does not have a negative effect on reproductive performances.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Freie Universität Berlin (Reference number 2347-A-3–2-2020).

Cows, Housing, and Feeding

The experiment was conducted from October 2020 until July 2021. Lactating Holstein cows (n = 1,056; 404 primiparous and 652 multiparous cows) from 1 commercial dairy farm in Northeast Germany were used in this study. The farm consisted of 1,600 Holstein Friesian cows with an average 305 ECM yield of 9,809 kg. Lactating cows were housed in a freestall barn with slatted floors and beds equipped with rubber mats. Group composition was dynamic, with cows entering and leaving the study barn depending on their calving dates. Cows were fed a TMR twice a day in a 12-h interval with free access to feed and water. The TMR consisted mainly of corn, alfalfa, and rye silage with brewer's grain, extruded flax, canola meal, and soda gain with an additional mineral mix. The rations were formulated to meet or exceed the requirements according to the NRC (2001). Lactating cows were milked 2 times daily, starting at 0730 and 2000 h.

Reproductive Management and Experimental Treatments

First postpartum AI was executed using a modified Presynch-Ovsynch protocol (PGF, 14 d later PGF$_{2\alpha}$, 12 d later start of an Ovsynch protocol) with cows receiving AI either at detected estrus after the first PGF treatment (50 ± 3 DIM) or after second PGF$_{2\alpha}$ treatment (64 ± 3 DIM). Cows not detected in estrus during this presynchronization (41.5%; n = 465) received TAI according to the following Ovsynch protocol (86 ± 3 DIM). For the subsequent AI, nonpregnant cows
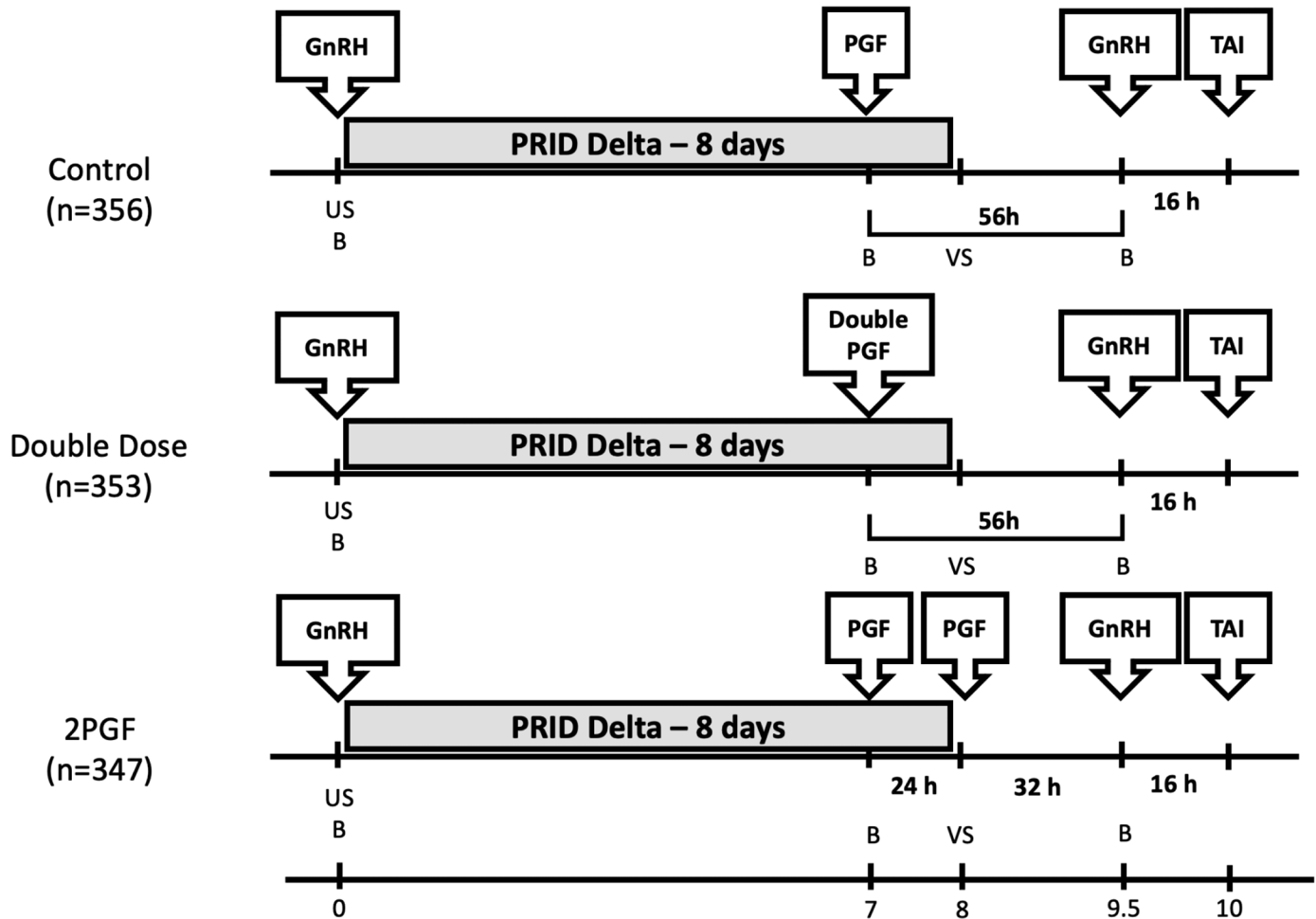
Figure 1. Schematic representation of the study design. Cows in the control group (n = 356) received an Ovsynch protocol with a single prostaglandin F$_{2\alpha}$ (PGF) treatment (control). Cows in the second group (n = 353) received an Ovsynch protocol with a double dose of PGF (DoubleDose). Cows in the third group (n = 347) received an Ovsynch protocol with a second PGF treatment 24 h after the first one (2PGF). All cows received a progesterone releasing intravaginal device (PRID) at the start of the Ovsynch protocol, which was removed at d 8. Approximately 16 h after the second GnRH treatment, all cows received timed AI (TAI). Blood samples were collected on d 0, 7, and 9, and ultrasound examination was performed on d 0 of each protocol. B = blood sample; US = ultrasonography; VS = vaginal discharge score.

not reinseminated based on estrus detection (46.0%; n = 591) received an Ovsynch protocol starting at the day of nonpregnancy diagnosis, at d 38 ± 3 post AI. All cows receiving TAI were randomly assigned within parity to 1 of 3 Ovsynch protocols on a weekly basis (Figure 1). Cows in the control group (n = 356) received an Ovsynch protocol with a single PGF$_{2\alpha}$ treatment (control: d 0, 100 µg of GnRH + PRID; d 7, 25 mg of dinoprost; d 8 PRID removal, d 9, 100 µg of GnRH). Cows in the second group (n = 353) received an Ovsynch protocol with a double dose of PGF$_{2\alpha}$ (**DoubleDose**: d 0, 100 µg of GnRH + PRID; d 7, 50 mg of dinoprost; d 8 PRID removal, d 9, 100 µg of GnRH). Cows in the third group (n = 347) received an Ovsynch protocol with a second PGF$_{2\alpha}$ treatment 24 h after the first one (**2PGF**: d 0, 100 µg of GnRH

+ PRID; d 7, 25 mg of dinoprost; d 8, 25 mg of dinoprost and PRID removal; d 9, 100 µg of GnRH). Cows that lost the PRID before scheduled removal (n = 56; control: 20; DoubleDose: 23; 2PGF: 13), cows that left the herd before pregnancy diagnosis (n = 47; control: 16; DoubleDose: 16; 2PGF: 15), and cows that were not treated or inseminated according to the protocol (n = 23; control: 14; DoubleDose: 6; 2PGF: 3) were excluded. Gonadotropin-Releasing-Hormon (gonadorelin diacetate tetrahydrate; Ovarelin 50 µg/mL), PGF$_{2\alpha}$ (dinoprost trometamol; Enzaprost T 5 mg/mL), and P4 devices (P4; PRID Delta 1.55g) were from Ceva Santé Animale. Approximately 16 h after the G2, all cows received TAI by a trained AI technician. Conventional semen of multiple Holstein sires chosen by the farmer was used for AI.

Body Condition Scoring, Ovarian Ultrasonography, Evaluation of Vaginal Discharge

On study d 0, transrectal ultrasound (Easi-Scan:GO, IMV Imaging) assessment of the ovaries was performed to determine the absence or presence and size of a CL. Luteal structures were identified based on their echogenicity (i.e., isoechogenic). Dimensions were estimated by visualization of the luteal tissue on a grid of 5-mm squares displayed in the background of the ultrasound device screen. Number and size of every CL on each ovary was recorded. Cavities within the CL filled with anechogenic texture were also recorded and subsequently subtracted from the CL total size. Body condition score was evaluated at enrollment using a 5-point scale with 0.25 increments whereas 1 = thin to 5 = fat (Ferguson et al., 1994). Vaginal discharge at the day of the PRID removal was evaluated and scored from 0 to 2, as described by Walsh et al. (2007) and modified by Santos et al. (2016b), consisting of 0 = no debris, 1 = small flecks of purulent debris, and 2 = abundant amount of purulent debris on the intravaginal device and vulva. Determination of BCS, vaginal discharge score (**VS**), and CL measurement were performed by the same, trained individual (M.H.) throughout the study.

