Interpretive Summary: Estrous synchronization and ovarian function. Herlihy

Effective regulation of the corpus luteum, ovulatory follicle development, and periovulatory circulating steroid hormone concentrations is critical for optimizing responses to synchronization treatments. This study compared ovarian follicular dynamics, corpus luteum growth, and circulating steroid hormone concentrations in lactating dairy cows treated with treatments to synchronize estrus or ovulation. Progesterone supplementation during synchronization and ovulation induction with GnRH on the day before timed AI impacted ovulatory follicle size, and periovulatory circulating concentrations of progesterone and estradiol. No differences in postovulatory progesterone concentrations or luteal volume were observed.

Effects of synchronization treatments on ovarian follicular dynamics, corpus luteum growth, and circulating steroid hormone concentrations in lactating dairy cows

M.M. Herlihy,*† M.A. Crowe,† M.G. Diskin,‡ and S.T. Butler*[1]

*Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland

†School of Agriculture, Food Science & Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

‡Teagasc, Animal and Grassland Research and Innovation Centre, Mellows Campus, Athenry, Co. Galway, Ireland

[1]Corresponding author: Stephen Butler, Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland Tel: + 353 25 42222 Fax: + 353 25 42310.

E-mail: Stephen.Butler@teagasc.ie

ABSTRACT

Lactating dairy cows (n=57) ≥ 45 d postpartum at first service were enrolled in a randomized complete block design study to evaluate treatments to synchronize estrus and ovulation. At 10 days before AI, animals were randomly assigned to: 1) d -10 GnRH (GnRH1; 10 μg i.m. Buserelin) and CIDR (Controlled Internal Drug Release insert, 1.38 g progesterone (P4)); d -3 $PGF_{2\alpha}$ (PGF; 25 mg i.m. dinoprost); d -2 CIDR out and AI at observed estrus (CIDR_OBS); 2) same as CIDR_OBS, but GnRH (GnRH2) 36 h after CIDR out and timed AI (TAI) 18 h later (CIDR_TAI) or 3) same as CIDR_TAI, but no CIDR (Ovsynch). Transrectal ultrasound was used to assess follicle size before ovulation and on d 4, d 8, and d 15 after the presumptive day of estrus (d 0) to measure the corpus luteum (CL). Blood samples were collected to determine concentrations of estradiol (E2) (d -10, d -9, d -3, d -2, d -1, d 0) and P4 (d -10, d -9, d -2, d -1, d 0, d 1, d 4, d 6, d 8, d 11, d 15). No treatment differences were observed in either circulating concentrations of P4 or the ovulatory response to GnRH1 at the onset of synchronization treatments. Circulating concentrations of P4 were greater for CIDR_OBS and CIDR_TAI compared with Ovsynch at 24 h after CIDR insertion (5.34 and 4.98 vs. 1.75 ng/mL) and immediately before CIDR removal (1.65 and 1.48 vs. 0.40 ng/mL). Peak circulating concentrations of E2 were greater for CIDR_OBS compared with Ovsynch (3.85 vs. 2.39 pg/mL), but CIDR_TAI (2.82 pg/mL) did not differ from either CIDR_OBS or Ovsynch. The interval from PGF to peak circulating E2 did not differ between CIDR_TAI and Ovsynch (52.1 vs.

49.8 h). Both CIDR_TAI and Ovsynch, however, had shorter intervals from PGF to peak circulating E2 concentrations compared with CIDR_OBS (67.8 h). Diameter of the dominant follicle before ovulation was greater for CIDR_OBS compared with Ovsynch (18.5 vs. 16.0 mm) but CIDR_TAI (17.1 mm) did not differ from either of the other treatments. The mean interval from PGF to ovulation was longer for CIDR_OBS (100.0 h) compared with CIDR_TAI and Ovsynch (84.4 and 83.2 h, respectively). Use of CIDR_OBS resulted in increased preovulatory follicle size and greater circulating concentrations of E2 due to a longer period of preovulatory follicle growth. Progesterone supplementation during synchronization and GnRH on the day before TAI impacted ovulatory follicle size, and periovulatory circulating concentrations of P4 and E2. No differences, however, in postovulatory P4 or luteal volume profiles were observed.

