Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine parameters in seasonal-calving, pasture-based Holstein-Friesian cows

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ABSTRACT

Fertility to timed AI (TAI) is profoundly affected by progesterone (P4) levels during hormonal synchronization protocols. Holstein-Friesian dairy cows managed in a seasonal-calving, pasture-based production system were randomly assigned to 2 treatments to manipulate P4 before TAI during growth of the preovulatory follicle. Cows in the first treatment (High P4; $n = 30$) were submitted to a Double-Ovsynch protocol {Pre-Ovsynch [GnRH; 7 d, PGF_{2α}; 3 d, GnRH] followed 7 d later by Breeding-Ovsynch [GnRH (G1); 7 d PGF_{2α}; 24 h, PGF_{2α}; 32 h, GnRH (G2); 16 h, TAI]}. Cows in the second treatment ($n = 30$; Low P4) received the same Double-Ovsynch protocol but with an additional PGF_{2α} treatment 24 h after G1. Overall, synchronization rate did not differ between treatments and was 92% (55/60). Unexpectedly, 37% of Low P4 cows were detected in estrus ~24 h before scheduled TAI and were inseminated ~16 h before scheduled TAI. Overall, P4 did not differ between treatments at G1, whereas High P4 cows had greater P4 concentrations at PGF_{2α} and G2 than Low P4 cows. High P4 cows had the smallest mean follicle diameter at G2, whereas Low P4 cows with no estrus before TAI had intermediate mean follicle diameter at G2, and Low P4 cows with estrus before TAI had the largest mean follicle diameter. Low P4 cows with estrus before TAI had larger corpora lutea 15 d after TAI than Low P4 cows without estrus before TAI or High P4 cows. In accordance with corpus luteum size on d 15, High P4 cows and Low P4 cows without estrus before TAI had lower P4 from 4 to 46 d after TAI than Low P4 cows with estrus before TAI. Relative mRNA

levels of the interferon-stimulated genes *ISG15*, *MX1*, *MX2*, and *OAS1* were greater for Low P4 than for High P4 cows, whereas relative mRNA levels of *RTP4* were greater for High P4 than for Low P4 cows 18 d after TAI. Treatment did not affect plasma pregnancy-associated glycoprotein concentrations after TAI; however, pregnancy-associated glycoprotein concentrations were affected by pregnancy status and parity. Treatment did not affect pregnancy per artificial insemination at 29, 39, or 60 d after TAI, and no pregnancy losses were observed from 39 to 60 d after TAI. We concluded that (1) Low P4 cows were more likely to express estrus than High P4 cows; (2) the subpopulation of Low P4 cows that expressed estrus had larger preovulatory follicles and greater P4 concentrations after TAI; and (3) regardless of estrus before TAI, all Low P4 cows had greater mRNA expression for 5 of 6 interferon-stimulated genes than High P4 cows 18 d after TAI.

Key words: progesterone, pregnancy-associated glycoprotein, interferon-stimulated genes, dairy cow

INTRODUCTION

Progesterone (P4) is the most biologically active progestogen in cattle and is produced and secreted primarily by the corpus luteum (CL) during the estrous cycle and the placenta during pregnancy. For cows inseminated to estrus, pregnancy per artificial insemination (P/AI) decreased 12.4 percentage points for every 1 ng/mL decrease in P4 concentration during the last half of the estrous cycle preceding the first AI (Fonseca et al., 1983). Fertility to timed artificial insemination (TAI) after hormonal synchronization protocols is profoundly affected by cyclicity status, as well as P4 concentration, at the onset of a synchronized breeding protocol. For example, cows with low P4 at the first GnRH treatment or at the PGF_{2α} treatment

Received March 28, 2016.

Accepted May 9, 2016.

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of an Ovsynch protocol have lower fertility than cows with high P4 (Fricke et al., 2003; Bisinotto et al., 2010; Carvalho et al., 2014). Increased P4 concentrations during the period of follicle growth before AI are associated with increased embryo quality (Cerri et al., 2009; Rivera et al., 2011) and increased fertility (Folman et al., 1990; Bisinotto et al., 2010; Dirandeh, et al., 2015b) in lactating dairy cows.

Steady-state P4 concentration in circulation is a balance between P4 production by the CL and P4 catabolism by the liver (Wiltbank et al., 2014). Because milk production is highly correlated ($r = 0.88$) with feed intake (Harrison et al., 1990), hepatic blood flow increases as milk production and feed intake increase, providing a potential physiological mechanism for decreased circulating P4 concentrations in high-producing lactating dairy cows (Sangsritavong et al., 2002; Vasconcelos et al., 2003). A potential mechanism by which low P4 during follicular growth could adversely affect embryo growth and development is overexposure of the oocyte to LH pulses during the final stages of follicular growth as a result of the low P4 environment (Kinder et al., 1996). Exposure of dominant follicles to increased LH pulses during the final stages of follicular growth before ovulation results in ovulation of a larger follicle (i.e., a persistent follicle; Ahmad et al., 1995), which generates a larger CL after ovulation that produces more P4 (Vasconcelos et al., 2001). Altering P4 before or after AI could in turn alter embryonic growth, thereby affecting maternal recognition of pregnancy or pregnancy loss. It is now possible to indirectly assess conceptus development 15 to 22 d after AI by measuring mRNA expression of interferon-stimulated genes (**ISG**) in circulating blood leukocytes (Stevenson et al., 2007; Gifford et al., 2008; Green et al., 2010) and by measuring pregnancy-associated glycoprotein (**PAG**) levels in blood or milk samples beginning approximately 25 d after AI (Green et al., 2000; Ricci et al., 2015).