Blood Collection and P4 Analysis

Blood samples were collected by venipuncture of the coccygeal vessels, using a 20-gauge, 1.5-inch hypodermic needle (Vacuette, Greiner Bio-One GmbH) at d 0, 7, and 9, to analyze the course of P4 concentration during the different protocols. Blood was extracted into a sterile, plastic, evacuated serum collection tube with no anticoagulant (8 mL, Vacuette, Greiner Bio-One GmbH), and centrifuged after collection on the dairy farm at 2,000 × g for 10 min at approximately 20°C, then stored on ice for transport to the laboratory of Clinic of Animal Reproduction, where serum was transferred into sterile vials (2 mL, Cryovial, Simport) and stored at −18°C until shipment to a commercial laboratory (Segalab, accreditation number L0295). Serum P4 concentrations were determined, using an Enzyme labeled chemiluminescent competitive immunoassay (Immulite Progesterone Enzym, Siemens Healthcare). Intra- and interassay coefficients of variation for 20 assays for repeated samples averaged 7.35 ± 2.44% and 8.08 ± 2.42%, respectively. The lower limit of detection was 0.2 ng/mL.

Pregnancy Diagnosis

Pregnancy diagnosis was performed using transrectal ultrasound d 38 ± 3 after AI and reconfirmed d 80 ± 7 after AI by a veterinarian using a portable ultrasound device (Easi-Scan:GO, IMV Imaging), equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of an embryo with a heartbeat. Nonpregnancy was based on absence of pregnancy at the day of examination or a rebreeding to an estrus before pregnancy diagnosis. Pregnancy loss included cows that experienced pregnancy loss from d 38 to d 80.

Statistical Analysis

The results of pregnancy diagnosis and additional breeding information such as parity and number of AI were obtained from the herd management software (HerdeW, dsp agrosoft GmbH) and transferred to Excel (Microsoft Office 2016, Microsoft Deutschland Ltd.). Based on a statistical power calculation using MedCalc (version 15.6.1, MedCalc Software), enrollment of 350 cows per treatment allowed for detection of a 10 percentage-point treatment difference in P/AI (95% confidence; 80% power) based on a 1-tailed test. All data were analyzed using SPSS for Windows (version 25.0, SPSS Inc., IBM). Logistic regression models for P/AI and pregnancy loss analysis were built using the GENLINMIXED procedure of SPSS. Cow was the experimental unit. Model building was conducted as recommended by (Dohoo et al., 2009), where each parameter was first analyzed separately in an univariable model. Only parameters resulting in univariable models with $P \leq 0.10$ were included in the final mixed model. Selection of the model that best fit the data was performed by using a backward stepwise elimination procedure that removed all variables with $P > 0.10$ from the model. The initial model included the following explanatory variables as fixed effects: treatment (control vs. DoubleDose vs. 2PGF), parity (primiparous vs. multiparous), BCS (≤ 2.75 vs. 2.75 to 3.5 vs. ≥ 3.5), AI number (first service vs. subsequent AI), and CL at G1 (yes vs. no), as well as interactions between these variables. Treatment and CL at G1 were forced to stay in the final model, regardless of their significance level. Insignificant effects and interactions were left out of the model. Therefore, the final model consisted of the following fixed effects: treatment, CL at G1, parity, and the interaction between treatment and CL at G1.

To evaluate the dynamics of P4 during the protocol (d 0, 7, 9), repeated measures ANOVA with first-order autoregressive covariance structure were performed using the GENLINMIXED procedure of SPSS. The outcome variable was serum P4 (ng/mL). The initial model contained the following explanatory variables as fixed effects: sampling time (d 0, 7, 9), treatment (control vs. DoubleDose vs. 2PGF), parity (primiparous vs. mul-
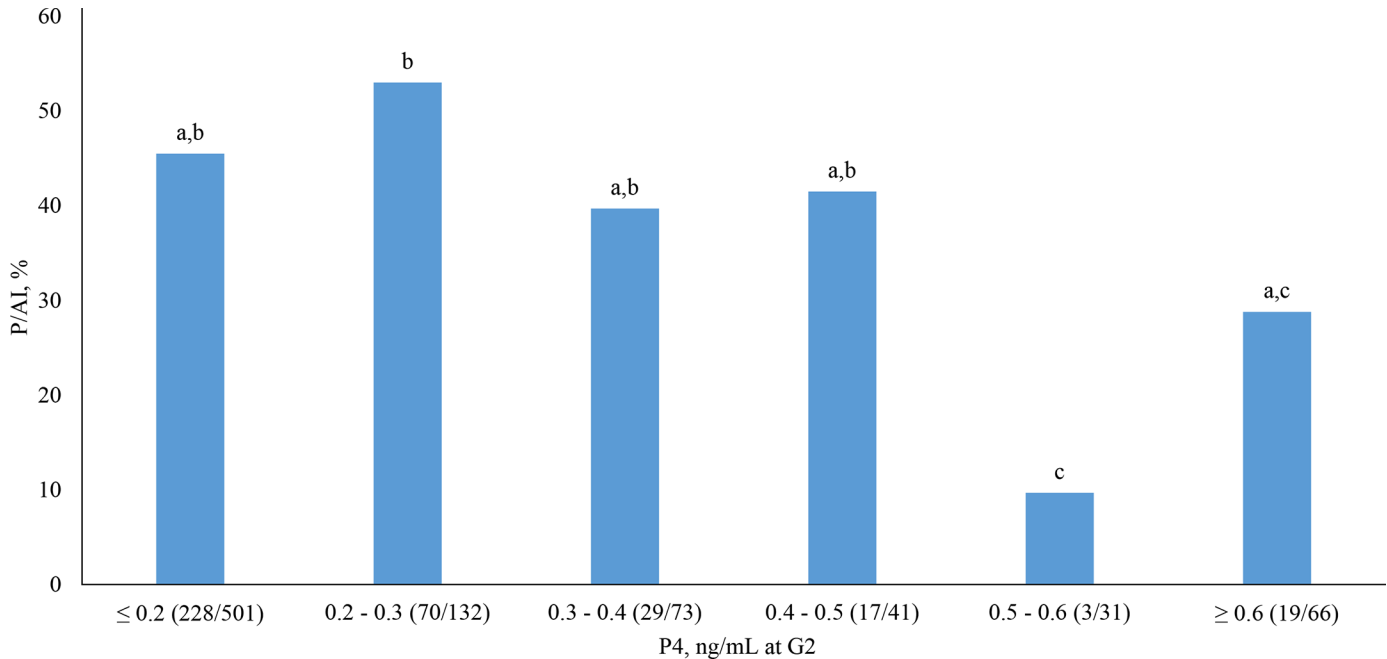
Figure 2. Comparison of pregnancy per AI (P/AI) among different progesterone (P4) concentrations at the second GnRH treatment (G2). Cows were stratified into 6 categories using P4 concentrations (≤0.2 to ≥0.6 ng/mL in 0.1 ng/mL increments) at G2. Pregnancy per AI was calculated for cows in each P4 section to determine a threshold with the greatest decrease in P/AI, using a chi-squared test. Bars with different letters (a–c) differed significantly ($P \leq 0.05$).

tiparous), AI number (first service vs. subsequent AI), CL at G1 (yes vs. no), and interactions between these variables. Insignificant effects and interactions were left out of the model. Therefore, the final model consisted of the following variables as fixed effects: treatment, CL at G1, parity, AI number, their interactions with sampling time, sampling time, and the interaction of CL at G1 and treatment. Due to a significant interaction of CL at G1 and treatment, we calculated 2 separate models for cows with and without a CL at G1.

A Bonferroni adjustment was used to account for multiple comparisons. Variables were declared to be significant when $P < 0.05$. A statistical tendency was declared when $P \geq 0.05$ and $P \leq 0.10$.

Cows were stratified into 6 categories (Figure 2) using P4 concentrations (≤0.2 to ≥0.6 ng/mL in 0.1 ng/mL increments) at G2. Pregnancy per AI was calculated for cows in each P4 section to determine a threshold with the greatest decrease in P/AI, using a chi-squared test. The threshold was used to categorize a cutoff value for incomplete luteal regression at G2.

The effect of VS on P/AI was evaluated using the GENLINMIXED procedure as described above. The initial model contained the following fixed effects: parity (primiparous vs. multiparous), BCS (≤2.75 vs. 2.75 to 3.5 vs. ≥ 3.5), AI number (first service vs. subsequent AI), CL at G1 (yes vs. no), and VS (0 vs. 1 vs.

2). Insignificant effects and interactions were left out of the final model; therefore, the final model consisted of VS and parity.

The effect of vaginal discharge on P4 dynamics was evaluated by repeated measures ANOVA using the GENLINMIXED procedure as described above. The initial model contained the following explanatory variables as fixed effects: sampling time (d 0, 7, 9), parity (primiparous vs. multiparous), AI number (first service vs. subsequent AI), CL at G1 (yes vs. no), VS (0 vs. 1 vs. 2), and interactions between these variables. Insignificant effects and interactions were left out of the model. Therefore, the final model consisted of the following variables as fixed effects: sampling time, VS, parity, AI number, CL at G1, and the interaction between VS and sampling time.