Keywords: estrous synchronization, Ovsynch, progesterone, dairy cow

INTRODUCTION

Reproductive management of lactating dairy cows is frequently compromised due to poor expression of estrus. Timed artificial insemination **(TAI)** programs can be implemented, allowing TAI before an induced ovulation, without requirement for detection of estrus (Thatcher et al., 2010). Treatments to synchronize estrus and ovulation based on GnRH and $PGF_{2\alpha}$ **(PGF)** (with/without progesterone **(P4)**) maximize the proportion of cows inseminated following the planned start of mating. Satisfactory conception rates are achieved when implemented in commercial dairy herds in seasonal calving, pasture-based milk production systems (Herlihy et al., 2011).

The Ovsynch treatment synchronizes follicle maturation coincident with regression of the corpus luteum **(CL)** before GnRH-induced ovulation and TAI. This treatment includes an injection of GnRH 7 d before **(GnRH1)** and 56 h after **(GnRH2)** an injection of PGF (Brusveen et al., 2008). Ovulation occurs within an 8 h interval, 24 to 32 h after GnRH2 (Pursley et al., 1995), facilitating TAI 16 h after GnRH2 (Pursley et al., 1998). Progesterone is an important regulator of the frequency of pulsatile secretion of LH, and hence plays an important regulatory role in preovulatory follicle development. Lesser concentrations of P4 in the cycle preceding ovulation may increase the risk of inferior oocyte quality before ovulation and poor embryo quality after fertilization (Ahmad et al., 1995; Revah and Butler, 1996). An ovulatory response to GnRH1 eliminates the risk of a persistent dominant follicle at the time of GnRH2, and ensures the presence of a responsive CL at the time of PGF. Administration of GnRH at random stages of the estrous cycle results in ovulation of a dominant follicle in 54 to 90% of dairy cows (Pursley et al., 1995; Vasconcelos et al., 1999; Bello et al., 2006), with emergence of a new follicular wave on average 2.5 d later (range 2 to 4 d) (Pursley et al., 1995).

Estradiol-17β **(E2)** is the endogenous hormone that induces estrous behavior (Allrich, 1994). Estradiol acts on the hypothalamus to induce release of a surge of GnRH that triggers the preovulatory LH surge (Gazal et al., 1998). Treatment with a bolus injection of a GnRH analogue induces an endogenous LH surge that peaks 2 h after GnRH injection and induces ovulation in follicles that have acquired ovulatory capacity (Sartori et al., 2001) 22 to 31 h after the LH surge (Komar et al., 2001). A rapid decline in blood concentrations of E2 is observed beginning at the peak of the gonadotropin surge (Haughian et al., 2004), reaching a nadir ~ 8-10 h before ovulation (Komar et al., 2001). Synthesis and secretion of P4 by the CL is essential for the establishment and maintenance of pregnancy (Spencer et al., 2004). Systemic concentrations of P4 in the estrous cycle preceding ovulation and during the early luteal phase of the cycle after AI affect embryo survival (Morris and Diskin, 2008). Greater P4 concentrations during early embryo development increase embryo size (Clemente et al., 2009; Forde et al., 2011).

We have recently reported the effects of TAI treatments (with/without P4) and a conventional CIDR-based estrous synchronization treatment on herd reproductive performance in seasonal calving lactating dairy cows (Herlihy et al., 2011). The objective of this study was to compare the effects of identical treatments on ovarian follicle and CL development and circulating concentrations of steroid hormones in lactating dairy cows. The present study was carried out to identify the underlying mechanisms responsible for the previously documented differences in fertility following use of treatments to synchronize estrus and ovulation in lactating dairy cows.