The objective of this experiment was to hormonally manipulate Holstein-Friesian dairy cows managed in a seasonal-calving, pasture-based system into a high or a low P4 environment during the growth of a synchronized preovulatory follicle. Our hypotheses were that cows with low P4 would (1) ovulate larger follicles; (2) have more P4 after TAI; and (3) have decreased early embryonic development based on ISG expression and PAG levels than cows with high P4.

MATERIALS AND METHODS

Cows, Housing, and Feeding

This study was conducted from March to June 2014 at the Animal and Grassland Research and Innovation

Centre at Teagasc, Moorepark (County Cork, Ireland). Holstein-Friesian dairy cows ($n = 60$) were managed as a single herd in a spring-calving, intensive rotational grazing system with a mating start date of May 1, 2014. Cows were housed in a freestall barn during the dry period. After parturition, cows were turned out to grass in early February and grazed under a rotational grazing system, as described by Dillon et al. (1995) in a predominantly perennial ryegrass (*Lolium perenne* L.) sward. Fresh pasture was allocated daily after morning milking by moving temporary fencing in the grazing paddocks. Cows were milked twice daily at 0730 and 1630 h throughout the experiment. All experimental procedures involving cows were approved by the Teagasc Animal Ethics Committee and authorized by the Health Products Regulatory Authority, which is the competent authority in Ireland responsible for the implementation of European Union legislation (Directive 2010/63/EU) for the protection of animals used for scientific purposes.

Experimental Design, Synchronization Treatments, and AI

Cows were enrolled in a completely randomized block experimental design and were blocked by lactation number and DIM at TAI (calculated based on the mating start date and calving dates of individual cows) as part of the randomization procedure. Holstein-Friesian cows ($n = 60$) were randomly assigned to each of 2 hormonal synchronization protocols to receive their first TAI (Figure 1). Synchronization protocols used i.m. treatments with GnRH (gonadorelin diacetate tetrahydrate; Ovarelin; Ceva Santé Animale, Libourne, France) and PGF_{2α} (dinoprost; Enzaprost; Ceva Santé Animale).

Cows ($n = 30$) randomized to the first treatment (**High P4**) were submitted for first TAI using a Double-Ovsynch protocol (Souza et al., 2008; Herlihy et al., 2012) with several modifications. For the first Ovsynch protocol (i.e., Pre-Ovsynch), cows were treated with 100 µg of GnRH, followed by 25 mg of PGF_{2α} 7 d later and 100 µg of GnRH 72 h after the PGF_{2α} treatment. For the second Ovsynch protocol (i.e., Breeding-Ovsynch), cows were treated with 200 µg of GnRH (i.e., a double dose, **G1**) followed by 25 mg of PGF_{2α} 7 d later, a second 25-mg PGF_{2α} treatment 24 h after the first, and 100 µg of GnRH 32 h later (i.e., 56 h after the first PGF_{2α} treatment, **G2**).

Cows ($n = 30$) randomized to the second treatment (**Low P4**) were submitted for first TAI using the same modified Double-Ovsynch protocol but with the addition of 25 mg of PGF_{2α} administered 24 h after the first GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (Figure 1).

Cows were scheduled to receive TAI approximately 16 h after the last GnRH treatment of the protocol using frozen-thawed semen from 1 of 3 high-genetic-merit Irish Holstein-Friesian AI sires, and sires were used equally to inseminate cows between the 2 treatments. Two experienced AI technicians who were blind to treatment performed all inseminations, and all TAI were completed within 1 h.

Transrectal Ultrasonography

Ovarian structures were monitored using transrectal ultrasonography (Aloka SSD-900; Aloka Ltd., Tokyo, Japan) fitted with a 7.5-MHz transrectal transducer. During each ultrasound examination, a sketch of the location and diameter of all ovarian follicles and CL >8 mm was recorded. For each ovarian structure, 2 diameter measurements (mm) assessed at perpendicular angles were recorded on a single frozen image of the apparent maximal diameter of the structure using the digital calipers of the ultrasound machine, and diameters were calculated as the mean of these 2 measurements.

The number of CL present on the ovaries of each cow was assessed at the first PGF_{2α} treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol to assess response to the synchronization treatments. Ovulatory response after the last GnRH treatment of the Double-Ovsynch protocol (G2) was determined by the presence of one or more ovulatory-sized follicles (>10 mm in diameter) at G2 and the absence of 1 (single ovulation) or 2 (double ovulation) of those follicles at an ultrasound examination 48 h later (Fricke et al., 1998). The presence of 1 or 2 CL at 15 d after TAI on the ovary where the ovulation(s) occurred was used to confirm ovulatory response to G2. The diameter of the CL was measured using transrectal ultrasonography and the procedure described previously.

Tail paint (FIL Detail; Gregg Care Ltd., Donegal, Ireland) was applied in a strip 5 cm wide by 20 cm

long over the coccygeal vertebrae of the tail head of all cows at the onset of the synchronization protocols and was assessed daily and refreshed as necessary to assess estrus during the protocol and return to estrus after TAI. Cows that failed to conceive to TAI and were detected in estrus visually or based on rubbed tail paint before the pregnancy diagnosis 39 d after TAI were re-inseminated. Pregnancy diagnosis was conducted using transrectal ultrasonography 39 d after TAI for all cows in both treatment groups, regardless of whether or not they were inseminated to estrus after TAI. A positive pregnancy diagnosis was based on the presence of a CL and an embryo with a visualized heartbeat located in the uterine horn ipsilateral to the ovary bearing the CL. Cows diagnosed as pregnant at the initial examination were re-examined 60 d after TAI to confirm a viable pregnancy and assess the incidence of pregnancy loss.