To determine the accuracy to identify a functional CL at the beginning of the Ovsynch protocol using transrectal ultrasound, we used a similar procedure as described by Sauls-Hiesterman et al. (2020). Accuracy, sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the visual ability of the veterinarian to detect a CL by transrectal ultrasonography. The threshold for comparison of a functional CL at G1 was defined as P4 above 1 ng/mL. Positive detection of a CL and serum P4 above 1 ng/mL was defined as a true positive (**TP**). Absence of a

Table 1. Comparison of descriptive data (±SEM) for cows enrolled in this experiment[1]

Item	Treatment[2]			P-value
	Control	DoubleDose	2PGF	
DIM at enrollment	141.6 ± 4.2	136.1 ± 4.0	143.4 ± 5.0	0.466
BCS at enrollment	2.89 ± 0.02	2.90 ± 0.02	2.91 ± 0.02	0.840
Primiparous cows, %	37.9	38	38.9	0.955
Mean lactation number	2.24 ± 0.07	2.21 ± 0.07	2.27 ± 0.08	0.513
First service, %	43.5	46.2	42.7	0.603
Mean service number	2.24 ± 0.08	2.20 ± 0.1	2.31 ± 0.11	0.704
Active CL at enrollment,[3] %	71.1	75.1	75.8	0.305

[1]Lactating Holstein cows were randomly assigned to receive (1) control: d 0, 100 µg of GnRH + progesterone releasing intravaginal device (PRID); d 7, 25 mg of dinoprost; d 8, PRID removal; d 9, 100 µg of GnRH; (2) DoubleDose: d 0, 100 µg of GnRH + PRID; d 7, 50 mg of dinoprost; d 8, PRID removal; d 9, 100 µg of GnRH; (3) 2PGF: d 0, 100 µg of GnRH + PRID; d 7, 25 mg of dinoprost; d 8, 25 mg of dinoprost and PRID removal; d 9, 100 µg of GnRH. All cows received timed AI approximately 16 h after the second GnRH treatment (G2).
[2]DoubleDose = an Ovsynch protocol with a double dose of $PGF_{2\alpha}$; 2PGF = an Ovsynch protocol with a second $PGF_{2\alpha}$ treatment 24 h after the first one.
[3]Blood progesterone ≥1 ng/mL at enrolment; CL = corpus luteum.

CL and serum P4 below 1 ng/mL was considered to be a true negative (**TN**). A false positive (**FP**) was defined as detection of a CL when P4 was below 1 ng/mL. A false negative (**FN**) was defined as absence of a CL when serum P4 was above 1 ng/mL. Accuracy was calculated by dividing the TP by all positives [TP/(TP + FP)]. Sensitivity was calculated by dividing the TP by the sum of the TP and FN [TP/(TP + FN)], whereas specificity was determined by dividing the TN by the sum of the FP and TN [TN/(FP + TN)]. The positive predictive value was determined by dividing the TP by all test positives [TP/(TP + FP)]. The negative predictive value was determined by dividing the TN by all test negatives [TN/(TN + FN)]. To define reference criteria for identifying cows with a functional CL based on the CL diameter, we used a receiver operating characteristic (**ROC**) analysis. The continuous variable was CL diameter, and the classification variable was P4 concentration above 1.0 ng/mL. The ROC curves compare sensitivity with 100 − specificity.

The point on the ROC curve with the highest combined sensitivity and specificity was considered the critical threshold. Interpretation of this critical threshold was based on the area under the curve (**AUC**), according to Swets (1988), as noninformative (AUC = 0.5), accurate (0.5 < AUC ≤ 0.7), very accurate (0.7 < AUC ≤ 0.9), highly accurate (0.9 < AUC < 1), and perfect (AUC = 1).

RESULTS

Milk Yield, BCS, and AI in Estrus

Descriptive data are summarized in Table 1. Average BCS did not differ among treatments (P = 0.840). Pri-

miparous cows had greater (P = 0.001) BCS compared with multiparous cows (3.09 ± 0.3 vs. 2.77 ± 0.4). The proportion of cows with low BCS (≤2.50) was higher in multiparous cows (37.5%) than in primiparous cows (6.6%). However, the distribution of cows with low BCS did not differ among treatments (P = 0.633). Average milk production did not differ among treatments (P = 0.788, control: 34.9 ± 0.4; DoubleDose: 34.6 ± 0.4; 2PGF: 34.5 ± 0.4). The proportion of cows receiving first postpartum AI, thus having presynchronization before the Ovsynch protocol, did not differ (P = 0.603) among treatments. Days in milk at enrollment did not differ among treatments (P = 0.466).

P4 Concentrations

In total, 1,011 cows had a complete set of P4 concentrations from all 3 time points (Table 2). Mean P4 serum concentrations at enrollment did not differ (P = 0.630) among treatments (control 2.31 ± 0.11 ng/mL; DoubleDose 2.38 ± 0.11 ng/mL; 2PGF 2.24 ± 0.11 ng/mL) or parity (P = 0.222; primiparous cows 2.37 ± 0.09 ng/mL; multiparous cows 2.25 ± 0.07 ng/mL). Cows receiving first AI had lower (P = 0.001) P4 concentrations at G1 (1.94 ± 0.08 ng/mL) compared with cows receiving subsequent AI (2.68 ± 0.08 ng/mL). Cows with a CL at G1 had greater (P = 0.001) P4 concentrations at initiation of the protocol (4.2 ± 0.06 ng/mL) compared with cows lacking a CL at G1 (0.41 ± 0.11 ng/mL). On study d 7, mean P4 concentrations did not differ (P = 0.282) among treatments (control 3.65 ± 0.14 ng/mL; DoubleDose 3.54 ± 0.14 ng/mL; 2PGF 3.33 ± 0.15 ng/mL), but differed among parity (P = 0.001), with primiparous cows having greater P4 concentrations (3.82 ± 0.13 ng/mL) than

Table 2. Comparison of progesterone (P4, ng/mL ± SEM) concentrations among treatments[1]

Item[2]	Treatment[3]			P-value[4]			
	Control	DoubleDose	2PGF	Trt	C1	C2	C3
P4 at G1, ng/mL	2.31 ± 0.11	2.38 ± 0.11	2.24 ± 0.11	0.630	0.612	0.649	0.340
P4 at PG, ng/mL	3.65 ± 0.14	3.54 ± 0.14	3.33 ± 0.15	0.282	0.586	0.115	0.305
P4 at G2, ng/mL	0.35 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.037	0.023	0.043	0.753

[1]Lactating Holstein cows were randomly assigned to receive (1) control: d 0, 100 µg of GnRH + progesterone releasing intravaginal device (PRID); d 7, 25 mg of dinoprost; d 8, PRID removal; d 9, 100 µg of GnRH; (2) DoubleDose: d 0, 100 µg of GnRH + PRID; d 7, 50 mg of dinoprost; d 8 PRID removal; d 9, 100 µg of GnRH; (3) 2PGF: d 0, 100 µg of GnRH + PRID; d 7, 25 mg of dinoprost; d 8, 25 mg of dinoprost and PRID removal; d 9, 100 µg of GnRH; all cows received timed AI approximately 16 h after the second GnRH treatment (G2).

[2]Progesterone (P4) concentrations were derived from the GENLINMIXED model including treatment, corpus luteum at d 0 of the protocol (G1), parity, sampling time, AI number, their interactions with sampling time and the interaction between treatment and corpus luteum at G1; PG = d 7 of the protocol.

[3]DoubleDose = an Ovsynch protocol with a double dose of PGF$_{2\alpha}$; 2PGF = an Ovsynch protocol with a second PGF$_{2\alpha}$ treatment 24 h after the first one.

[4]Trt = treatment; C1 = pairwise comparison between the control group and DoubleDose group; C2 = pairwise comparison between the control group and 2PGF group; C3 = pairwise comparison between the 2PGF group and DoubleDose group.

multiparous cows (3.12 ± 0.1 ng/mL). Cows receiving first AI had greater ($P = 0.001$) P4 concentrations on study d 7 (4.24 ± 0.11 ng/mL) compared with cows receiving subsequent AI (2.8 ± 0.11). Cows with a CL at initiation of the protocol had greater ($P = 0.001$) P4 concentrations on study d 7 (4.34 ± 0.09 ng/mL) compared with cows lacking a CL at G1 (2.68 ± 0.14 ng/mL). Distribution of cows having P4 concentrations above 1.0 ng/mL, as considered a cutoff level for presence of a CL, at first PGF$_{2\alpha}$ application did not differ ($P = 0.904$) among treatments (control 85.7%; DoubleDose 84.7%; 2PGF 85.4%), but was greater ($P = 0.001$) for primiparous cows (359/389; 93.3%) compared with multiparous cows (504/622; 81.0%), and also ($P = 0.001$) for cows receiving first AI (402/443; 90.7%) compared with cows receiving subsequent AI (461/568; 81.2%). Proportion of cows with luteolysis differed among treatments ($P = 0.025$; control 84.8%; DoubleDose 91%; 2PGF 92.6%) considering only cows with P4 concentrations above 1.0 ng/mL (indicative of presence of a CL) at first PGF$_{2\alpha}$ (863/1011) and 0.5 ng/mL at G2 (indicative of complete luteolysis). Cows in the 2PGF group ($P = 0.010$) and in the DoubleDose group ($P = 0.033$) had a greater proportion of cows with complete luteolysis compared with control cows. The proportion of cows with complete luteolysis did not differ between DoubleDose and 2PGF cows ($P = 0.525$). The proportion of cows with complete luteal regression did not differ among parity ($P = 0.229$) or luteal status at protocol initiation ($P = 0.267$). We found, however, an interaction between treatment and CL at G1 ($P = 0.045$), with a greater proportion of cows with complete luteolysis in the 2PGF group ($P = 0.06$) and DoubleDose group ($P = 0.017$) compared with control cows, when no CL was present at G1. The proportion of cows with complete luteolysis differed (P

