Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine outcomes in high-producing multiparous Holstein cows

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ABSTRACT

Our objective was to evaluate the effect of manipulating progesterone (P4) concentrations before timed artificial insemination (TAI) on reproductive and endocrine outcomes in high-producing Holstein cows. Multiparous lactating Holstein cows ($n = 80$) were synchronized for first TAI using a Double-Ovsynch protocol and were randomly assigned to receive 25 mg of PGF_{2α} 1 d after the first GnRH treatment of the Breeding-Ovsynch protocol that included a once-used P4 insert (low-P4 group) or to receive 2 new P4 inserts during the Breeding-Ovsynch protocol (high-P4 group). Blood samples were collected thrice weekly from –10 to 32 d relative to TAI for all cows and from 32 to 67 d after TAI for pregnant cows and were analyzed for P4 and pregnancy-specific protein B (PSPB) concentrations. Expression of IFN γ -stimulated gene 15 (*ISG15*) was assessed in blood leukocytes 18 and 20 d after TAI. As expected, P4 concentrations were greater for high-P4 cows than for low-P4 cows from 3 to 8 d before TAI. Incidence of double ovulation was 3-fold greater for low-P4 cows than for high-P4 cows (33 vs. 10%), which resulted in more twin pregnancies 32 d after TAI for low-P4 cows than for high-P4 cows (29 vs. 0%). Low-P4 cows had larger preovulatory follicles at the last GnRH treatment of the Double-Ovsynch protocol and greater P4 concentrations than high-P4 cows after TAI. Relative expression of *ISG15* mRNA 18 and 20 d after TAI was greater for low-P4 cows than for high-P4 cows and for pregnant cows than for nonpregnant cows. Overall, PSPB concentrations tended to be greater for low-P4 cows than for high-P4 cows, and pregnant cows had greater P4 concentrations than nonpregnant cows. In summary, cows with low P4 before TAI had increased preovulatory follicle diameter, PSPB concentrations, relative expression of *ISG15* mRNA 18 and 20 d after

TAI, double ovulations, and twinning compared with cows with high P4 before TAI. Increasing P4 before TAI may effectively decrease double ovulation and twinning in high-producing multiparous Holstein cows. **Key words:** progesterone, double ovulation, twinning, dairy cow

INTRODUCTION

Modern high-producing dairy cows experience several reproductive problems, including a high incidence of double ovulation (Wiltbank et al., 2000; Lopez et al., 2005a), increased twinning over time (Silva del Río et al., 2007), and increased pregnancy loss (Santos et al., 2004; Wiltbank et al., 2016). A possible physiologic mechanism that could explain these problems is that high-producing dairy cows experience increased hepatic metabolism of progesterone (P4) due to high feed intake, resulting in decreased circulating P4 concentrations (Sangsritavong et al., 2002; Vasconcelos et al., 2003). Circulating P4 concentrations in dairy cows at the onset of a fertility protocol have profound effects on fertility to timed AI (TAI) in which cows with low P4 at the first GnRH treatment or at the PGF_{2α} treatment of an Ovsynch protocol have lower fertility than cows with high P4 (Fricke et al., 2003; Bisinotto et al., 2010; Carvalho et al., 2014). More recently, high-producing Holstein cows manipulated to have low P4 concentrations at the first GnRH treatment of an Ovsynch protocol that included a second PGF_{2α} treatment given 24 h after the first to complete luteal regression had a dramatically increased incidence of double ovulation and subsequent pregnancy loss (Martins et al., 2018). To further complicate matters, cows with double ovulations initially have increased fertility to TAI compared with cows with single ovulations (Fricke and Wiltbank, 1999), but cows that conceive twins, particularly unilateral twins, have a 3-fold increase in subsequent pregnancy losses compared with cows that conceive singletons (López-Gatius and Hunter, 2005; Silva del Río et al., 2009).

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With these effects of P4 on reproduction in mind, an experiment was conducted in which primiparous and multiparous Holstein-Friesian cows managed in a seasonal-calving pasture-based dairy system in Ireland were manipulated to have either high or low P4 concentrations during growth of a synchronized follicular wave during an Ovsynch protocol (Fricke et al., 2016). In that experiment, the effect of manipulating P4 before TAI on early embryo development was assessed by measuring circulating P4, 4 different IFN-stimulated genes (**ISG**), and plasma pregnancy-associated glycoprotein (**PAG**) concentrations. Low-P4 cows had larger ovulatory follicles and more P4 after TAI than high-P4 cows; however, this effect was manifested primarily for a subgroup of low-P4 cows that expressed estrus approximately 16 h before TAI, likely due to the low P4 concentrations near the end of the protocol as a consequence of the low-P4 treatment. In contrast to the stated hypothesis in that study, there was no effect of treatment on early embryo development based on plasma PAG concentrations and pregnancy outcomes based on transrectal ultrasonography or on the incidence of double ovulation, twinning, or pregnancy loss (Fricke et al., 2016). We speculated that Holstein-Friesian cows managed in a seasonal-calving pasture-based system with moderate milk production (i.e., <25 kg/d near peak production) may not present problems associated with hepatic P4 metabolism similar to high-producing Holsteins housed in confinement-based systems (Fricke et al., 2016).