Blood Sample Collection

Blood samples for analysis of P4 and PAG were collected via puncture of the coccygeal vessels into evacuated tubes containing lithium heparin as an anti-coagulant (Becton Dickinson, Plymouth, UK). Within 60 min after collection, blood samples were placed in a centrifuge for 15 min at $1,500 \times g$ at 4°C, and plasma was decanted into individual polypropylene tubes and stored at -20°C. Blood samples for analysis of P4 were collected at -10, -8, -6, -3, and -0.5 d relative to TAI (i.e., during the Breeding-Ovsynch portion of the Double-Ovsynch protocol), and 3 times per week (Monday, Wednesday, Friday) from 1 to 39 d after TAI and at 46 d after TAI. Blood samples collected from 20 to 36 d after TAI were split into 2 aliquots before freezing; 1 aliquot was used for analysis of P4, the other for analysis of PAG concentrations.

Blood samples (3 mL) were collected 18 d after TAI from the coccygeal vessels into Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA) containing 6 mL of RNA stabilization solution. Tubes were

Figure 1. Schematic diagram of the modified Double-Ovsynch hormonal treatment protocols used to manipulate progesterone during growth of the preovulatory follicle and for submission of cows for first timed AI (TAI). 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.

shaken vigorously by hand for 20 s immediately after blood collection, and whole blood was stored at -80°C .

Analysis of Progesterone

Concentration of P4 was determined in all plasma samples using a solid-phase, no-extraction radioimmunoassay (Coat-a-count; Diagnostic Products Corporation, Los Angeles, CA). The average sensitivity for the P4 assays was 0.05 ng/mL. The intraassay and interassay coefficients of variation based on low, medium, and high quality control samples were 5.56% and 3.84%, respectively.

Analysis of PAG

Plasma PAG levels were determined using a commercial ELISA kit (Idexx Visual Read Pregnancy Test; Idexx Laboratories, Westbrook, ME) on all samples collected from 20 to 36 d. Plasma PAG ELISA kits were run according to the manufacturer's instructions. Briefly, a microtiter plate format was configured by coating an anti-PAG monoclonal antibody onto the plate. The PAG monoclonal antibody was raised against the PAG-55 protein fraction, comprising PAG-4, PAG-6, PAG-9, PAG-16, PAG-18, and PAG-19 (Nagappan et al., 2009). After incubation of the diluted test sample in the coated well, captured PAG was detected with a PAG-specific antibody (detector solution) and horseradish peroxidase conjugate. Unbound conjugate was washed away, and 3,3',5,5'-Tetramethylbenzidine substrate was added to the wells. Color development was proportional to the amount of PAG in the sample and was measured using a spectrophotometer. Results were calculated from the optical density (OD) of the sample (S) minus the OD of the negative control (N) at 450 nm (with OD values for each well corrected by subtracting the 630 nm reference wavelength) to generate an $S - N$ value for each unknown. Each microplate included negative and positive controls.

The commercial ELISA kit used in the present experiment was a qualitative assay that we modified to be semiquantitative. A standard curve was created by diluting late-pregnant cow plasma with nonpregnant cow plasma. Standard curve points were 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0%. Each plate contained a standard curve performed in duplicate. The OD for wells containing the standard curve and unknown samples was measured by using a microtiter plate reader (ELx808; BioTek, Winooski, VT). Data for unknown samples were analyzed using the Gen5 microplate reader software (BioTek) and expressed as a percentage of that of late-pregnant cow.

Analysis of ISG mRNA Expression

RNA Extraction and cDNA Synthesis. The RNA was extracted from whole blood collected into Tempus tubes using the Tempus Spin RNA Isolation Reagent Kit (Applied Biosystems) according to manufacturer's instructions and including the optional DNase step. A NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to quantify the RNA. The quality of the RNA was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Dublin, Ireland). All samples had an RNA integrity number of between 9.0 and 10.0. The cDNA was synthesized in a 20- μL reaction, according to the manufacturer's instructions, from 1 μg of total RNA per sample, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was then stored at -20°C .

Real-Time Quantitative PCR. Primer sequences for candidate genes were obtained from the literature (Table 1) and were commercially synthesized (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Serial dilutions of pooled cDNA samples were used to determine amplification efficiencies using the equation $E = -1 + 10(-1/\text{slope})$. The slope was calculated by plotting the linear curve of cycle threshold (C_q) values against the log dilutions (Pfaffl, 2001). Primers had PCR amplification efficiencies of between 80% and 110%. Five reference genes, β -actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), ribosomal protein L19 (*RPL19*), and cyclophilin A (*cycloA*), known to have stable expression in circulating bovine lymphocytes, were used in this study. Expression of *RPL19* was greater for one of the experimental treatments and was excluded as a reference gene. For the remaining 4 reference genes, an average stability (M) value of 0.56 was calculated based on average pairwise variations using GenEx software (MultiD Analyses AB, Göteborg, Sweden). The geometric mean of the reference genes was used to calculate a normalization factor that was used to normalize expression of each gene of interest. One microliter of the optimal concentration of cDNA (determined when calculating efficiencies using serial dilutions) was added to 19 μL of master mix (10 μL of Fast SYBR Green 1 master mix; Applied Biosystems), 8 μL of nuclease-free water, and 0.5 μL each of forward and reverse primers at individually optimized concentrations. Real-time quantitative (q)PCR was used to measure gene expression of the reference genes and the target genes (Table 1) according to Minimum Information for Publication of Quantitative Real-Time PCR