= 0.032) among AI number (first AI 87.7%; subsequent AI 91.7%). Mean P4 concentrations at G2 differed ($P = 0.037$) among treatments (control 0.35 ± 0.02 ng/mL; DoubleDose 0.29 ± 0.02 ng/mL; 2PGF 0.30 ± 0.02 ng/mL) and were lower for 2PGF cows ($P = 0.043$) and DoubleDose cows ($P = 0.023$) compared with cows in the control group. Progesterone at G2 did not differ for DoubleDose and 2PGF cows ($P = 0.753$). Progesterone concentrations at G2 did not differ among parity ($P = 0.425$; multiparous 0.27 ± 0.01 ng/mL; primiparous 0.29 ± 0.02 ng/mL). Cows receiving first AI tended ($P = 0.087$) to have greater P4 concentrations at G2 (0.36 ± 0.01 mg/mL), compared with cows receiving subsequent AI (0.26 ± 0.03 ng/mL). Luteal status at G1 had no influence on P4 concentrations ($P = 0.700$). We found, however, an interaction between treatment and CL at G1 ($P = 0.087$). Cows without a CL at initiation of the protocol had decreased ($P = 0.014$) P4 concentrations when treated with a double dose of PGF$_{2\alpha}$ and tended ($P = 0.062$) to have a decreased P4 concentration at G2 when receiving 2 consecutive treatments 24 h apart.

P/AI and Pregnancy Loss

Overall P/AI at d 38 ± 3 was 42.3%. When all 3 treatments were considered in the model, an effect of treatment on P/AI at d 38 ± 3 (Table 3) was not observed ($P = 0.119$). In pairwise comparison, however, cows that received an additional treatment 24 h apart had greater P/AI (46.5%) compared with DoubleDose cows (37.7%; $P = 0.045$), but did not differ compared with cows in the control group (40.3%; $P = 0.144$). Treatment effect for cows receiving a double dose compared with control cows was not significant ($P = 0.548$). Primiparous cows (44.6%; 185/404) tended (P

Table 3. Comparison of pregnancy per AI [P/AI, % (number of pregnant cows, n/number of all cows, N)] among treatments within luteal status at d 0[1]

Treatment	No CL, %			CL, %			P-value[3]			
	Control	DoubleDose	2PGF	Control	DoubleDose	2PGF	Trt	C1	C2	C3
P/AI at d 38 ± 3 (n/N)	36.1 (37/103)	32.7 (29/88)	47.9 (39/84)	44.5 (111/253)	43.0 (113/265)	45.2 (118/263)	0.119	0.548	0.144	0.045
P/AI at d 80 ± 7 (n/N)	35.5 (36/102)	30.9 (27/87)	45.8 (37/84)	40.0 (98/250)	40.7 (105/261)	42.4 (110/262)	0.134	0.620	0.139	0.055

[1]Lactating Holstein cows were randomly assigned to receive (1) control: d 0, 100 μg of GnRH + progesterone releasing intravaginal device (PRID); d 7, 25 mg of dinoprost; d 8 PRID removal; d 9, 100 μg of GnRH; (2) DoubleDose: d 0, 100 μg of GnRH + PRID; d 7, 50 mg of dinoprost; d 8, PRID removal; d 9, 100 μg of GnRH; (3) 2PGF: d 0, 100 μg of GnRH + PRID; d 7, 25 mg of dinoprost; d 8, 25 mg of dinoprost and PRID removal; d 9, 100 μg of GnRH. All cows received timed AI approximately 16 h after the second GnRH treatment.

[2]Percentages for P/AI were derived from the GENLINMIXED model including treatment, parity, CL at first GnRH treatment (G1), and the interaction of CL at G1 × treatment; no CL = lack of a corpus luteum at d 0 based on a progesterone threshold of 1 ng/mL at d 0; CL = presence of a corpus luteum at d 0 based on a progesterone threshold of 1 ng/mL at d 0; DoubleDose = an Ovsynch protocol with a double dose of $PGF_{2\alpha}$; 2PGF = an Ovsynch protocol with a second $PGF_{2\alpha}$ treatment 24 h after the first one.

[3]Trt = treatment; C1 = pairwise comparison between the control group and DoubleDose group; C2 = pairwise comparison between the control group and 2PGF group; C3 = pairwise comparison between the 2PGF group and DoubleDose group.

= 0.051) to have greater P/AI at d 38 ± 3 post AI than multiparous cows (38.4%; 262/652). Service number had no effect ($P = 0.867$) on P/AI at d 38 ± 3 post AI.

Pregnancy per AI at d 80 ± 7 after AI was significantly greater for primiparous cows (42.9%) compared with multiparous cows (35.4%; $P = 0.015$). Treatment did not affect ($P = 0.134$) P/AI at d 80 ± 7 post AI. However, pairwise comparison indicated that cows in the 2PGF group tended to have greater (44.1%) P/AI than cows in the DoubleDose group (35.6%; $P = 0.055$) but did not differ compared with control cows (37.7%; $P = 0.139$). Pregnancy per AI did not differ between cows in the control group and DoubleDose cows ($P = 0.620$). Service number had no effect ($P = 0.993$) on P/AI at d 80 ± 7 post AI.

Treatment had no effect on pregnancy loss [$P = 0.471$; control 6.9% (10/144) vs. DoubleDose 3.6% (5/137) vs. 2PGF 5.8% (9/156)]. Parity tended to affect pregnancy loss ($P = 0.09$). Primiparous cows tended to have reduced pregnancy loss compared with multiparous cows [3.3% (6/181) vs. 7.0% (18/256)]. Service number had no effect ($P = 0.514$) on pregnancy loss.

Effect of Presence of a CL at G1

Of all the cows included in this study, 74% (781/1056) had a functional CL, based on serum P4 levels of 1.0 ng/mL or above at G1. Distribution of CL among treatments ($P = 0.305$) did not differ (Table 1). Distribution of CL did not differ ($P = 0.133$) between primiparous and multiparous cows [76% (307/404) vs. 72.7 (474/652)] and also ($P = 0.315$) between cows receiving first service and subsequent AI [73.1%

(340/465) vs. 74.6% (441/591)]. Overall P/AI at d 38 ± 3 after AI did not differ ($P = 0.121$) between cows with and without a CL at G1. For cows with a CL at G1, P/AI did not differ among treatments ($P = 0.868$). For cows without a CL at G1, an effect of treatment on P/AI was not observed ($P = 0.110$). Pairwise comparison, however, indicated that cows within the 2PGF group had greater (47.9%; $P = 0.043$) P/AI when compared with DoubleDose cows (32.7%), but did not differ compared with control cows (36.1%; $P = 0.109$). Pregnancy per AI did not differ between DoubleDose and control cows ($P = 0.623$). Presence of a CL had no significant effect on P/AI at d 80 ± 7 after AI ($P = 0.280$) as well as pregnancy loss ($P = 0.203$). Pregnancy per AI at d 80 ± 7 did not differ among treatments when considering the presence ($P = 0.854$) or absence ($P = 0.130$) of a CL. Cows in the 2PGF group, however, had greater P/AI ($P = 0.047$) if no CL was present at G1 compared with cows in the DoubleDose group, but did not differ for cows in the control group ($P = 0.162$). Pregnancy per AI did not differ when comparing cows in the DoubleDose group with control cows ($P = 0.504$).

Accuracy to Detect a Functional CL

The overall accuracy was 87.2% to identify a functional CL using transrectal ultrasound at the beginning of the protocol. The AUC was 0.901 and can be considered highly accurate. The optimum cutoff was a 20-mm diameter of the CL. Sensitivity and specificity was 89.6 and 80.0%, respectively. The positive predictive value was 92.8%. The negative predictive value was 73.1%.
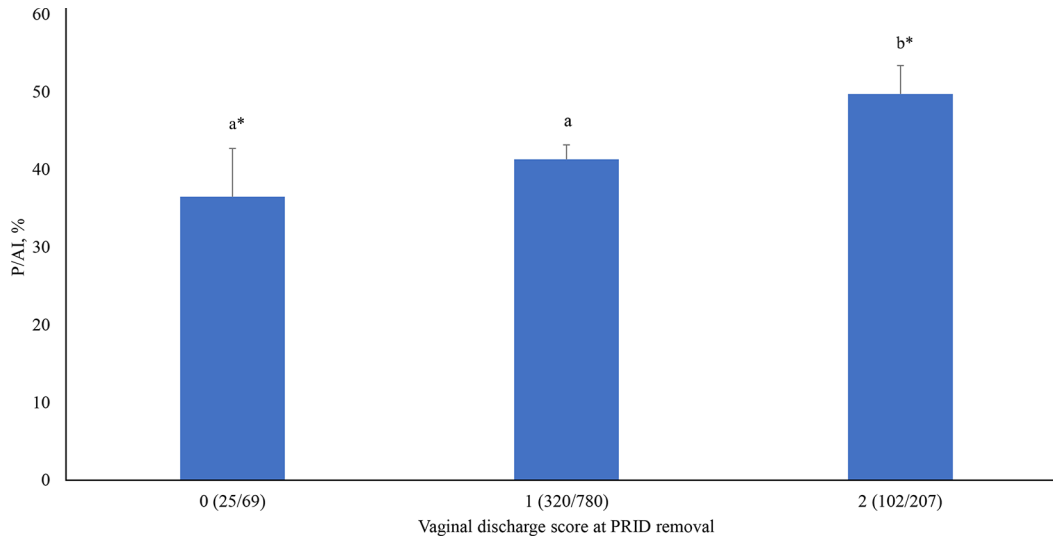
Figure 3. Comparison of pregnancy per AI (P/AI; $\pm$ SEM) at d 38 $\pm$ 3 post AI, depending on vaginal discharge score at removal of the progesterone releasing intravaginal device (PRID). Vaginal discharge was evaluated in all cows at PRID removal using a scale of 0 to 2. A score of 0 indicated clear or no debris on the PRID (6.6% of cows), a score of 1 indicated small flecks of purulent debris on the PRID (73.8% of cows), and a score of 2 indicated abundant purulent mucus on the PRID (19.6% of cows). Treatment had no effect on vaginal discharge score ($P = 0.265$). Percentages for P/AI were derived from the GENLINMIXED model including vaginal discharge score and parity. Bars with different letters (a, b) differed significantly ($P \leq 0.05$). Bars with * tended to differ ($P \leq 0.1$).