The objective of this experiment, therefore, was to use an experimental model similar to that used by Fricke et al. (2016) to hormonally manipulate high-producing Holstein cows typical of confinement-based dairy systems in North America into either a high-P4 or a low-P4 environment during growth of a synchronized follicular wave during an Ovsynch protocol to assess the effect of P4 before TAI on the incidence of double ovulation and twinning as well as on IFN-stimulated gene 15 (**ISG15**) mRNA expression 18 and 20 d after TAI and serum pregnancy-specific protein B (**PSPB**) and P4 concentrations after TAI. Our hypotheses were that cows with low P4 during growth of a synchronized follicular wave would (1) ovulate larger follicles, (2) have increased **ISG15** mRNA expression and **PSPB** concentrations after TAI, and (3) have more double ovulations and twinning than cows with high P4.

MATERIALS AND METHODS

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

Cows, Housing, and Feeding

This study was conducted at the University of Wisconsin–Madison Emmons Blaine Dairy Cattle Research Center located near Arlington, Wisconsin, from April to October 2017. Lactating Holstein cows ($n = 80$) were milked twice daily at approximately 12-h intervals and were fed a TMR once daily 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. Only multiparous cows were enrolled in this experiment to minimize variation among cows and to study a uniform group of cows with high milk production without the confounding effects of parity (primiparous vs. multiparous). The rolling herd average was 13,880 kg and average daily milk production was 41.6 kg/cow per day with 3.8% fat and 3.2% protein for the herd during this experiment.

Treatments

All cows were submitted to a Double-Ovsynch protocol for first TAI (Figure 1) as described by Souza et al. (2008) and modified by Brusveen et al. (2009). The GnRH (100 µg/dose of gonadorelin hydrochloride, Factrel), PGF_{2α} (25 mg/dose of dinoprost tromethamine, Lutalyse), and controlled internal drug release (**CIDR**) inserts (1.38 g of P4/insert) were manufactured by Zoetis (Madison, NJ). Briefly, cows received the first GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol starting at 53 ± 3 DIM, followed by treatment with PGF_{2α} 7 d later and treatment with GnRH 72 h after treatment with PGF_{2α}. Seven days later, cows began the Breeding-Ovsynch portion of the Double-Ovsynch protocol in which cows received a GnRH treatment (**G1**) followed by 2 PGF_{2α} treatments administered 7 and 8 d later and the last GnRH treatment (**G2**) administered 56 h after the first PGF_{2α} treatment followed by TAI 16 to 20 h after G2. One day after G1, cows were randomly assigned to 1 of 2 treatments: 2 new CIDR inserts for 7 d (referred to as the high-P4 group; $n = 40$) or 25 mg of PGF_{2α} and a once-used CIDR insert for 7 d (referred to as the low-P4 group; $n = 40$; Figure 1). To create once-used CIDR inserts, CIDR inserts were inserted into multiparous lactating Holstein cows at G1 of a Double-Ovsynch protocol and were removed at the first PGF_{2α} treatment (i.e., 7 d later) of the Breeding-Ovsynch portion of a Double-Ovsynch protocol. Once-used CIDR inserts were then scrubbed with a mild disinfectant diluted in hot water (Nolvasan Solution, Zoetis, Madison, NJ)

Figure 1. Schematic diagram of the treatment protocols used to manipulate lactating multiparous Holstein cows into a high-progesterone (high-P4) versus a low-progesterone (low-P4) environment before timed AI (TAI) and timing of blood sampling after TAI to assess serum progesterone (P4) concentration, IFN-stimulated gene 15 (*ISG15*) mRNA expression in blood leukocytes, and serum pregnancy-specific protein B (PSPB) concentration. G1 = first GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol; G2 = last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol; CIDR = controlled internal drug release; US = ultrasound.

and allowed to air dry. After drying, CIDR inserts were autoclaved and stored in plastic bags in the dark until used in the present experiment. Multiple sires with high genetic merit and proven fertility were used for TAI, and sires were equally balanced between treatments.

Transrectal Ultrasonography

Number of corpus luteum (CL) was evaluated at the time of the first PGF_{2α} treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (Figure 1) by transrectal ultrasonography using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer to determine response to the treatment protocols. On the day of G2, number and size of follicles ≥ 10 mm in diameter were also recorded. Ovulation was confirmed by disappearance of 1 (single ovulation) or 2 (double ovulation) follicles ≥ 10 mm in diameter and appearance of 1 or more CL in the same locations as the preovulatory follicles 6 d after TAI (Figure 1).

Pregnancy diagnosis and measurement of embryonic crown-rump length were performed using a portable scanner (Ibex Pro, E. I. Medical Imaging) equipped

with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a CL on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant 32 d after TAI was reconfirmed weekly until 67 d after TAI. Any cow diagnosed pregnant and subsequently diagnosed not pregnant at a pregnancy confirmation was considered to have undergone pregnancy loss. Images were frozen when the crown-rump length was determined visually to be at its maximal size, and the length was then measured using the built-in digital calipers of the ultrasound machine.

Blood Sample Collection

Blood samples to assess P4 and PSPB concentrations were collected via puncture of the median caudal veins or arteries into 8-mL evacuated serum collection tubes (Vacutette, Greiner Bio-One North America Inc., Monroe, NC) thrice weekly beginning at G1 (i.e., immediately before GnRH treatment) until 32 d after TAI for all cows and then weekly until 67 d for cows diagnosed pregnant 32 d after TAI (Figure 1). Blood samples were immediately placed on ice after collection

and were centrifuged ($1,600 \times g$, 4°C) for 20 min within 2 h. Serum was harvested and stored at -20°C until assayed for P4 and PSPB. Blood samples (3 mL) for analysis of *ISG15* mRNA expression were collected 4, 18, and 20 d after TAI from the coccygeal vessels into Tempus Blood RNA tubes containing 6 mL of RNA stabilization solution (Applied Biosystems, Foster City, CA). Tubes were shaken vigorously by hand for 20 s immediately after collection, and whole blood was stored in the Tempus tubes at -20°C until the mRNA isolation procedure.