MATERIALS AND METHODS

Animals

This study was conducted at the Animal and Grassland Research and Innovation Centre at Teagasc, Moorepark, County Cork, Ireland from November 2008 to January 2009. Lactating dairy cows (n=64) were managed as a single herd, housed indoors in a freestall barn, and allocated 20 kg DM/cow per d in a TMR consisting of 7.4 kg grass silage, 4.0 kg of maize silage, 0.6 kg of straw, 7.0 kg of concentrate, and 1.0 kg of molasses on a DM basis throughout the experimental period. All experimental procedures involving animals were licensed in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the University College Dublin Animal Research Ethics Committee.

Experimental Design, Synchronization Treatments, and Artificial Insemination

Cows were enrolled in a completely randomized block experimental design, and the experiment was completed in 3 replicates. All cows were ≥ 35 DIM (mean = 58; range 35 to 82 DIM) at the initiation of synchrony treatments, resulting in synchronized estrus/ovulation at ≥ 45 DIM (mean = 68; range = 45 to 92 DIM). Within each replicate, cows were blocked on the basis of parity, cumulative milk yield from wk 2 to 5 of lactation, and body condition score 1 wk before treatment initiation and randomly assigned to one of the 3 treatments illustrated in Figure 1. The **CIDR_OBS** treatment was an estrous synchronization treatment, whereas **CIDR_TAI** and **Ovsynch** were ovulation synchronization treatments. All treatments were initiated at a random stage of the estrous cycle. The i.m. GnRH agonist injections contained 10 µg buserelin (Receptal; Intervet Ireland, Dublin, Ireland). The Controlled Internal Drug Release **(CIDR)** device used contained 1.38 g of progesterone **(P4**; Pfizer Ireland, Dublin, Ireland). The i.m. $PGF_{2\alpha}$

contained 25 mg dinoprost tromethamine (Lutalyse; Pfizer Ireland, Dublin, Ireland). Cows assigned to CIDR_OBS were inseminated using the a.m./p.m. rule (Nebel et al., 1994) following detection of estrus with the aid of tail paint. Detection of estrus was performed four times per 24 h period for 20 min (i.e., 6 h intervals). All cows on the CIDR_TAI and Ovsynch treatments received TAI 18 h after GnRH2, which was administered 60 h after PGF. All cows were moved to a clean stand-off woodchip pad 3 d before the presumptive day of estrus until ovulation was confirmed, in order to enhance expression of estrous behavior. All inseminations were performed by a single experienced technician.

Insert Figure 1 here

Transrectal Ultrasonography

Ovarian structures were examined by linear array ultrasonography using a 7.5-MHz transrectal transducer (Aloka SSD-900; Aloka Ltd., Tokyo, Japan). The reproductive tracts of all cows were examined immediately before initiation of synchronization treatments and cows were assigned an ultrasound reproductive tract score describing the volume and echogenicity of fluid contained within the uterus (Mee et al., 2009). Cows that were classified as endometritic were not included in the study. Ultrasound examinations were carried out on all cows according to the following schedule: at treatment initiation to determine follicle and CL diameter; 4 d after treatment initiation to determine the ovulatory response to GnRH1; 2 d before the presumptive day of estrus to determine dominant follicle diameter; every 8 h commencing 80 h after PGF (22 h after GnRH2 for CIDR_TAI and Ovsynch) until 128 h after PGF, and again at 152 h after PGF, until ovulation was confirmed. Ultrasound examinations ceased in animals that failed to ovulate when a new follicular wave had emerged. Ovulation was assumed to have occurred mid-

way between two 8 h observation periods, or in the case of later ovulating animals, between the two 24 h observation periods. For cows with follicles that ovulated before the first scan on the presumptive day of estrus (80 h after PGF) it was assumed that ovulation occurred at 76 h after PGF. Further ultrasound examinations were completed on d 4, d 8, and d 15 after the presumptive day of estrus to determine CL diameter post ovulation.