Effect of Vaginal Discharge at PRID Removal on P/AI and P4

Overall, 6.5% (69/1056) of cows had a VS of 0, 73.9% (780/1056) had a VS of 1, and 19.5% (207/1056) had a VS of 2 (Figure 3). The distribution of VS at PRID removal did not differ among treatments ($P = 0.265$) or luteal status at G1 ($P = 0.579$). The distribution of VS was affected by parity ($P = 0.001$; primiparous cows: 3.7% VS 0, 72.3% VS 1, 24.0% VS 2 vs. multiparous cows: 8.3% VS 0, 74.8% VS 1, 16.9% VS 2) and service number ($P = 0.046$; first AI: 6.5% VS 0, 70.5% VS 1, 23% VS 2 vs. subsequent AI: 6.6% VS 0, 76.5% VS 1, 16.9% VS 2).

Vaginal discharge score had a significant association ($P = 0.001$) with P4 concentrations at the first PGF$_{2\alpha}$ treatment (Figure 4). Cows assigned to a VS of 2 had greater (4.3 $\pm$ 0.2 ng/mL) serum P4 levels than cows assigned to score 0 (3.0 $\pm$ 0.3 ng/mL; $P = 0.001$) and 1 (3.7 $\pm$ 0.1 ng/mL; $P = 0.002$). Progesterone concentration at d 7 was greater ($P = 0.027$) for cows assigned to VS 1 compared with cows assigned to VS 0. Vaginal discharge score at PRID removal tended to have an association with P/AI at d 38 after AI (Figure 3; $P = 0.072$). Pregnancy per AI did not differ ($P = 0.459$) among cows scored with a VS of 0 and 1 [36.5% (25/69); 41.3% (320/780)], but was significantly greater [49.7% (102/207)] for cows having a VS of 2, compared with VS 1 ($P = 0.039$), and tended to be

greater compared with VS 0 ($P = 0.066$). Parity had a significant effect ($P = 0.032$), with primiparous cows having greater (45.9%) P/AI at d 38 $\pm$ 3 post AI compared with multiparous cows (39%).

Vaginal discharge had no relationship with P/AI at d 80 $\pm$ 7 after AI ($P = 0.116$). Pregnancy per AI did not differ ($P = 0.848$) among cows scored with a VS of 0 and 1 [37% (22/65); 38.2% (287/754)], but was greater [46.5% (91/196)] for cows having a VS of 2 compared with VS 1 ($P = 0.045$). Pregnancy per AI did not differ for cows with a VS of 0 and 2 ($P = 0.190$) and a VS of 1 and 2 ($P = 0.123$). Parity had a significant effect ($P = 0.008$) on P/AI at d 80 $\pm$ 7 post AI, with primiparous cows having greater (44.9%) P/AI compared with multiparous cows (36.2%).

Pregnancy loss was not affected by VS ($P = 0.461$).

DISCUSSION

Outcomes from this study provide additional evidence on the effect of different PGF$_{2\alpha}$ modifications in reproductive programs, using dinoprost tromethamine and the inclusion of a PRID. Failure of complete luteal regression (P4 above 0.3–0.5 ng/mL at G2) is a major problem that occurs in 12 to 21% of cows in TAI protocols with a single PGF$_{2\alpha}$ treatment (Brusveen et al., 2009; Heidari et al., 2017; Barletta et al., 2018). This has a deleterious effect on P/AI (Borchardt et al., 2018). Especially cows without a CL at the initiation of

the protocol are prone to incomplete luteolysis. Cows, experiencing a low P4 environment, have a greater amplitude of LH surge induced by the G1 (Giordano et al., 2012), leading to a higher ovulatory response (Giordano et al., 2013; Carvalho et al., 2015b) and a newly formed CL. The latter needs at least 5 d to be susceptible to a PGF$_{2\alpha}$ dose for induction of luteolysis (Pursley et al., 1995; Tsai and Wiltbank, 1998; Levy et al., 2000). The young CL remains reluctant to regression, although PGF$_{2\alpha}$ receptors with high PGF$_{2\alpha}$ affinity already exist within d 2 after ovulation (Pursley et al., 1995; Wiltbank et al., 1995). It is assumed that pulsatile release rather than the amplitude of PGF$_{2\alpha}$ contributes to luteolysis, which may explain the greater P/AI results in cows that received 2 consecutive treatments of PGF$_{2\alpha}$ (McCracken et al., 1999; Ginther et al., 2009). This finding was supported by the pattern of PGF$_{2\alpha}$-induced gene expression being more distinct for luteolysis after multiple treatments compared with 1 single application (Atli et al., 2012).

In our study, cows benefitted most of the Ovsynch modification with a second PGF$_{2\alpha}$ treatment. The positive effect on reproductive performance can especially be seen in cows without a CL at G1, with an 11.8 percentage-points increase in P/AI compared with the control group. This supports the notion that a CL at the beginning of an Ovsynch protocol does influence treatment effects. However, these results need to be assessed cautiously because of the small subgroup of cows without a CL (n = 275) at the beginning of the protocol. Barletta et al. (2018) provided similar results for cows without a CL at initiation of a resynch protocol. These authors reported an increase of 19 percentage-points ($P = 0.09$) in cows treated with 2 consecutive doses of dinoprost tromethamine and also acknowledged that the lack of statistical significance might be due to the relatively small subgroup of cows without a CL at G1 (n = 66). In a recent study, Tippenhauer et al. (2021) compared a standard Ovsynch protocol including 1 PGF treatment (0.5 mg cloprostenol) on d 7 with a double PGF$_{2\alpha}$ dose (1 mg) and 2 treatments (0.5 mg cloprostenol) 24 h apart on 8 different dairy farms. The results showed that 2 consecutive cloprostenol sodium treatments increased P/AI by 8.5 percentage-points compared with the control group, whereas a higher PGF$_{2\alpha}$ dose increased P/AI by 1.6 percentage-points, indicating the inferiority of the latter. On the contrary, Minela et al. (2021) showed that both modifications achieve similar results when using cloprostenol sodium, considering the different metabolization leading to an enhanced half-life of approximately 3 h for cloprostenol sodium compared with approximately 8 to 9 min for dinoprost tromethamine (Reeves, 1978; Veronese et al., 2019).

These cows were, however, synchronized using a Double-Ovsynch or a GnRH administration 7 d before Ovsynch for subsequent AI. Regarding the addition of a second PGF$_{2\alpha}$ treatment in a 7-d Ovsynch protocol, both types of prostaglandin have been shown to be effective (Barletta et al., 2018; Tippenhauer et al., 2021).

Different presynchronization protocols might explain differences between studies as fertility protocols including GnRH (e.g., Double-Ovsynch, G6G) increase the proportion of cows with a functional CL at the beginning of the protocol and with an accessory CL at the time of PGF$_{2\alpha}$ treatment. Although incomplete luteolysis is an issue related to this accessory CL, the simultaneous presence of an older CL, associated with higher P4 concentrations at protocol initiation or at the time of PGF$_{2\alpha}$ induced luteolysis (Martins et al., 2011), seems to have a beneficial effect on luteal regression in the accessory CL (Carvalho et al., 2018). In contrast to that, using a presynchronization with PGF (i.e., Presynch-Ovsynch) and AI after spontaneous estrus (i.e., cherry picking) might lead to a greater proportion of cows without an active CL at G1. We observed no difference in the proportion of cows with an active CL at G1 for cows receiving first AI (26.9%) compared with cows receiving subsequent AI (25.4%). However, we found a difference in the average P4 concentration at G1 for cows receiving first AI (1.94 ± 0.08 ng/mL) compared with cows receiving subsequent AI (2.68 ± 0.08 ng/mL). We did not observe an interaction of treatment by service number regarding fertility. Therefore, it remains questionable whether the treatment effect of a PGF$_{2\alpha}$ modification is different among various presynchronization protocols.