Analysis of P4 and PSPB

Serum P4 concentrations were determined using a solid-phase, no-extraction RIA (MP Biomedicals, Solon, OH). The average sensitivity for the 3 assays was 0.027 ng/mL. The average intra-assay coefficient of variation (CV) was 5.6%, and the interassay CV was 6.9% based on a quality control sample (2.50 ng/mL of P4) that was replicated within each P4 assay. Concentrations of PSPB were determined using a quantitative ELISA (Biopryn, BioTracking LLC, Moscow, ID). The average intra-assay CV was 5.3%, and the interassay CV was 7.4% based on a quality control sample (0.9 ng/mL of PSPB) that was replicated within each PSPB assay.

Analysis of *ISG15*

Total RNA was extracted from blood leukocytes using the Tempus Spin RNA Isolation Kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions (<http://tools.thermofisher.com/content/sfs/manuals/4382075b.pdf>), including the optional DNase step. Extracted RNA (1 μg) was reverse transcribed to cDNA (Bio-Rad iScript Reverse Transcription Supermix no. 1708840; Bio-Rad, Hercules, CA). Quantitative real-time PCR was conducted with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Reaction mixtures and cycling conditions were performed as described elsewhere (Carvalho et al., 2017). Amplification efficiencies of primers were accepted within a range of 95 to 105% efficiency, and primer specificity was assessed by the presence of a single temperature dissociation peak. Cyclophilin-A (CycloA; NM_178320; forward: 5'-CACCGTGGTTCGACATCG-3'; reverse: 5'-ACAGCTCAAAGAGACGCGG-3') was used as a housekeeping gene, and all samples were analyzed for *ISG15* (NM_174366; forward: 5'-GGTATGAGCTGAAGCAGTT-3'; reverse: 5'-ACCTCCCTGCTGTCAAGGT-3'). Analysis was conducted using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001). Blood samples collected 4 d after TAI served as a reference to calculate

fold-change in *ISG15* mRNA expression 18 and 20 d after TAI.

Statistical Analyses

The experiment was conducted as a completely randomized design, and cows were randomly assigned to treatments. All statistical analyses were performed using SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). Analyses of binary response data [pregnancies per AI (P/AI), pregnancy loss, number of ovulations, and twins] were performed by logistic regression using the GLIMMIX procedure of SAS. The model included the fixed effects of treatment and double ovulation. Incidence of double ovulation was also analyzed by logistic regression with a model containing the fixed effect of treatment.

Continuous variables, including concentrations of P4 and PSPB, CL volume, and ovulatory follicle size, were analyzed by ANOVA using the MIXED procedure of SAS. The initial model included the fixed effect of treatment, time, double ovulation (only for P4 after TAI), and their interaction. Due to repeated measures within each cow, a repeated statement was used to account for autocorrelations by modeling the spatial power between sequential measurements. For P4 concentrations from -10 to -1 d, the model contained the fixed effect of treatment, time, and their interaction. For P4 concentrations from 1 to 15 d, the model contained the fixed effect of treatment, time, double ovulation, and the interaction between time and double ovulation. For P4 concentrations between 18 and 67 d after TAI, only cows that maintained pregnancy were included and the model included the fixed effects of treatment and time. Progesterone concentrations were also compared between 15 and 32 d after TAI between pregnant cows and open cows with low and high PSPB 27 d after TAI. The model contained the fixed effect of pregnancy status and time. For PSPB concentrations in pregnant cows, the model contained the fixed effect of treatment, time, and pregnancy type (twins vs. singleton). Fold-change in *ISG15* mRNA from 4 to 18 or 20 d after TAI was analyzed by ANOVA using the MIXED procedure of SAS. The model contained the fixed effects treatment, pregnancy status 32 d after TAI (pregnant vs. open), and double ovulation. In a secondary analysis, pregnant cows were subdivided into 3 groups based on ovulatory response and pregnancy type: (1) single ovulation with a singleton pregnancy, (2) double ovulation with a singleton pregnancy, and (3) double ovulation with a twin pregnancy. In addition, cows diagnosed not pregnant 32 d after TAI were subdivided into 2 groups based on PSPB concentrations 27 d after

TAI. A receiver operating characteristic curve was generated with the LOGISTIC procedure of SAS using the outcome of pregnancy status at 32 d after TAI. The threshold of 0.77 ng/mL of PSPB was selected as a PSPB cutoff because it resulted in the highest Youden's J statistic (area under the curve = 0.929, sensitivity = 1.0, and specificity = 0.80). Thus, the analysis was based on 2 groups of cows: nonpregnant cows with low PSPB concentrations 27 d after TAI and nonpregnant cows with high PSPB concentrations 27 d after TAI. The correlation between follicle diameter before TAI and CL volume after TAI was determined using the CORR procedure of SAS.

A significant difference between 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 least squares means $\pm$ standard error of the means obtained from the MIXED procedure of SAS.