Determination of Luteal Tissue Volume

An image of the CL was frozen on screen at its maximal area, vertical and horizontal diameters were measured using the integral electronic calipers to determine the mean maximum cross-sectional diameter of the CL. Luteal volume (mm^3) was calculated as $V = 4/3 \times \pi \times r^3$, where the radius (r) was calculated as 0.5 x mean maximum cross-sectional CL diameter. For CL with a fluid filled cavity, volume of the cavity was calculated in an identical manner, and subtracted from the total volume of the CL.

Blood Sampling

Blood samples were collected on d -10, d -9, d -3, d -2 (x 2; 12 h intervals), d -1 (x 4 at 6 h intervals), d 0 (x 4 at 6 h intervals), and d 1 (x 1 for cows with follicles that had ovulated; x 3 at 6 h intervals for cows with follicles that had not ovulated by 18 h after animals were bred by TAI), d 4, d 6, d 8, d 11, and d 15 relative to the presumptive day of estrus (d 0). For sampling timepoints that coincided with timing of treatments, blood samples were collected immediately before administration of treatments or CIDR insertion/removal. Blood was collected in lithium heparin vacutainer tubes (Becton Dickinson, Plymouth, United Kingdom) by puncture of coccygeal vessels, centrifuged at 2,000 × *g* for 15 minutes at 5 ^{o}C, the plasma was harvested and stored at -20 ^{o}C until later analysis. Concentrations of E2 were analyzed on d -10, d -9, d -3, d -2 (x 2 at 12 h

intervals), d -1 (x 4 at 6 h intervals), d 0 (x 4 at 6 h intervals), and d 1 (x 3 at 6 h intervals for cows with follicles that had not ovulated by 18 h after animals were bred by TAI). Concentrations of P4 were analyzed on d -10, d -9, d -2 (x 2; 12 h intervals), d -1, d 0, d 1, d 4, d 6, d 8, d 11, and d 15.

Hormonal Assays

Concentrations of E2 in plasma were determined by radioimmunoassay following extraction using E2 MAIA kits (BioStat Diagnostic Systems, UK) as previously described (Prendiville et al., 1995). The mean interassay and intra-assay coefficients of variation for high quality control samples were 1.84%, and 6.84% with a mean concentration of 3.95 pg/mL, respectively. The minimum detectable limit for this assay was 0.30 pg/mL.

Concentrations of P4 in plasma were determined using a commercially available solid-phase radioimmunoassay (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angles, CA). The mean interassay and intra-assay coefficients of variation for high quality control samples were 7.62% and 6.70% with a mean concentration of 6.85 ng/mL, respectively. The minimum detectable limit for this assay was 0.12 ng/mL.

Milk Yield, Composition, Bodyweight, and Body Condition Score

Cows were milked twice daily at 0700 and 1600 h. Individual milk yields (kg) were recorded automatically at each milking (Dairymaster, Causeway, Co. Kerry, Ireland). Milk fat, protein, and lactose concentrations were calculated weekly from one successive evening and morning milk sample for each animal. Near-infrared reflectance spectroscopy (Milkoscan 203; Foss Electric, Hillerød, Denmark) was used to determine the concentrations of constituents in the milk. All cows were weighed weekly. Body condition

score was recorded weekly, by one experienced independent observer, on a scale from 1 (emaciated) to 5 scale (extremely obese) with increments of 0.25 (Edmonson et al., 1989).

Data Handling

When an animal had more than one follicle ≥ 10 mm present on the ovary at onset of synchronization treatments, only the diameter of the follicle (≥ 10 mm) that ovulated was included in the analysis. When an animal had more than one follicle ≥ 10 mm present on the ovary at onset of synchronization treatments and failed to ovulate to GnRH1 of synchronization treatments, the diameter of the largest follicle (≥ 10 mm) was included in the analysis. When an animal had more than one CL present on the ovary at onset of synchronization treatments, the diameter of the largest CL was included in the analysis of CL diameter. When an animal had more than one ovulation at synchronized estrous, the combined volume of all CL were included in the analysis of postovulatory CL volume.