In the literature, the effect of parity on P/AI is inconsistent when comparing a single PGF$_{2\alpha}$ dose at d 7 with a modified PGF$_{2\alpha}$ schedule. Although primiparous cows may have greater luteal regression compared with multiparous cows when submitted to an Ovsynch protocol (Martins et al., 2011; Giordano et al., 2012b), P/AI results of various studies differ. A recent meta-analysis by Borchardt et al. (2021) showed that in primiparous and multiparous cows, an additional dose of PGF$_{2\alpha}$ on d 8 of the protocol yielded a 4.24 (95% CI = 0.31–8.17) and 5.31 (95% CI = 2.75–7.87) risk difference in P/AI, respectively. In our study, reproductive performance tended to be improved in primiparous cows. However, we found a potential confounding through BCS, with multiparous cows having overall lower BCS and more cows having a BCS below 2.5. A study by Atanasov et al. (2021) showed that thinner ($\leq$2.75) cows were less successful in luteal regression and had lower P/AI compared with cows having a BCS above 2.75, whereas no difference was observed between treatments. On

the contrary, Stevenson and Phatak (2010) stated that cows with a BCS below 2.5 had significantly greater luteolysis.

Progesterone concentrations below 1.0 ng/mL during the follicle growth phase result in lower P/AI (Carvalho et al., 2018) and greater risk for double ovulation (Carvalho et al., 2019). In this study, the relatively low pregnancy losses could be explained by the addition of the PRID device to the protocol. The insert provides P4 during the follicular growth phase in cows without a CL at G1, which has a beneficial effect on oocyte quality due to a decreased LH pulse frequency that would otherwise cause overstimulation (Kinder et al., 1996; Santos et al., 2016a). This assumption is supported by the outcomes of previous studies (Bisinotto et al., 2015) observing decreased pregnancy loss or greater P/AI after TAI protocols with intravaginal P4 inserts. Herlihy et al. (2011) demonstrated that P4 inserts in combination with an Ovsynch protocol can increase conception rates when the P4 insert was applied from d 0 to d 8 of the protocol as implemented in our study. Furthermore, Lauber et al. (2021) observed a decrease of early estrus expression when delaying the removal of the P4 insert from d 5 until d 6 in a modified 5-d Cosynch protocol in dairy heifers. Whether the extended time of PRID insertion has an influence on fertility and cyclic synchronization, ultimately affecting reproductive performance and oocyte quality, needs to be addressed in further studies.

It has been shown in a recent meta-analysis (Bisinotto et al., 2015) that the benefit of P4 supplementation was observed primarily in cows without CL at the initiation of the synchronization protocol. Therefore, the accuracy of detecting a functional CL is critical to maximize the effect of P4 supplementation. In our study, we observed a sensitivity of 89.6% and a specificity of 80%. The sensitivity is in the range with 2 other studies (Bicalho et al., 2008; Sauls-Hiesterman et al., 2020). Specificity seems a bit higher than what has been observed in these studies. Sauls-Hiesterman et al. (2020) described a discrepancy between different AI technicians in terms of specificity, ranging from 20 to 70% with an average of 39.8%, and Bicalho et al. (2008) reported a specificity of 45.7%. The accuracy of a technician to identify cows with a functional CL needs to be taken into account to maximize the potential of P4 supplementation in cows with a CL.

Whereas high P4 levels during the follicle development can be advantageous, slight elevations of P4 (Giordano et al., 2012b; Carvalho et al., 2015b) near AI can cause a dramatic reduction in fertility (Brusveen et al., 2009; Wiltbank et al., 2012; Borchardt et al., 2018). This effect might be mediated through altered oviductal and uterine contractility (Hunter, 2005), reduced endome-

trial thickness (Souza et al., 2011), and a reduced magnitude of the LH surge after G2 (Stevenson and Pulley, 2016). Considering this, complete luteal regression in TAI protocols is crucial for fertility in dairy cows. Research by Barletta et al. (2018) described a tendency for cows with complete luteolysis when treated with 2 separate PGF$_{2\alpha}$ doses (94%), which also resulted in a tendency for greater P/AI results, where the increased dose of PGF$_{2\alpha}$ did not show any differences in luteal regression (88%) or P/AI compared with a control group (88%). Our results confirm those findings as cows in the 2PGF group had significantly greater risk for luteolysis (92.6%) compared with control cows (84.8%). The DoubleDose cows, however, also had greater risk for luteal regression, but this was not accompanied by an improvement in fertility.

An interesting observation was the association between VS at PRID removal and P/AI. To the best of our knowledge, this is the first study, where we observed greater P4 levels at d 7 of the protocol in cows with a greater VS (Figure 4). The higher P4 levels observed in those cows with more local inflammation, based on mechanical irritation (Ahmadi et al., 2007), may be due to higher vaginal blood flow and thereby an increased hormonal absorption. In addition to that, however, the results observed in our study may be influenced by service number and parity. Several studies did not show a negative or positive influence of VS after vaginal P4 insert removal on reproductive performance and P4 concentrations. Walsh et al. (2008) could not find an association between vaginal discharge caused by intravaginal devices and systemic inflammation. This indicates that inflammation caused by a P4 insert is locally limited to the vagina. Considering the results of the aforementioned authors, we conclude that intravaginal P4 inserts have no negative effect on P/AI. In our study, this local inflammation in the vagina was actually associated with greater P4 levels leading to greater P/AI.

The study was limited by the total number of cows enrolled, creating relatively small subgroups. The beneficial effect of constantly high P4 levels during the follicular growth phase, regarding pregnancy loss, remains hypothetical, as it was not the scope of the study to validate an effect of P4 on pregnancy loss.

CONCLUSIONS

A second treatment of dinoprost at d 8 of the Ovsynch protocol combined with a PRID increased overall P/AI by 6.1 percentage-points. An increased dinoprost dose did not result in better fertility. Particularly, cows without a CL at G1 benefitted from a second treatment with dinoprost, whereas cows with a CL at G1 did not
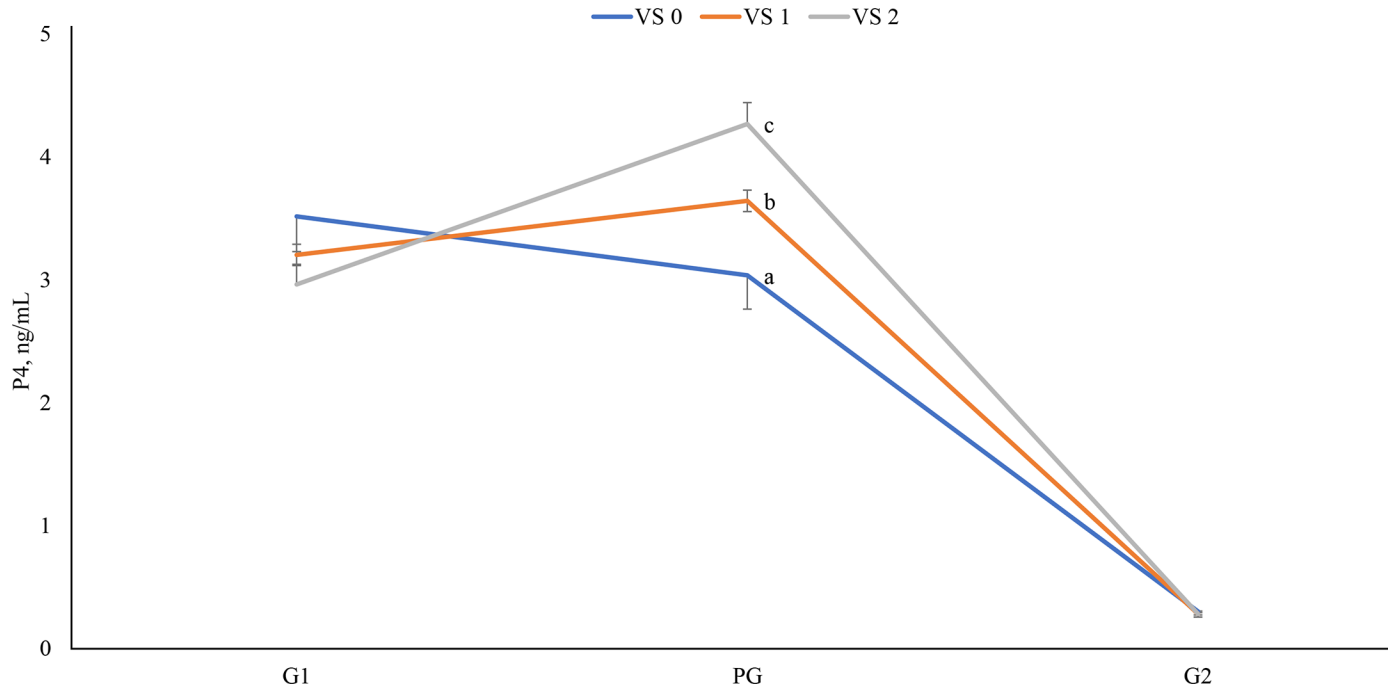
Figure 4. Comparison of progesterone concentration (P4, ng/mL ± SEM) at 3 different time points during a 7-d Ovsynch protocol depending on vaginal discharge score (VS) at removal of the P4-releasing intravaginal device (PRID). Vaginal discharge was evaluated in all cows at PRID removal using a scale of 0 to 2. A score of 0 (n = 69) indicated clear or no debris on the PRID (6.6% of cows), a score of 1 (n = 780) indicated small flecks of purulent debris on the PRID (73.8% of cows), and a score of 2 (n = 207) indicated abundant purulent mucus on the PRID (19.6% of cows). Treatment had no effect on VS ($P = 0.265$). Progesterone concentrations were derived from the GENLINMIXED model including sampling time, VS, parity, AI number, corpus luteum at G1, and the interaction between VS and sampling time. G1 = d 0 of the protocol; PG = d 7 of the protocol; G2 = d 9 of the protocol. Lines with different letters (a–c) differ significantly ($P \leq 0.05$) within each sampling time (i.e., G1, PG, G2).

show improved fertility. Two consecutive dinoprost treatments decreased P4 levels near TAI as well as doubling the dose of dinoprost. Vaginal discharge score at d 8 of the protocol, after PRID removal, was associated with higher P4 concentrations at d 7 and greater P/AI.