RESULTS AND DISCUSSION

The objective of this experiment was to revisit the hypotheses tested by Fricke et al. (2016) in moderate-producing Holstein-Friesian dairy cows managed in a seasonal-calving pasture-based system in Ireland, but in high-producing Holstein cows typical of confinement-based dairy systems in North America. To test our hypotheses related to the effect of P4 during growth of a synchronized follicular wave, we used a Double-Ovsynch protocol for first TAI as described by Souza et al. (2008) with several modifications as described by Fricke et al. (2016), which included an increased dose of GnRH at G1 (i.e., 200 μ g) to increase ovulatory response to G1 (Giordano et al., 2013) as well as a second PGF_{2 α} treatment administered 24 h after the first during the Breeding-Ovsynch portion of the Double-Ovsynch protocol to induce complete luteal regression by G2 (Brusveen et al., 2009; Carvalho et al., 2018). We further modified the protocols described by Fricke et al. (2016) in the present experiment by including exogenous P4 provided through intravaginal P4 inserts (i.e., CIDR inserts). Because almost one-third of the cows in the low-P4 treatment described by Fricke et al. (2016) expressed estrus before G2 and therefore failed to synchronize properly, low-P4 cows received a once-used CIDR insert for 7 d during the protocol (Figure 1) to suppress early expression of estrus, thereby increasing synchrony of cows subjected to low P4 during the synchronized follicular wave. Further, because high-producing dairy cows have decreased circulating P4 concentrations due to hepatic metabolism of P4 as a result of high feed intake, thereby increasing hepatic blood flow (Sangsritavong et al., 2002; Vasconcelos et

al., 2003), high-P4 cows received 2 new CIDR inserts for 7 d during the protocol to achieve high circulating P4 concentrations during growth of the synchronized follicular wave (Figure 1). Supplementation with 2 CIDR inserts increased P4 in plasma by an additional 1.85 ng/mL compared with controls, resulting in concentrations of 2.77 ng/mL during development of the ovulatory follicle, which restored fertility in dairy cows lacking CL to a level similar to that of cows in diestrus (Bisinotto et al., 2015). These modifications allowed us to test our hypotheses related to low versus high P4 during growth of a synchronized follicular wave before TAI under experimental protocols in high-producing Holstein cows that allowed for a high rate of synchrony.

Milk Production and BCS

Average milk yield during the first 4 mo after calving did not differ ($P = 0.74$) between treatments and was 51.7 ± 0.8 and 52.0 ± 0.8 kg/d for high-P4 and low-P4 cows, respectively. For comparative purposes, milk production of multiparous cows in the present experiment was more than twice that of the primiparous and multiparous Holstein-Friesian cows managed in a seasonal-calving pasture-based system (24.7 vs. 24.9 kg/d for high-P4 vs. low-P4 cows, respectively) at a similar stage of lactation (Fricke et al., 2016). On the day of enrollment, BCS did not differ ($P = 0.82$) between treatments (2.76 vs. 2.75 for high-P4 vs. low-P4 cows, respectively), and BCS in the present study was similar to that of Holstein-Friesian cows reported by Fricke et al. (2016). The proportion of cows with BCS ≤ 2.5 did not differ ($P = 0.81$) between treatments [33% (13/40) vs. 35% (14/40) for high-P4 vs. low-P4 cows, respectively].

Effect of Treatment on Ovarian Structures

To manipulate cows into a high-P4 environment during growth of the preovulatory follicle, we used a Double-Ovsynch protocol as described by Fricke et al. (2016) including the modifications described above. Ideally, cows that ovulate a follicle to the last GnRH treatment of the Pre-Ovsynch portion and G1 should have 2 CL at the first PGF_{2 α} treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol. This then creates a high-P4 environment during growth of a synchronized follicular wave. To further increase P4 during growth of the preovulatory follicle for high-P4 cows, we included 2 new CIDR inserts to achieve a high-P4 environment. To manipulate cows into a low-P4 environment during growth of the preovulatory follicle, we administered a luteolytic dose of PGF_{2 α} 24 h after G1. Because the early CL is refractory to PGF_{2 α} -induced

Table 1. Effect (mean $\pm$ SEM unless otherwise noted) of treatment on number and size of corpus luteum (CL) at the first PGF_{2 α} treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol, number and size of ovulatory follicles at the last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (G2), incidence of double ovulation after G2, and CL volume 7 d after timed AI (TAI) in multiparous Holstein cows manipulated to have high versus low progesterone (P4) during growth of a synchronized follicular wave before TAI

Item	Treatment ¹		P-value
	High P4	Low P4	
CL at PGF _{2α}	1.9 $\pm$ 0.1	1.2 $\pm$ 0.1	<0.01
Ovulatory follicles at G2	1.2 $\pm$ 0.1	1.4 $\pm$ 0.1	0.01
Ovulatory follicle size at G2	14.8 $\pm$ 0.3	16.4 $\pm$ 0.5	<0.01
Double ovulation (% no./no.)	10 (4/40)	33 (13/40)	<0.01
CL volume 6 d after TAI (mm ³)	5,550 $\pm$ 341	8,129 $\pm$ 637	<0.01

¹Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation to receive first TAI. One day after G1, cows were randomly assigned to receive 2 new controlled internal drug-release (CIDR) inserts (high P4) or a PGF treatment and a used CIDR insert (low P4).

luteolysis until about 7 d after ovulation (Nascimento et al., 2014), a CL induced by the last GnRH treatment of the Pre-Ovsynch portion should regress, whereas the newly forming CL induced by G1 administered 24 h before this PGF_{2 α} treatment should not (Nascimento et al., 2014). As expected, number of CL in the present experiment was greater ($P < 0.01$) for high-P4 cows than for low-P4 cows at the time of the first PGF_{2 α} treatment during the Breeding-Ovsynch portion of the Double-Ovsynch protocol (Table 1), demonstrating that the experimental protocols successfully manipulated CL number during growth of the synchronized follicular wave.