Table 1. Primer sequences for interferon-stimulated gene mRNA expression analysis

Gene	Accession No.	Primer sequence
Target genes		
<i>ISG15</i>	NM_174366	Forward: 5'-GGTATGAGCTGAAGCAGTT-3' Reverse: 5'-ACCTCCCTGCTGTCAAGGT-3'
<i>MX2</i>	NM_173941	Forward: 5'-CTTCAGAGACGCCTCAGTCG-3' Reverse: 5'-TGAAGCAGCCAGGAATAGTG-3'
<i>OAS1</i>	NM_001040606	Forward: 5'-ACCCTCTCCAGGAATCCAGT-3' Reverse: 5'-GATTCTGGTCCCAGGTCTGA-3'
<i>MX1</i>	AF04769	Forward: 5'-GTACGAGCCGAGTTCTCCAA-3' Reverse: 5'-ATGTCCACAGCAGGCTCTTC-3'
<i>RTP4</i>	BC105539	Forward: 5'-CACATGTACCTGGAGAACCAGA-3' Reverse: 5'-AGGTAGCTCTGAAACCTTCCTG-3'
Reference genes		
<i>CycloA</i>	NM_178320	Forward: 5'-CACCGTGTTCCTTCGACATCG-3' Reverse: 5'-ACAGCTCAAAAGAGACGCGG-3'
<i>ACTB</i>	AY141970	Forward: 5'-CTGGACTTCGAGCAGGAGAT-3' Reverse: 5'-GGATGTCGACGTCACACTTC-3'
<i>RPL19</i>	NM_001040516	Forward: 5'-ATCGATCGCCACATGTATCA-3' Reverse: 5'-GCGTGCTTCCTTGGTCTTAG-3'
<i>GADPH</i>	DQ402990	Forward: 5'-GGGTCATCATCTCTGCACCT-3' Reverse: 5'-GGTCATAAGTCCCTCCACGA-3'
<i>YWHA</i>	NM_174814.2	Forward: 5'-GCATCCCACAGACTATTTCC-3' Reverse: 5'-GCAAAGACAATGACAGACCA-3'

Experiments (MIQE) guidelines (Bustin et al., 2009). Applied Biosystems 7500 FAST RT-PCR equipment v2.0.1 was used (Applied Biosystems). The conditions applied were as follows: 95°C for 20 s; 40 cycles of 95°C for 3 s and 60°C for 30 s; and finishing with amplicon dissociation at 95°C for 15 s, 60°C for 1 min, increasing 1°C per cycle until 95°C was reached for 15 s, and then 60°C for 15 s.

Real-Time qPCR Data Normalization. The C_q values were imported into GenEx software v.5.2.7.44 (MultiD Analyses AB). A modified Grubbs test was used to remove outliers from replicate wells at a $P < 0.05$ confidence interval for replicates differing from the mean by a standard deviation of greater than 0.25 cycles. Adjustments were performed to account for inter-plate variation using the inter-plate calibrator sample included on all plates. The C_q values were adjusted for amplification efficiencies, and replicates were averaged. The resulting values were normalized to the reference genes, and relative quantities of mRNA were calculated to the highest C_q value.

Statistical Analyses

The experimental design was a complete randomized block design, using parity (primiparous vs. multiparous) and days postcalving at initiation of the synchronization protocols as blocking factors. Analyses of binary response data (P/AI, pregnancy loss, ovulatory response to GnRH, luteal response to PGF_{2α}, synchronization rate) were performed by logistic regression

using the GLIMMIX procedure in SAS (version 9.2; SAS Institute Inc., Cary, NC); the model included the fixed effects of treatment, parity, and days postcalving.

Differences in mean lactation number and DIM at TAI were determined by ANOVA using the MIXED procedure in SAS; the model contained the fixed effect of treatment. Differences in BCS, milk yield, preovulatory follicle diameter, and CL volume after TAI were also determined by ANOVA using the MIXED procedure in SAS; the model included the fixed effects of treatment, parity, and DIM at TAI. Differences in P4 concentrations before and after TAI were determined by ANOVA using the MIXED procedure in SAS; the model contained the fixed effects of treatment, time, treatment by time interaction, parity, and DIM at TAI. Because of repeated measures within each cow, we used a repeated statement to account for autocorrelations by modeling the spatial power between sequential measurements. Differences in plasma PAG levels were determined by ANOVA using the MIXED procedure in SAS; the model contained the fixed effects of treatment, time, treatment by time interaction, parity, and DIM at TAI. Because of repeated measures within each cow, we used a repeated statement to account for autocorrelations by modeling the spatial power between sequential measurements.