Animals Removed From Data Analysis

Initially, 64 animals were enrolled in the study. Animals (n=3) treated with CIDR_OBS that failed to ovulate on the presumptive day of estrus or within 3 d after the presumptive day of estrus were subsequently removed from the data analysis. Animals treated with CIDR_TAI (n=2) and Ovsynch (n=2) that failed to undergo complete luteal regression (P4 ≥ 1.0 ng/mL 48 h after PGF) in response to PGF on d 7 of the synchronization treatment were subsequently removed from the analysis. After these exclusions, the final dataset included 57 cows (26 multiparous and 31 primiparous). The numbers of animals reported per treatment were as follows: CIDR_OBS (n=19), CIDR_TAI (n=18), and Ovsynch (n=20).

Statistical Analyses

All statistical analyses were completed using SAS (SAS Inst. Inc., Cary, NC, 2006). Data were checked for normality and homogeneity of variance using histograms, qqplots, and formal statistical tests in the UNIVARIATE and GLM procedures of SAS. Variables with a non-normal distribution were log transformed before analysis. Continuous variables including dominant follicle diameter, CL diameter, luteal volume, interval to ovulation following PGF, and individual timepoints for circulating concentrations of P4 and E2 were analyzed using mixed models, with treatment (n=3), parity (1, >1), replicate (n=3) and calving date as fixed effects, and block as a random effect. Biologically plausible interactions were tested for significance in the model for each dependent variable. Fixed effects ($P > 0.05$) and interactions ($P > 0.10$) not associated with the dependent variables were removed by backward elimination with the exception of treatment and parity, which were forced into each model. Differences between treatments were declared significant when $P \leq 0.05$, and a tendency towards significance was assumed when $0.05 < P \leq 0.10$. Differences between least squares means were compared using the Tukey option to adjust for multiple comparisons. Preplanned contrasts were used to compare treatments to synchronize estrus with treatments to synchronize ovulation for specific individual timepoints of interest. All results are reported as least squares means and SEM for untransformed data and back transformed least squares means and 95% confidence intervals for log transformed data.

Linear interpolation was performed using the TRANSREG procedure of SAS and was used to calculate values for every day of the study for postovulatory P4 concentrations and postovulatory luteal volume. Animals (n=4) with missing values at any time point for postovulatory luteal volume were removed. Circulating postovulatory P4 and preovulatory E2 concentrations and luteal volume profiles for each treatment were

analyzed using mixed models with repeated measures. Treatment (n=3), time, parity (1, >1), replicate (n=3), and calving date were included as fixed effects and block as a random effect. All models with repeated measures included the effect of time and the interaction between treatment and time. The Satterthwaite adjustment was used to calculate denominator degrees of freedom and a first order autoregressive covariance structure (AR1) was used. The appropriate covariance structure for each repeated measures analysis was identified based on Akaike's Information Criterion (AIC) model fit statistic. Binomial response data including presence or absence of a CL at GnRH1, ovulation rate to GnRH1, and cumulative proportion of cows with follicles ovulating at different intervals following PGF were compared using Fisher's Exact Test.

Multiple linear regression (PROC REG) and the stepwise variable selection procedure was used to generate a model containing independent variables that were most effective at predicting peak circulating concentrations of E2 and preovulatory follicle diameter. The independent variables tested for peak circulating concentrations of E2 related to treatment, parity, DIM, milk production, bodyweight, body condition score, ovarian status at treatment initiation, preovulatory dominant follicle diameter, preovulatory concentrations of P4, and timing of ovulation. The independent variables tested for preovulatory dominant follicle diameter were identical to those tested for peak circulating concentrations of E2 with the exception that the dominant follicle independent variables were removed, and independent variables relating to preovulatory concentrations of E2 were included. The significance level for entry and the significance level to stay in the model were both set at $P \leq 0.05$. Non-linear associations between continuous variables and both peak circulating concentrations of E2 and preovulatory follicle diameter were also tested. Multicollinearity between predictor variables in the final model was examined using the variance inflation factor test.