One of our hypotheses was that low-P4 cows would ovulate larger follicles. Progesterone affects growth of a dominant follicle through a negative feedback on LH pulses from the anterior pituitary in which low P4 is associated with dramatically increased LH pulse frequency and amplitude (Kinder et al., 1996). Exposure of dominant follicles to increased LH pulses during growth of a dominant follicle before ovulation results in ovulation of a larger follicle (i.e., a persistent follicle; Ahmad et al., 1995), which in turn produces a larger CL after ovulation that produces more P4 (Vasconcelos et al., 2001). In agreement with our hypothesis, mean ovulatory follicle size in the present experiment was 1.6 mm greater ($P < 0.01$) for low-P4 cows than for high-P4 cows (Table 1). This increase in ovulatory follicle size resulted in an average CL volume 6 d after AI that was 2,579 mm³ greater for low-P4 cows than for high-P4 cows (Table 1). Overall, as size of the ovulatory follicle increased, CL volume 6 d after TAI also increased ($r = 0.60$; $P < 0.01$).

Another hypothesis in the present study was that high-producing Holstein cows subjected to a low-P4 environment during growth of a synchronized follicular wave would have an increased incidence of double

ovulation. Low P4 during growth of ovulatory follicles in high-producing cows is associated with an increased incidence of double ovulation (Wiltbank et al., 2000). Further, cows in which the preovulatory follicle develops in the absence of P4 from a CL have a greater incidence of codominant follicles, resulting in double ovulations (Stevenson et al., 2007; Hayashi et al., 2008), likely due to increased LH pulses that result from the low-P4 environment that occur during selection of the dominant follicle from the cohort of growing follicles in the follicular wave (Lopez et al., 2005b). In the present experiment, low-P4 cows had more ($P = 0.01$) preovulatory follicles at G2 than high-P4 cows (Table 1), and the proportion of cows that double ovulated after G2 was 3-fold greater ($P < 0.01$) for low-P4 cows than for high-P4 cows (Table 1).

Low P4 concentrations at the first GnRH treatment of an Ovsynch protocol that included a second PGF_{2 α} treatment given 24 h after the first to complete luteal regression dramatically increased the incidence of double ovulation and subsequent pregnancy loss in high-producing Holstein cows (Martins et al., 2018). Despite the high circulating P4 concentrations in the high-P4 cows in the present experiment, 10% of the cows still double ovulated. Similar to the present study, 12% of cows manipulated to have high P4 throughout a synchronized follicular wave double ovulated (Martins et al., 2018). Thus, the subgroup of cows that double ovulated in these 2 experiments despite high P4 during growth of a synchronized follicular wave likely did so for reasons independent of circulating P4 concentrations. Although the incidence of double ovulation in low-P4 cows in the present study was only 33% compared with the 49% incidence of double ovulation in the low-P4 cows reported by Martins et al. (2018), both studies agree from a physiologic perspective that low P4 during growth of a synchronized follicular wave in

high-producing dairy cows dramatically increases the incidence of double ovulation. By contrast, only 2 (1 cow in each treatment) of the moderate-producing Holstein-Friesian cows managed under a seasonal-calving pasture-based system had double ovulations (Fricke et al., 2016). The incidence of double ovulation is a sensitive endpoint affected by circulating P4 during growth of a follicular wave, and the lack of double ovulations in the study by Fricke et al. (2016) in moderate-producing Holstein-Friesian cows that were manipulated into a low-P4 environment is intriguing as well as difficult to explain physiologically.

P4 Before TAI

Similar to the study by Fricke et al. (2016), which used a similar experimental model, the treatments imposed in the present experiment successfully manipulated cows into divergent low-P4 and high-P4 environments during growth of the synchronized follicular wave before ovulation (Figure 2, upper left panel). On d -10, P4 concentrations did not differ ($P = 0.18$) between treatments (2.8 ± 0.4 vs. 2.2 ± 0.3 ng/mL for high-P4 vs. low-P4 cows, respectively). By contrast, from 3 to 8 d before TAI, P4 concentrations were about 5 ng/mL greater ($P < 0.01$) for high-P4 cows than for low-P4 cows (6.5 ± 0.2 vs. 1.7 ± 0.1 ng/mL, respectively). After induction of luteolysis during the protocol, P4 concentrations did not differ ($P = 0.88$) between treatments (0.04 ± 0.01 vs. 0.1 ± 0.1 for high-P4 cows vs. low-P4 cows, respectively) 1 d before TAI. Thus, complete luteal regression for cows in both treatments occurred by G2 based on a P4 cutoff of <0.4 ng/mL used in other experiments (Brusveen et al., 2009; Giordano et al., 2013; Carvalho et al., 2018). Further, inclusion of CIDR inserts during the protocols prevented cows from displaying estrus and failing to synchronize before scheduled TAI as reported by Fricke et al. (2016).

P4 After TAI

Ovulation of a larger follicle forms a larger CL that results in greater concentrations of circulating P4 after ovulation (Vasconcelos et al., 2001; Fricke et al., 2016). Concentrations of P4 in the present study increased ($P < 0.01$) over time for both treatments. Concentrations of P4 at 1 and 4 d after TAI were 0.04 ± 0.0 and 0.6 ± 0.05 ng/mL, respectively, and did not differ ($P > 0.1$) between treatments (Figure 2, upper right panel); however, from 6 to 15 d after TAI, P4 concentrations were greater ($P < 0.01$) for low-P4 cows than for high-P4 cows (Figure 2, upper right panel). In addition, cows with double ovulations had greater P4 concentrations than cows with single ovulations (4.0 ± 0.3 vs. $2.6 \pm$

0.1 ng/mL, respectively) due to the presence of 2 CL. For cows that maintained pregnancy until 67 d after TAI, P4 concentrations tended to be greater ($P = 0.06$) for low-P4 cows than for high-P4 cows (Figure 2, lower right panel). Furthermore, P4 concentrations were greater from 32 to 67 d after TAI than from 18 to 29 d after TAI (Figure 2, lower right panel).