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

Table 2. Synchronization rate and characteristics of Holstein-Friesian dairy cows enrolled in the experiment

Item	Treatment ¹	
	High P4	Low P4
Cows enrolled (no.)	30	30
Synchronization rate (%; no./no.)	90 (27/30)	93 (28/30)
Primiparous cows (%; no./no.)	33 (9/27)	32 (9/28)
Lactation number (mean $\pm$ SEM)	2.8 $\pm$ 0.3	2.8 $\pm$ 0.3
BCS (mean $\pm$ SEM)	2.71 $\pm$ 0.02	2.74 $\pm$ 0.01
Milk yield (kg/d; mean $\pm$ SEM), in week of TAI	24.7 $\pm$ 1.2	24.9 $\pm$ 0.9
DIM at TAI (mean $\pm$ SEM)	88.5 $\pm$ 3.1	89.1 $\pm$ 2.9

¹Cows were randomly assigned to 1 of 2 treatments (High P4 vs. Low P4) to manipulate progesterone (P4) before timed AI (TAI) during growth of the preovulatory follicle. Items did not differ statistically between treatments.

RESULTS AND DISCUSSION

To test our hypotheses related to the effect of P4 during growth of the preovulatory follicle, we hormonally manipulated cows using 2 synchronization protocols for TAI that allowed for differential manipulation of P4 and for all cows to receive TAI on the mating start date. To accomplish this, we used a Double-Ovsynch protocol for first TAI as described by Souza et al. (2008) with several modifications. Because a high degree of synchrony to each of the treatments of the Breeding-Ovsynch portion of the Double-Ovsynch protocol was critical for testing our hypotheses, we used 2 modifications for both treatments. First, because ovulatory response to G1 was critical for cows in both treatments, we increased the dose of GnRH at G1 from 100 to 200 μ g to increase ovulatory response, as we have reported (Giordano et al., 2013). Ovulatory response to G1 affects fertility to timed AI, with higher rates of P/AI as ovulatory response to G1 increases (Bisinotto and Santos, 2011; Carvalho et al., 2015b). Second, we included an additional PGF_{2 α} treatment 24 h after the first during the Breeding-Ovsynch portion of the Double-Ovsynch protocol, as first described by Brusveen et al., (2009) to induce complete luteal regression by G2. Incomplete luteal regression, measured as P4 $\geq$ 0.4 ng/mL at G2, is associated with decreased fertility at first and second and greater TAI, and cows that received a second PGF_{2 α} treatment 24 h after the first during an Ovsynch protocol for first or second and greater TAI had decreased incomplete luteal regression and increased fertility to TAI (Carvalho et al., 2015a; Wiltbank et al., 2015).

Synchronization Rate and Incidence of Estrus Before TAI

The experimental model we used resulted in a high synchronization rate (Table 2). To be considered

synchronized, cows had to undergo complete luteal regression (<0.4 ng/mL plasma P4 concentration) and ovulate a follicle within 48 h after G2. The overall synchronization rate was 92% (55/60) and did not differ between treatments (Table 2). Cows that failed to synchronize to the protocols (3 High P4 cows and 2 Low P4 cows) were removed from subsequent analyses. Cows were blocked by lactation number and DIM at TAI as part of the randomization procedure, and these parameters did not differ between treatments, nor did mean milk production during the week of TAI (Table 2).

All cows in both treatments were assessed for estrus twice daily at milking based on rubbed tail paint. Unexpectedly, 37% (11/30) of the Low P4 cows had rubbed tail paint at the morning milking 48 h after the first PGF_{2 α} treatment (i.e., 24 h before the scheduled TAI). Because the timing of AI relative to ovulation plays a critical role in fertility (Roelofs et al., 2006), all cows with rubbed tail paint 48 h after the first PGF_{2 α} treatment received G2 as scheduled at the afternoon milking but were inseminated $\sim$ 16 h before the scheduled TAI. Because of the potential effect of estrus before TAI on experimental endpoints, Low P4 cows were further classified based on expression of estrus before TAI, and data are presented separately.

Low P4 cows with estrus before TAI had less P4 at the first PGF_{2 α} treatment than Low P4 cows without estrus before TAI (Figure 2), a finding that could partially explain why these cows came into estrus before TAI. Another explanation for why the Low P4 cows expressed estrus before TAI was preovulatory follicle size at G2 (Table 3). High P4 cows had the smallest mean follicle diameter at G2 (15.6 $\pm$ 0.4 mm); Low P4 cows with no estrus before TAI had an intermediate mean follicle diameter at G2 (16.7 $\pm$ 0.5 mm), and Low P4 cows with estrus before TAI had the largest mean follicle diameter (17.1 $\pm$ 0.4 mm). The size of the ovulatory follicle in cattle affects the size of the resulting

Table 3. Effect of treatment and expression of estrus before timed AI (no or yes) on mean ($\pm$ SEM) preovulatory follicle diameter at G2¹ and mean ($\pm$ SEM) corpus luteum (CL) volume 15 d after timed AI (TAI)

Item	Treatment ¹		
	High P4	Low P4	
	No	No	Yes
Cows (no.)	28	16	11
Preovulatory follicle diameter at G2 ² (mm)	15.6 ^a $\pm$ 0.4	16.7 ^{ab} $\pm$ 0.5	17.1 ^b $\pm$ 0.4
CL volume 15 d after TAI (mm ³)	9,632 ^a $\pm$ 810	9,531 ^a $\pm$ 750	12,203 ^b $\pm$ 1,383

^{a,b}Means within a row with different superscripts differ ($P < 0.01$).

¹Cows were randomly assigned to 1 of 2 treatments (High P4 vs. Low P4) to manipulate progesterone (P4) before TAI during growth of the preovulatory follicle.

²G2 = last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol.