REFERENCES

Ahmadi, M. R., S. Nazifi, J. Sajedianfard, and G. Moattari. 2007. Impact of estrous synchronization methods on cellular proportions in cervical mucus and serum hormone concentrations. Theriogenology 67:598–604. https://doi.org/10.1016/j.theriogenology.2006.09.030.

Atanasov, B., T. Dovenski, I. Celeska, and J. S. Stevenson. 2021. Luteolysis, progesterone, and pregnancy per insemination after modifying the standard 7-day Ovsynch program in Holstein-Friesian and Holstein cows. J. Dairy Sci. 104:7272–7282. https://doi.org/10.3168/jds.2020-19922.

Atli, M. O., R. W. Bender, V. Mehta, M. R. Bastos, W. Luo, C. M. Vezina, and M. C. Wiltbank. 2012. Patterns of gene expression in the bovine corpus luteum following repeated intrauterine infusions of low doses of prostaglandin F2alpha. Biol. Reprod. 86:130. https://doi.org/10.1095/biolreprod.111.094870.

Barletta, R. V., P. D. Carvalho, V. G. Santos, L. F. Melo, C. E. Consentini, A. S. Netto, and P. M. Fricke. 2018. Effect of dose and timing of prostaglandin F2α treatments during a Resynch protocol on luteal regression and fertility to timed artificial insemination in lactating Holstein cows. J. Dairy Sci. 101:1730–1736. https://doi.org/10.3168/jds.2017-13628.

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. https://doi.org/10.3168/jds.S0022-0302(06)72378-5.

Bicalho, R. C., K. N. Galvão, C. L. Guard, and J. E. P. Santos. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. Theriogenology 70:199–207. https://doi.org/10.1016/j.theriogenology.2008.03.015.

Bisinotto, R. S., L. O. Castro, M. B. Pansani, C. D. Narciso, N. Martinez, L. D. P. Sinedino, T. L. C. Pinto, N. S. van de Burgwal, H. M. Bosman, R. S. Surjus, W. W. Thatcher, and J. E. P. Santos. 2015a. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. J. Dairy Sci. 98:2515–2528. https://doi.org/10.3168/jds.2014-9058.

Bisinotto, R. S., I. J. Lean, W. W. Thatcher, and J. E. P. Santos. 2015. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. J. Dairy Sci. 98:2472–2487. https://doi.org/10.3168/jds.2014-8954.

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. P. Santos. 2010. 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. https://doi.org/10.3168/jds.2010-3516.

Bisinotto, R. S., E. S. Ribeiro, and J. E. P. Santos. 2014. Synchronisation of ovulation for management of reproduction in dairy cows. Animal 8:151–159. https://doi.org/10.1017/S1751731114000858.

Borchardt, S., A. Pohl, P. D. Carvalho, P. M. Fricke, and W. Heuwieser. 2018. Short communication: Effect of adding a second prostaglandin F2α injection during the Ovsynch protocol on luteal regression and fertility in lactating dairy cows: A meta-analysis. J. Dairy Sci. 101:8566–8571. https://doi.org/10.3168/jds.2017-14191.

Borchardt, S., C. M. Tippenhauer, P. M. Fricke, and W. Heuwieser. 2021. Economic impact of adding a second prostaglandin F2α treatment during an Ovsynch protocol using a meta-analytical assessment and a stochastic simulation model. J. Dairy Sci. 104:12153–12163. https://doi.org/10.3168/jds.2021-20295.

Brusveen, D. J., A. H. Souza, and M. C. Wiltbank. 2009. Effects of additional prostaglandin F$_{2\alpha}$ and estradiol-17β during Ovsynch in lactating dairy cows. J. Dairy Sci. 92:1412–1422. https://doi.org/10.3168/jds.2008-1289.

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. https://doi.org/10.3168/jds.S0022-0302(06)72522-X.

Carvalho, P. D., M. J. Fuenzalida, A. Ricci, A. H. Souza, R. V. Barletta, M. C. Wiltbank, and P. M. Fricke. 2015. 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. J. Dairy Sci. 98:8741–8752. https://doi.org/10.3168/jds.2015-9719.

Carvalho, P. D., V. G. Santos, H. P. Fricke, L. L. Hernandez, and P. M. Fricke. 2019. Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine outcomes in high-producing multiparous Holstein cows. J. Dairy Sci. 102:7509–7521. https://doi.org/10.3168/jds.2019-16536.

Carvalho, P. D., V. G. Santos, J. O. Giordano, M. C. Wiltbank, and P. M. Fricke. 2018. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. Theriogenology 114:165–172. https://doi.org/10.1016/j.theriogenology.2018.03.037.

Carvalho, P. D., M. C. Wiltbank, and P. M. Fricke. 2015b. Manipulation of progesterone to increase ovulatory response to the first GnRH treatment of an Ovsynch protocol in lactating dairy cows receiving first timed artificial insemination. J. Dairy Sci. 98:8800–8813. https://doi.org/10.3168/jds.2015-9968.

Dohoo, I., S. Martin, and H. Stryhn. 2009. Veterinary Epidemiologic Research. 2nd ed. University of Prince Edward Island.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77:2695–2703. https://doi.org/10.3168/jds.S0022-0302(94)77212-X.

Fricke, P. M., P. D. Carvalho, M. C. Lucy, F. Curran, M. M. Herlihy, S. M. Waters, J. A. Larkin, M. A. Crowe, and S. T. Butler. 2016. Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine parameters in seasonal-calving, pasture-based Holstein-Friesian cows. J. Dairy Sci. 99:6780–6792. https://doi.org/10.3168/jds.2016-11229.

Galvão, K. N., M. F. Sá Filho, and J. E. P. Santos. 2007. Reducing the interval from presynchronization to initiation of timed artificial insemination improves fertility in dairy cows. J. Dairy Sci. 90:4212–4218. https://doi.org/10.3168/jds.2007-0182.

Ginther, O. J., R. R. Araujo, M. P. Palhão, B. L. Rodrigues, and M. A. Beg. 2009. Necessity of sequential pulses of prostaglandin F2alpha for complete physiologic luteolysis in cattle. Biol. Reprod. 80:641–648. https://doi.org/10.1095/biolreprod.108.072769.

Giordano, J. O., P. M. Fricke, J. N. Guenther, G. Lopes Jr., M. M. Herlihy, A. B. Nascimento, and M. C. Wiltbank. 2012. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. J. Dairy Sci. 95:3781–3793. https://doi.org/10.3168/jds.2011-5155.

Giordano, J. O., M. C. Wiltbank, P. M. Fricke, S. Bas, R. Pawlisch, J. N. Guenther, and A. B. Nascimento. 2013. Effect of increasing GnRH and PGF2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology 80:773–783. https://doi.org/10.1016/j.theriogenology.2013.07.003.

Giordano, J. O., M. C. Wiltbank, J. N. Guenther, R. Pawlisch, S. Bas, A. P. Cunha, and P. M. Fricke. 2012b. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. J. Dairy Sci. 95:639–653. https://doi.org/10.3168/jds.2011-4418.

Heidari, F., E. Dirandeh, Z. Ansari Pirsaraei, and M. G. Colazo. 2017. Modifications of the G6G timed-AI protocol improved pregnancy per AI and reduced pregnancy loss in lactating dairy cows. Animal 11:2002–2009. https://doi.org/10.1017/S1751731117000520.

Herlihy, M. M., D. P. Berry, M. A. Crowe, M. G. Diskin, and S. T. Butler. 2011. Evaluation of protocols to synchronize estrus and ovulation in seasonal calving pasture-based dairy production systems. J. Dairy Sci. 94:4488–4501. https://doi.org/10.3168/jds.2010-4126.

Herlihy, M. M., J. O. Giordano, A. H. Souza, H. Ayres, R. M. Ferreira, A. Keskin, A. B. Nascimento, J. N. Guenther, J. M. Gaska, S. J. Kacuba, M. A. Crowe, S. T. Butler, and M. C. Wiltbank. 2012. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. J. Dairy Sci. 95:7003–7014. https://doi.org/10.3168/jds.2011-5260.

Hunter, R. H. F. 2005. The Fallopian tubes in domestic mammals: How vital is their physiological activity? Reprod. Nutr. Dev. 45:281–290. https://doi.org/10.1051/rnd:2005020.

Kinder, J. E., F. N. Kojima, E. G. Bergfeld, M. E. Wehrman, and K. E. Fike. 1996. Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. J. Anim. Sci. 74:1424–1440. https://doi.org/10.2527/1996.7461424x.

Lauber, M. R., E. M. Cabrera, V. G. Santos, P. D. Carvalho, C. Maia, B. Carneiro, A. Valenza, V. E. Cabrera, J. J. Parrish, and P. M. Fricke. 2021. Comparison of reproductive management programs for submission of Holstein heifers for first insemination with conventional or sexed semen based on expression of estrus, pregnancy outcomes, and cost per pregnancy. J. Dairy Sci. 104:12953–12967. https://doi.org/10.3168/jds.2021-20617.