Lactating dairy cows experience high rates of pregnancy loss before pregnancy status is diagnosed 32 d after AI. For example, at least half of the nonpregnant cows that maintained their CL until 32 d after TAI but were diagnosed not pregnant using transrectal ultrasonography were initially pregnant but underwent early pregnancy loss based on increased plasma PAG levels at 25 and 32 d after TAI (Ricci et al., 2017). To evaluate early pregnancy loss in the present study, a receiver operating characteristic curve was generated to determine a PSPB cutoff threshold to determine pregnancy status based on pregnancy outcomes determined using transrectal ultrasonography 32 d after TAI. Based on a threshold of 0.77 ng/mL PSPB, we divided cows into 3 subgroups according to their pregnancy status: (1) cows diagnosed pregnant based on transrectal ultrasonography 32 d after TAI, (2) cows diagnosed not pregnant based on transrectal ultrasonography that had high (>0.77 ng/mL) PSPB concentrations 27 d after TAI, and (3) cows diagnosed not pregnant based on transrectal ultrasonography that had low (<0.77 ng/mL) PSPB concentrations 27 d after TAI. For cows diagnosed pregnant 32 d after TAI, P4 concentrations remained constant over time and did not differ ($P > 0.10$) from those of cows diagnosed not pregnant with high PSPB concentrations, which also remained constant over time (Figure 2, lower left panel). By contrast, P4 concentrations for cows diagnosed not pregnant with low PSPB concentrations 27 d after TAI began to decrease 18 d after TAI and remained lower than for cows diagnosed not pregnant with high PSPB concentrations 27 d after TAI or for cows diagnosed pregnant (Figure 2, lower left panel). Thus, some cows are pregnant long enough to initiate maternal recognition of pregnancy by preventing luteolysis but subsequently undergo early pregnancy loss. These results agree with the observation that cows have high rates of pregnancy loss that can be determined based on extended luteal phases and the presence of circulating PAG concentrations such as PSPB before establishing pregnancy status using transrectal ultrasonography (Ricci et al., 2017).

Effect of Treatment, Ovulation Number, and Pregnancy Type on ISG15 mRNA Expression

The bovine blastocyst initiates the production and release of IFN τ to prevent secretion of uterine PGF $_{2\alpha}$

Figure 2. Mean $\pm$ SEM serum progesterone (P4) concentrations in lactating multiparous Holstein cows. Effect of treatment (Treat) on serum P4 concentrations during the Breeding-Ovsynch portion of the Double-Ovsynch protocol (upper left panel). Effect of treatment on serum P4 concentrations after timed AI (TAI; upper right panel). Effect of pregnancy (Preg) status and low versus high pregnancy-specific protein B (PSPB) concentration 32 d after TAI on serum P4 concentrations from 15 to 32 d after TAI (lower left panel). Effect of treatment on serum P4 concentrations from 18 to 67 d after TAI for cows that maintained pregnancy until 67 d after TAI (lower right panel). Within a panel, means ($\pm$ SEM) with different letters (a–d) differ ($P < 0.01$). D.Ov = double ovulation.

pulses, thereby preventing luteolysis and initiating maternal recognition of pregnancy (Spencer and Bazer, 2004). The production of IFN τ by the conceptus is detectable by d 7 in bovine blastocysts (Farin et al., 1990; Kubisch et al., 1998) and is then dramatically up-regulated concomitant to blastocyst elongation (Mann and Lamming, 1999, 2001; Rizos et al., 2012) due to its role in the regulation of genes that affect implantation and conceptus growth (Spencer et al., 2007).

In the present study, we assessed the presence of IFN τ indirectly through detection of *ISG15* (Han et al., 2006). We assessed the effect of both treatment and pregnancy status based on fold-change in *ISG15* mRNA expression in blood leukocytes from baseline blood samples collected 4 d after TAI and that of blood samples collected 18 and 20 d after TAI. By 18 d after

TAI, pregnant cows had a 4.8-fold increase ($P = 0.05$) in relative *ISG15* mRNA expression (Figure 3; upper panel); however, relative *ISG15* mRNA expression did not differ between treatments ($P = 0.23$), and there was no treatment $\times$ pregnancy status interaction ($P = 0.45$). Overall, cows with double ovulations had increased ($P < 0.01$) relative *ISG15* mRNA expression compared with cows with single ovulations. At 20 d after TAI, pregnant cows had an 8-fold increase ($P < 0.01$) in *ISG15* mRNA expression (Figure 3, upper panel). Relative *ISG15* mRNA expression did not differ ($P = 0.32$) between treatments for pregnant or non-pregnant cows 20 d after TAI (Figure 3; upper panel); however, cows with double ovulations had greater ($P < 0.01$) relative mRNA expression than cows with single ovulations.