CL and fertility (Vasconcelos et al., 2001). Thus, the presence of larger follicles at PGF_{2 α} treatment could account for the early expression of estrus. When CL volume was measured 15 d after TAI, Low P4 cows with estrus before TAI had larger ($P < 0.01$) CL than Low P4 cows without estrus before TAI or High P4 cows (Table 3). Our first hypothesis was that cows with low P4 during the growth of the preovulatory follicle would ovulate larger follicles because of increased follicle growth when exposed to increased LH pulses in the low P4 environment (Kinder et al., 1996). Based on data from our experiment, we can only partially accept this hypothesis, because the effect of low P4 during growth of the preovulatory follicle was manifested primarily in Low P4 cows with estrus before TAI, and the resulting CL 15 d after TAI was only larger for Low P4 cows with estrus before AI.

Progesterone Before Timed AI

To manipulate cows into a high P4 environment during growth of the preovulatory follicle, we used a Double-Ovsynch protocol including the 2 modifications discussed above. Ideally, cows that ovulate a follicle to the last GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol and G1 of the Breeding-Ovsynch portion of the Double-Ovsynch protocol should have 2 CL at the first PGF_{2 α} treatment of the protocol, resulting in 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 luteolysis until about 7 d after ovulation (Nascimento et al., 2014), a CL induced by the last GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol should regress, whereas the newly forming CL induced by G1 of the Breeding-Ovsynch portion of the Double-Ovsynch protocol administered 24 h before PGF_{2 α} treatment should

not (Nascimento et al., 2014). As expected, High P4 cows had more ($P < 0.001$) CL at the first PGF_{2 α} treatment during the Breeding-Ovsynch portion of the Double-Ovsynch protocols (1.93 ± 0.09 vs. 1.04 ± 0.04). Further, P4 (ng/mL) did not differ between treatments at G1 (3.8 ± 0.3 vs. 4.1 ± 0.4) before cows were differentially treated during the protocols, whereas High P4 cows had greater ($P < 0.001$) circulating P4 at PGF_{2 α} (8.5 ± 0.9 vs. 1.6 ± 0.4) and G2 (0.30 ± 0.04 vs. 0.08 ± 0.02) than Low P4 cows (Figure 2). Complete luteal regression for cows in both treatment groups occurred by G2 based on a P4 cutoff of <0.4 ng/mL used in previously reported experiments (Brusveen et al., 2009; Giordano et al., 2013).

Progesterone After Timed AI

Our second hypothesis was that cows with Low P4 during development of the ovulatory follicle would ovu-

Figure 2. Effect (mean $\pm$ SEM) of treatment and expression of estrus before timed AI (TAI) on plasma progesterone (P4) levels (ng/mL) during growth of the preovulatory follicle. Circled points denote no statistical difference between the data points. Data points with different letters (c-f) differ ($P < 0.05$).

late larger follicles, resulting in increased P4 concentrations after TAI. In contrast to this hypothesis, P4 levels for cows diagnosed pregnant 39 d after TAI (Figure 3) corresponded to mean CL size 15 d after TAI (Table 3) instead of treatment group. High P4 cows and Low P4 cows without estrus before TAI had less P4 from 4 to 46 d after TAI than Low P4 cows with estrus before TAI ($P < 0.01$). These results support the hypothesis that the main factor affecting P4 after TAI in the present study was the size of the preovulatory follicle, which in turn affected the size of the CL 15 d after TAI and subsequent plasma P4 concentrations.

Steady-state P4 concentrations in circulation represent a balance between P4 production by the CL and P4 catabolism by the liver (Wiltbank et al., 2014), which can confound direct relationships between CL size and circulating P4 in high-producing dairy cows. For example, Sartori et al. (2004) reported that maximal serum P4 concentration was less for lactating Holstein cows than for nonlactating heifers (5.6 ± 0.5 vs. 7.3 ± 0.4 ng/mL), whereas maximal CL volume was greater for lactating cows than for nonlactating heifers ($11,120 \pm 678$ vs. $7,303 \pm 308$ mm³). They concluded that the greater incidence of reproductive anomalies in lactating dairy cows compared with nonlactating heifers, including poor fertility, ovulation failure, delayed ovulation, and multiple ovulations, may be due to decreased circulating steroid concentrations (Sartori et al., 2004). The Holstein-Friesian cows managed in a grazing-based system used in the present experiment produced ~25 kg/d of milk during the week of TAI (Table 2), substantially less than high-producing Holstein cows housed in confinement, which can exceed 45 kg/d. It is possible that hepatic steroid metabolism is less important in Irish Holstein-Friesian dairy cows than in high-producing US Holstein cows.

One of the most sensitive biological endpoints affected by P4 levels during growth of follicles in a follicular wave is the selection of co-dominant follicles, resulting in double ovulations (Wiltbank et al., 2000). When the ovulatory follicle arises in a follicular wave that develops in the absence of P4 (i.e., no active CL), co-dominant follicles resulting in double ovulations occur more frequently (Wiltbank et al., 2000; Hayashi et al., 2008). This is particularly the case for anovular cows at first postpartum ovulation, which had a 41% double-ovulation rate, compared with 4% for cycling cows (Gümen et al., 2003). The increase in twinning in the US Holstein population over time has been related to a concomitant increase in double ovulations (Silva del Río et al., 2007), which cause many reproductive problems in US Holsteins, including increased pregnancy loss (Silva-del-Río et al., 2009). Surprisingly, only 1 cow in each treatment group from the present experiment

Figure 3. Effect (mean $\pm$ SEM) of treatment and expression of estrus before timed AI (TAI) on plasma progesterone (P4) levels (ng/mL) after TAI for dairy cows diagnosed pregnant 39 d after TAI.

had a double ovulation after G2. This observation is in contrast to a recent report in which high-producing US Holsteins were manipulated into a low P4 environment before TAI, resulting in a double ovulation rate that approached 50% (Martins et al., 2015). Taken together, it may be that the Irish Holstein-Friesian cows used in the present study did not present a physiological scenario in which hepatic P4 metabolism driven by high feed intake and high milk production negatively affected or altered the preovulatory follicle before TAI or P4 levels after TAI.