Levy, N., S. Kobayashi, Z. Roth, D. Wolfenson, A. Miyamoto, and R. Meidan. 2000. Administration of prostaglandin F$_{2\alpha}$ during the early bovine luteal phase does not alter the expression of ET-1 and of its type A receptor: A possible cause for corpus luteum refractoriness. Biol. Reprod. 63:377–382. https://doi.org/10.1095/biolreprod63.2.377.

Martins, J. P. N., R. K. Policelli, L. M. Neuder, W. Raphael, and J. R. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin F2α of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. J. Dairy Sci. 94:2815–2824. https://doi.org/10.3168/jds.2010-3652.

Martins, J. P. N., D. Wang, N. Mu, G. F. Rossi, A. P. Martini, V. R. Martins, and J. R. Pursley. 2018. Level of circulating concentrations of progesterone during ovulatory follicle development affects timing of pregnancy loss in lactating dairy cows. J. Dairy Sci. 101:10505–10525. https://doi.org/10.3168/jds.2018-14410.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: A neuroendocrine-mediated event. Physiol. Rev. 79:263–323. https://doi.org/10.1152/physrev.1999.79.2.263.

Minela, T., A. Santos, E. J. Schuurmans, E. L. Middleton, and J. R. Pursley. 2021. The effect of a double dose of cloprostenol sodium on luteal blood flow and pregnancy rates per artificial insemina-

tion in lactating dairy cows. J. Dairy Sci. 104:12105–12116. https://doi.org/10.3168/jds.2020-20113.

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. https://doi.org/10.3168/jds.S0022-0302(01)74600-0.

Nascimento, A. B., A. H. Souza, A. Keskin, R. Sartori, and M. C. Wiltbank. 2014. Lack of complete regression of the day 5 corpus luteum after one or two doses of PGF2α in nonlactating Holstein cows. Theriogenology 81:389–395. https://doi.org/10.1016/j.theriogenology.2013.10.009.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press.

Peters, M. W., and J. R. Pursley. 2002. Fertility of lactating dairy cows treated with Ovsynch after presynchronization injections of PGF$_{2\alpha}$ and GnRH. J. Dairy Sci. 85:2403–2406. https://doi.org/10.3168/jds.S0022-0302(02)74322-1.

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. https://doi.org/10.3168/jds.S0022-0302(97)75938-1.

Pursley, J. R., M. O. Mee, and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 44:915–923. https://doi.org/10.1016/0093-691X(95)00279-H.

Reeves, P. R. 1978. Distribution, elimination, and residue studies in the cow with the synthetic prostaglandin estrumate. J. Agric. Food Chem. 26:152–155. https://doi.org/10.1021/jf60215a001.

Revah, I., and W. R. Butler. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. Reproduction 106:39–47. https://doi.org/10.1530/jrf.0.1060039.

Rheinberger, J. M., D. D. Colson, D. S. Beggs, P. D. Mansell, M. A. Stevenson, R. J. Rheinberger, and M. F. Pyman. 2020. Effect of a second treatment of prostaglandin F2α during the Ovsynch program on fixed-time artificial insemination conception rates and luteolysis in split-calving, pasture-fed dairy cows. Aust. Vet. J. 98:190–196. https://doi.org/10.1111/avj.12918.

Ribeiro, E. S., R. S. Bisinotto, M. G. Favoreto, L. T. Martins, R. L. A. Cerri, F. T. Silvestre, L. F. Greco, W. W. Thatcher, and J. E. P. Santos. 2012. Fertility in dairy cows following presynchronization and administering twice the luteolytic dose of prostaglandin F2α as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78:273–284. https://doi.org/10.1016/j.theriogenology.2012.01.012.

Santos, J. E. P., R. S. Bisinotto, and E. S. Ribeiro. 2016a. Mechanisms underlying reduced fertility in anovular dairy cows. Theriogenology 86:254–262. https://doi.org/10.1016/j.theriogenology.2016.04.038.

Santos, V. G., P. D. Carvalho, C. Maia, B. Carneiro, A. Valenza, P. M. Crump, and P. M. Fricke. 2016b. Adding a second prostaglandin F2α treatment to but not reducing the duration of a PRID-Synch protocol increases fertility after resynchronization of ovulation in lactating Holstein cows. J. Dairy Sci. 99:3869–3879. https://doi.org/10.3168/jds.2015-10557.

Sauls-Hiesterman, J. A., B. E. Voelz, and J. S. Stevenson. 2020. A shortened resynchronization treatment for dairy cows after a non-pregnancy diagnosis. Theriogenology 141:105–112. https://doi.org/10.1016/j.theriogenology.2019.09.013.

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. https://doi.org/10.1016/j.theriogenology.2008.03.014.

Souza, A. H., E. P. B. Silva, A. P. Cunha, A. Gümen, H. Ayres, D. J. Brusveen, J. N. Guenther, and M. C. Wiltbank. 2011. Ultrasonographic evaluation of endometrial thickness near timed AI as a predictor of fertility in high-producing dairy cows. Theriogenology 75:722–733. https://doi.org/10.1016/j.theriogenology.2010.10.013.

Stevenson, J. S. 2016. Physiological predictors of ovulation and pregnancy risk in a fixed-time artificial insemination program. J. Dairy Sci. 99:10077–10092. https://doi.org/10.3168/jds.2016-11247.

Stevenson, J. S., and A. P. Phatak. 2010. Rates of luteolysis and pregnancy in dairy cows after treatment with cloprostenol or dinoprost. Theriogenology 73:1127–1138. https://doi.org/10.1016/j.theriogenology.2010.01.014.

Stevenson, J. S., and S. L. Pulley. 2016. Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. J. Dairy Sci. 99:3003–3015. https://doi.org/10.3168/jds.2015-10091.

Stevenson, J. S., J. A. Sauls, L. G. D. Mendonça, and B. E. Voelz. 2018. Dose frequency of prostaglandin F2α administration to dairy cows exposed to presynchronization and either 5- or 7-day Ovsynch program durations: Ovulatory and luteolytic risks. J. Dairy Sci. 101:9575–9590. https://doi.org/10.3168/jds.2018-14653.

Swets, J. A. 1988. Measuring the accuracy of diagnostic systems. Science 240:1285–1293. https://doi.org/10.1126/science.3287615.

Tippenhauer, C. M., I. Steinmetz, W. Heuwieser, P. M. Fricke, M. R. Lauber, E. M. Cabrera, and S. Borchardt. 2021. Effect of dose and timing of prostaglandin F2α treatments during a 7-d Ovsynch protocol on progesterone concentration at the end of the protocol and pregnancy outcomes in lactating Holstein cows. Theriogenology 162:49–58. https://doi.org/10.1016/j.theriogenology.2020.12.020.

Tsai, S. J., and M. C. Wiltbank. 1998. Prostaglandin F$_{2\alpha}$ regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. Biol. Reprod. 58:346–352. https://doi.org/10.1095/biolreprod58.2.346.

Veronese, A., O. Marques, R. Moreira, A. L. Belli, T. R. Bilby, and R. C. Chebel. 2019. Estrous characteristics and reproductive outcomes of Holstein heifers treated with 2 prostaglandin formulations and detected in estrus by an automated estrous detection or mounting device. J. Dairy Sci. 102:6649–6659. https://doi.org/10.3168/jds.2018-15957.

Walsh, R. B., S. J. LeBlanc, T. D. Duffield, D. F. Kelton, J. S. Walton, and K. E. Leslie. 2007. Synchronization of estrus and pregnancy risk in anestrous dairy cows after treatment with a progesterone-releasing intravaginal device. J. Dairy Sci. 90:1139–1148. https://doi.org/10.3168/jds.S0022-0302(07)71600-4.

Walsh, R. B., S. J. LeBlanc, E. Vernooy, and K. E. Leslie. 2008. Safety of a progesterone-releasing intravaginal device as assessed from vaginal mucosal integrity and indicators of systemic inflammation in postpartum dairy cows. Can. J. Vet. Res. 72:43–49.

Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin F2α during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. J. Dairy Sci. 98:8644–8654. https://doi.org/10.3168/jds.2015-9353.

Wiltbank, M. C., T. F. Shiao, D. R. Bergfelt, and O. J. Ginther. 1995. Prostaglandin F$_{2\alpha}$ receptors in the early bovine corpus luteum. Biol. Reprod. 52:74–78. https://doi.org/10.1095/biolreprod52.1.74.

Wiltbank, M. C., A. H. Souza, P. D. Carvalho, A. P. Cunha, J. O. Giordano, P. M. Fricke, G. M. Baez, and M. G. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8:70–81. https://doi.org/10.1017/S1751731114000585.

Wiltbank, M. C., A. H. Souza, J. O. Giordano, A. B. Nascimento, J. M. Vasconcelos, M. H. C. Pereira, P. M. Fricke, R. S. Surjus, F. C. S. Zinsly, P. D. Carvalho, R. W. Bender, and R. Sartori. 2012. Positive and negative effects of progesterone during timed AI protocols in lactating dairy cattle. Anim. Reprod. 9:231–241.

ORCIDS

M. Hölper ⦿ https://orcid.org/0000-0003-3295-2172

L. Bretzinger ⦿ https://orcid.org/0000-0003-4072-9851

F. Randi ⦿ https://orcid.org/0000-0001-6400-7429

W. Heuwieser ⦿ https://orcid.org/0000-0003-1434-7083

S. Borchardt ⦿ https://orcid.org/0000-0003-3937-5777