Figure 3. Mean $\pm$ SEM relative fold change in IFN-stimulated gene 15 (*ISG15*) mRNA concentrations in blood leukocytes 18 and 20 d after timed AI (TAI) in lactating multiparous Holstein cows. Upper panel: effect of treatment (Treat) and pregnancy (Preg) status 32 d after TAI on *ISG15*. Middle panel: effect of ovulatory response (SOV = single ovulation; DOV = double ovulation) and pregnancy type (singleton vs. twins) on *ISG15*. Bottom panel: effect of pregnancy status 27 d after TAI and low versus high serum pregnancy-specific protein B (PSPB) concentrations 27 d after TAI on *ISG15*. Within a day after TAI, means ($\pm$ SEM) with different superscripts (a–c) differ ($P < 0.01$). P4 = progesterone.

Because treatment also affected the incidence of double ovulation and the proportion of cows diagnosed with twin pregnancies, pregnant cows were further divided into 3 subgroups: (1) cows with a single ovulation after G2 and a singleton pregnancy 32 d after TAI, (2) cows with double ovulations after G2 and a singleton pregnancy 32 d after TAI, and (3) cows with double ovulations after G2 and a twin pregnancy 32 d after TAI (Figure 3, middle panel). At both 18 ($P < 0.01$) and 20 ($P < 0.01$) d after TAI, relative *ISG15* mRNA expression was greatest ($P < 0.01$) for cows with double ovulations and a twin pregnancy, intermediate ($P < 0.01$) for cows with double ovulations and a singleton pregnancy, and least ($P < 0.01$) for cows with single ovulations and a singleton pregnancy (Figure 3, middle panel). We are not aware of any studies that have reported *ISG15* levels in cows with twin versus singleton pregnancies; however, it is likely that differences in *ISG15* mRNA expression due to pregnancy type in the present experiment can be explained simply based on the number of conceptuses present during maternal recognition of pregnancy 18 and 20 d after TAI in each of these subgroups. In addition, circulating P4 has been positively associated with IFN γ synthesis in cattle (Kerbler et al., 1997), and the effect of treatment or increased incidence of double ovulations on circulating P4 during conceptus growth in the present study may also have affected *ISG15* mRNA expression in the present study.

In a final analysis, nonpregnant cows were further divided based on the PSPB cutoff threshold defined previously (0.77 ng/mL). Thus, 2 subgroups of nonpregnant cows were analyzed: (1) nonpregnant cows with low (<0.77 ng/mL) PSPB concentrations 27 d after TAI and (2) nonpregnant cows with high (>0.77 ng/mL) PSPB concentrations 27 d after TAI. Nonpregnant cows with high PSPB concentrations 27 d after TAI had a 3.7- and 5.3- fold increase in relative *ISG15* mRNA expression 18 and 20 d after TAI compared with nonpregnant cows with low PSPB concentrations 27 d after TAI (Figure 3, lower panel). Taken together with the analysis of low versus high PSPB concentrations on circulating P4 (Figure 2, lower left panel), it is clear that high-producing Holstein cows experience a high rate of early pregnancy loss before pregnancy status is normally assessed using transrectal ultrasonography based on an embryonic heartbeat 32 d after TAI.

Effect of Treatment and Pregnancy Type on PSPB Concentrations

Serum PSPB concentrations have been used as an indicator of pregnancy (Sasser et al., 1986) and to

evaluate pregnancy loss (Giordano et al., 2012). Further, cows carrying twins have increased circulating PSPB, or PAG concentrations, or both early during pregnancy (López-Gatius et al., 2007; Giordano et al., 2012). Experimentally decreasing serum P4 after TAI in lactating Holstein cows decreased PSPB concentrations in pregnant cows by decreasing embryonic growth (Carvalho et al., 2017), whereas increasing P4 after TAI or in vitro fertilization embryo transfer in nulliparous Holstein heifers had no effect on PSPB concentrations during early pregnancy (Niles et al., 2019). By contrast, increasing serum P4 concentrations early after AI can advance embryonic growth by increasing expression of endometrial genes responsible for histotroph composition (Forde et al., 2009).

Serum PSPB concentrations were evaluated for cows that maintained pregnancies until 67 d after TAI and

are summarized in Figure 4. Overall, serum PSPB concentrations did not differ between treatments; however, a treatment $\times$ time interaction ($P = 0.05$) indicated that serum PSPB concentrations did not differ ($P > 0.10$) between treatments between 18 and 22 d and between 53 and 67 d after TAI, whereas serum PSPB concentrations were greater ($P < 0.01$) for low-P4 cows than for high-P4 cows between 25 and 46 d after TAI (Figure 4, upper panel).

Results from the present study with regard to serum PSPB concentrations are in contrast to those reported by Fricke et al. (2016) in which circulating PAG concentrations in Holstein-Friesian cows managed in a seasonal-calving pasture-based system in Ireland did not differ despite dramatic differences in P4 due to treatment both before and after TAI. In addition, PSPB concentrations in the present study did not differ ($P = 0.88$) between cows with singleton versus twin pregnancies; however, there was an interaction ($P = 0.03$) between number of conceptuses and time (Figure 4, lower panel). The comparison in circulating PSPB concentrations based on pregnancy type for cows that maintained pregnancies until 67 d after TAI in the present study should be interpreted with caution because of dramatic differences in embryo survival from an initial pregnancy diagnosis 32 d after TAI until 67 d after TAI between twin versus singleton pregnancies. By contrast, several studies have reported increased circulating PSPB concentrations, PAG concentrations, or both early during pregnancy for cows carrying twins versus singleton pregnancies (López-Gatius et al., 2007; Giordano et al., 2012).

Figure 4. Mean $\pm$ SEM serum pregnancy-specific protein B (PSPB) concentrations in lactating multiparous Holstein cows that maintained pregnancy until 67 d after timed AI (TAI). Upper panel: effect of treatment (Treat) on PSPB concentrations. Lower panel: effect of pregnancy type (singleton vs. twins) on PSPB concentrations. P4 = progesterone.