Effect of Treatment on ISG mRNA Expression

Interferon-tau is a member of the type I interferon family (Roberts et al., 1997), is produced by trophoblast cells of the blastocyst, and is the signal for maternal recognition of pregnancy in ruminants (Spencer and Bazer, 2004). Production of IFN-tau during maternal recognition of pregnancy induces expression of a large number of ISG in the endometrium (Gray et al., 2006) and in circulating blood leukocytes (Stevenson et al., 2007; Gifford et al., 2008; Green et al., 2010). Several ISG have been used to determine pregnancy status at ~18 d after AI, including interferon-stimulated gene 15 (*ISG15*; Johnson et al., 2000), myxovirus resistance 1 (*MX1*) and myxovirus resistance 2 (*MX2*; Gifford et al., 2007), 20,50-oligoadenylate synthetase (*OAS1*; Johnson et al., 2001), and chemosensory receptor transporter protein 4 (*RTP4*; Gifford et al., 2008).

Our third hypothesis was that Low P4 cows would have altered early embryonic development arising from ovulation of a larger follicle, resulting in more P4. In the present study, we assessed the effect of P4 before

TAI on the relative mRNA expression of ISG 18 d after TAI. Relative mRNA levels of *ISG15*, *Mx1*, *Mx2*, and *OAS1* were greater for Low P4 than for High P4 cows 18 d after TAI, but relative mRNA levels of *RTP4* were greater for High P4 than for Low P4 cows (Figure 4). In contrast to our hypothesis, the greater mRNA expression of 4 of the 5 ISG in Low P4 cows may be a proxy for increased embryo size or advanced stage of development. In agreement with this idea, anovular (i.e., low P4) cows had longer concepti 15 d after AI than cyclic (i.e., high P4) cows (Bisinotto et al., 2015). It is unclear why *RTP4* expression differed by treatment compared with the other 4 ISG. The *RTP4* gene is unique among the ISG assessed in the present experiment because it belongs to a larger family of genes, several members of which appear to be specifically expressed in olfactory neurons (Saito et al., 2004).

Although P4 is required for maintenance of pregnancy, results from manipulative and observational experiments are equivocal regarding the relationship between P4 concentrations after AI and fertility in dairy cows. Pregnant cows have more circulating P4 from 4 to 11 d after AI than nonpregnant cows (Larson et al., 1997; Mann and Lamming, 1999; Gümen et al., 2003). Because early embryos express P4 receptors, the P4 environment early after AI may affect embryo growth and development (Clemente et al., 2009). Several experiments using embryo transfer in beef cattle, however, support a direct role for circulating P4 in the first 7 d after ovulation, inducing changes in the uterine environment that advance conceptus elongation (Carter et al., 2008, 2010; Larson et al., 2011). Indeed, lactating Holstein cows in which P4 was experimentally decreased from 6 to 11 d after TAI had decreased mRNA expression of *ISG15* 20 d after TAI, less circulating pregnancy-specific protein B 25 to 67 d after TAI, and smaller embryos 46 d after TAI than cows with normal P4 (Carvalho et al., 2016). However, it is important to note that treatment effects on ISG mRNA expression in the present experiment appear to be a direct effect of treatment (i.e., P4 during growth of the preovulatory follicle) rather than an effect of P4 after TAI (Figure 3). It may be that the effects of P4 during growth of the preovulatory follicle on ISG mRNA expression superseded any potential effects of P4 after TAI on embryo development. Further experiments are needed to clarify the biology underlying these results.

PAG

Assays for detecting PAG levels in maternal circulation originating from mono- and binucleated cells of the embryonic trophoblast have been developed and

Figure 4. Effect (mean $\pm$ SEM) of treatment and expression of estrus before timed AI (TAI) on relative interferon-stimulated gene (*ISG15*, *MX1*, *MX2*, *OAS1*, and *RTP4*) mRNA expression 18 d after TAI for dairy cows diagnosed pregnant 39 d after TAI.

commercialized to determine pregnancy status in dairy cattle (Sasser et al., 1986; Green et al., 2000). Mean PAG concentrations in cattle increase from about 15 to 35 d in gestation; however, variation in plasma PAG levels among cows precludes PAG testing as a reliable indicator of pregnancy until about 26 to 30 d after AI (Zoli et al., 1992; Humblot, 2001). In the present study, we measured relative plasma PAG levels from 20 to 36 d after TAI. Treatment did not affect ($P = 0.58$) PAG levels; however, PAG levels were greater ($P < 0.01$) from 25 to 36 d after TAI for cows diagnosed pregnant versus not pregnant 39 d after TAI, and were greater ($P < 0.02$) from 25 to 36 d after TAI for pregnant primiparous versus pregnant multiparous cows (Figure 5). Variation in PAG concentrations among individual cows during early gestation (Ricci et al., 2015) may have precluded the detection of a treatment effect. The effect of parity and pregnancy status in the present experiment agree with results from an experiment in which stage of gestation, parity, pregnancy loss, and milk production were associated with plasma and milk PAG levels after TAI in Holstein dairy cows (Ricci et al., 2015).

Pregnancy Outcomes

Pregnancy outcomes were assessed based on PAG levels 29 d after TAI and transrectal ultrasonography 39 and 60 d after TAI (Table 4). Overall, the fertility of the Irish Holstein-Friesian cows used in the pres-

Figure 5. Effect (mean $\pm$ SEM) of pregnancy status (open vs. pregnant; upper panel) and parity (primiparous vs. multiparous; lower panel) on plasma pregnancy-associated glycoprotein (PAG) levels (relative to that of late-pregnant cows) after timed AI (TAI) for dairy cows diagnosed pregnant 39 d after TAI.

ent experiment was slightly greater than the mean of 57% reported for Holstein dairy heifers in the United States (Kuhn et al., 2006) and was high relative to US Holsteins submitted to a Double-Ovsynch protocol for first TAI that included a second PGF_{2 α} treatment 24

h after the first (Brusveen et al., 2009; Carvalho et al., 2015b; Wiltbank et al., 2015). Treatment did not affect P/AI 29 d after TAI based on a PAG test or at 39 and 60 d after TAI based on transrectal ultrasonography. Although this experiment was not statistically powered to detect treatment effects on fertility or pregnancy loss, overall fertility was high for both treatments and the number of cows diagnosed pregnant at 39 and 60 d after TAI in each treatment group was the same. It is noteworthy that no pregnancy losses were observed from 39 to 60 d after TAI in either treatment group of the present experiment. In a summary of 14 studies (Santos et al., 2004), pregnancy loss from 27 to 31 d and 38 to 50 d of gestation averaged 13 percentage points based on transrectal ultrasonography, and the losses we observed were less than the 6.1% pregnancy loss reported by Dirandeh et al. (2015a).

The lack of an effect of P4 during the growth of the preovulatory follicle on pregnancy outcomes is inconsistent with other studies (Fricke et al., 2003; Bisinotto et al., 2010; Carvalho et al., 2014), but a larger experiment statistically powered to assess P/AI would be required to replicate this outcome and make broader conclusions. One important aspect of our experimental design was the inclusion of a second PGF_{2 α} treatment 24 h after the first in the Breeding-Ovsynch portion of the Double-Ovsynch protocol. Cows initiating the first GnRH treatment of an Ovsynch protocol in a low P4 environment have a high ovulatory response due to the lack of circulating P4 (Gümen et al., 2003), resulting in an early CL at the PGF_{2 α} treatment of the Ovsynch protocol that fails to fully regress and leading to poor fertility to TAI (Giordano et al., 2012). Addition of a second PGF_{2 α} treatment 24 h after the first dramatically decreases the proportion of cows with incomplete luteolysis, thereby increasing P/AI after first TAI using a Double-Ovsynch protocol (Wiltbank et al., 2015) and increasing P/AI to Resynch TAI (Carvalho et al., 2015a; Santos et al., 2016). Thus, the association of low P4 at the onset of or during the Ovsynch protocol

Table 4. Effect of treatment on pregnancies per AI and pregnancy loss

Item	Treatment ¹		P-value
	Low P4	High P4	
Pregnancies per AI (%; no./no.)			
29 d after TAI	70 (19/27)	64 (18/28)	0.63
39 d after TAI	63 (17/27)	61 (17/28)	0.87
60 d after TAI	63 (17/27)	61 (17/28)	0.87
Pregnancy loss (%; no./no.)			
29 to 39 d after TAI	11 (2/19)	6 (1/18)	—
39 to 60 d after TAI	0 (0/17)	0 (0/17)	—

¹Cows were randomly assigned to 1 of 2 treatments (High P4 vs. Low P4) to manipulate progesterone (P4) before timed AI (TAI) during growth of the preovulatory follicle.

with poor fertility to TAI may be a result of incomplete luteal regression rather than an effect on the preovulatory follicle, and our results would support this idea.

In conclusion and in partial agreement with our hypothesis, Low P4 cows had larger ovulatory follicles and greater P4 after TAI than High P4 cows; however, this effect was manifested primarily for a subgroup of Low P4 cows that expressed estrus ~16 h before TAI. Cows that ovulated larger follicles had larger CL 15 d after TAI, which resulted in greater P4 concentrations after TAI. Treatment affected ISG mRNA expression 18 d after TAI, and this appeared to be a direct effect of treatment (i.e., high vs. low P4 before TAI) rather than an effect of P4 after TAI. In contrast to our hypothesis, we found no evidence for an effect of high vs. low P4 before TAI on early embryo development based on plasma PAG concentrations or on pregnancy outcomes based on transrectal ultrasonography. We speculate that Irish Holstein-Friesian cows managed in a grazing-based system with moderate milk production may not present the problems associated with hepatic P4 metabolism found in high-producing US Holsteins housed in confinement-based systems.

ACKNOWLEDGMENTS

We thank J. P. Murphy and the Moorepark farm staff for management and care of the cows enrolled in this study. The authors thank J. Kenneally (Teagasc Moorepark) and Matt McCabe (Teagasc Grange) for their technical assistance. This research was supported by the Irish Department of Agriculture, Food and Marine under competitive research grant RSF 13/S/528 and the USDA National Institute of Food and Agriculture (Washington, DC) Hatch project 231440.

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