P/AI, Pregnancy Loss, and Twin Pregnancies

At 32 d after TAI, P/AI did not differ ($P = 0.97$) between treatments; however, cows with double ovulations had more ($P = 0.02$) P/AI than cows with single ovulations (Table 2). Further, neither treatment ($P = 0.49$) nor number of ovulations ($P = 0.11$) affected pregnancy loss from 32 to 67 d after TAI (Table 2). Twinning in dairy cattle is an undesirable trait that reduces the overall profitability of a dairy operation through negative effects on cows calving twins as well as on calves born as twins (Fricke, 2001). The incidence of twin pregnancies in the present study was greater ($P < 0.01$) for low-P4 cows than for high-P4 cows, and cows with double ovulations had a greater ($P < 0.01$) incidence of twin pregnancies than cows with single ovulations (Table 2).

We chose not to conduct this study as a field trial on a commercial dairy farm with a larger number of cows to assess the effect of treatment on P/AI or pregnancy

Table 2. Effect (%; no./no. in parentheses) of treatment and ovulation response (single vs. double) on pregnancies per AI from 32 to 67 d after timed AI (TAI), pregnancy loss from 32 to 67 d after TAI, and twin pregnancies 32 d after TAI in multiparous Holstein cows

Item	Treatment ¹		Ovulation		<i>P</i> -value	
	High P4	Low P4	Single	Double	Treatment	Ovulation
Day after TAI						
32	45 (18/40)	53 (21/40)	41 (26/63)	77 (13/17)	0.97	0.02
39	40 (16/40)	45 (18/40)	37 (23/63)	65 (11/17)	0.90	0.05
46	38 (15/40)	40 (17/40)	37 (23/63)	47 (8/17)	0.99	0.45
53	38 (15/40)	40 (14/40)	37 (23/63)	47 (8/17)	0.99	0.45
60	35 (14/40)	40 (14/40)	35 (22/63)	47 (8/17)	0.83	0.42
67	35 (14/40)	40 (14/40)	35 (22/63)	47 (8/17)	0.83	0.42
Pregnancy loss	22 (4/18)	24 (5/21)	15 (4/26)	39 (5/13)	0.49	0.11
Twins	0 (0/17)	29 (6/31)	0 (0/26)	46 (6/13)	<0.01	<0.01

¹Cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation to receive first TAI. One day after G1, cows were randomly assigned to receive 2 new controlled internal drug-release (CIDR) inserts (high P4) or a PGF treatment and a used CIDR insert (low P4).

loss due to the potential of dramatically increasing twinning and pregnancy loss for cows manipulated to have low P4. Rather, we conducted an intensive study with frequent blood sampling and transrectal ultrasonography in our university research herd. Although the present study lacked statistical power to fully assess the effect of treatment on P/AI and pregnancy loss, our results agree with a study in which cows were manipulated into a high- or low-P4 environment during growth of the ovulatory follicle (Martins et al., 2018). In that study, cows that were manipulated into a low-P4 environment during growth of the ovulatory follicle had a 49% incidence of double ovulation, P/AI of 66.4%, and pregnancy loss from d 23 to calving of 33% (Martins et al., 2018). Although cows with double ovulations have more P/AI at the initial pregnancy diagnosis than cows with single ovulations (Fricke and Wiltbank, 1999; Martins et al., 2018), pregnancy loss from an initial pregnancy diagnosis to a pregnancy reconfirmation based on transrectal ultrasonography was 3-fold greater for cows diagnosed with twins compared with cows diagnosed with singletons (Silva del Río et al., 2009). Pregnancy loss before 90 d in gestation was greater for cows with unilateral twins (35%) than for cows with bilateral twins (8%) in one experiment (López-Gatius and Hunter, 2005), and cows with unilateral double ovulations experienced greater pregnancy loss than cows with bilateral double ovulations (Martins et al., 2018). Taken together, these data support the idea that twinning due to double ovulations that results in unilateral twin pregnancies may be a significant cause of pregnancy loss in high-producing lactating dairy cows.

CONCLUSIONS

In agreement with our hypotheses, low P4 concentrations before TAI increased the incidence of double ovulation and twin pregnancies, serum PSPB concen-

trations, and relative expression of *ISG15* in blood leukocytes at 18 and 20 d after TAI in high-producing multiparous Holstein cows. Our results agree with and extend results from a study in which low P4 concentrations before TAI dramatically increased the incidence of double ovulation, P/AI, and the incidence of pregnancy loss, particularly for cows with unilateral double ovulations (Martins et al., 2018). Perhaps the most intriguing aspect of the present study is the contrast in reproductive outcomes from an earlier study that used a similar experimental model but was conducted using moderate-producing Holstein-Friesian cows managed in a seasonal-calving pasture-based system in Ireland (Fricke et al., 2016). The striking dichotomy in reproductive outcomes between these 2 experiments suggests that effects of genotype, milk production, hepatic steroid metabolism, or other factors independent of circulating P4 before or after TAI may play a physiologic role in controlling these reproductive outcomes. Finally, our results support the idea that manipulating high-producing multiparous Holstein cows into a high-P4 environment during a synchronized follicular wave before TAI can decrease the incidence of double ovulation and twinning. By contrast, high-producing multiparous Holstein cows in which a synchronized follicular wave occurs in a low-P4 environment before TAI have a dramatically increased incidence of double ovulation and twinning.

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