RESULTS

Ovarian Status at Treatment Initiation

The details of the ovarian structures present and the ovulatory response to GnRH1 is summarized in Table 1. A difference ($P = 0.03$) between treatments in dominant follicle diameter at GnRH1 was observed. Dominant follicle diameter was larger ($P = 0.04$) for CIDR_OBS compared with Ovsynch, and tended ($P = 0.06$) to be larger for CIDR_OBS compared with CIDR_TAI. However, no differences ($P = 0.3$) were observed between synchronization treatments in the ovulatory response to GnRH1. Across all cows on the study, 72% of animals ovulated in response to GnRH1. No difference ($P = 0.3$) was observed between treatments in the proportion of cows with a CL present or in CL diameter ($P = 0.3$) at GnRH1. The diameter of the CL at treatment initiation was lesser ($P = 0.009$) in animals treated with CIDR_TAI that ovulated in response to GnRH1.

Preovulatory Circulating Progesterone Concentrations

No differences ($P = 0.2$) between treatments in mean circulating P4 concentrations (95% confidence interval) were observed at the onset of synchronization treatments (2.96 (1.21,7.21), 0.94 (0.38,2.36), and 1.52 (0.64,3.64) ng/mL, CIDR_OBS, CIDR_TAI, and Ovsynch, respectively). By 24 h after CIDR insertion, circulating P4 concentrations were greater ($P = 0.007$) for CIDR-based treatments compared with Ovsynch (5.34 (2.85,10.00) and 4.98 (2.62,9.48) vs. 1.75 (0.93,3.28) ng/mL, CIDR_OBS and CIDR_TAI vs. Ovsynch, respectively). Similarly, by 24 h after PGF (immediately before CIDR removal) circulating concentrations of P4 were greater ($P < 0.001$) for CIDR-based treatments compared with Ovsynch (1.65 (1.25,2.18) and 1.48 (1.11,1.97) vs. 0.40 (0.30,0.53) ng/mL, CIDR_OBS and CIDR_TAI vs. Ovsynch, respectively). By 12 h after CIDR removal, no differences ($P = 0.8$) between treatments in circulating concentrations of P4 were observed

(0.16 (0.10,0.26), 0.13 (0.08,0.21), and 0.13 (0.08,0.21) ng/mL, CIDR_OBS, CIDR_TAI, and Ovsynch, respectively). Circulating concentrations of P4 were not different (P = 0.2) between treatments on the day of presumptive estrus (0.06 (0.04,0.10), 0.03 (0.02,0.05), and 0.04 (0.03,0.07) ng/mL in CIDR_OBS, CIDR_TAI, and Ovsynch, respectively).

Dominant Follicle Diameter

The effect of synchronization treatment on dominant follicle measurements is summarized in Table 2. Synchronization treatment had no effect (P = 0.9) on dominant follicle diameter 24 h after PGF. Dominant follicle diameter at 80 h after PGF (22 h after GnRH2 for CIDR_TAI and Ovsynch) was greater (P = 0.05) for CIDR_TAI compared with Ovsynch, but CIDR_OBS was not different from CIDR_TAI (P = 0.6) or Ovsynch (P = 0.2). Peak preovulatory follicle diameter was greater (P = 0.02) for CIDR_OBS compared with Ovsynch, but neither treatment differed from CIDR_TAI.

Preovulatory Circulating Estradiol Concentrations

The effect of synchronization treatment on mean circulating E2 concentrations is summarized in Table 3 and Figure 2. Circulating E2 concentrations were greater ($P < 0.05$) in CIDR_OBS at all time points from 36 h after PGF compared with Ovsynch, and was greater ($P < 0.001$) at 72 h after PGF compared with the CIDR_TAI treatment. Peak circulating concentrations of E2 were greater (P = 0.004) for CIDR_OBS compared with Ovsynch, and tended (P = 0.10) to be greater compared with CIDR_TAI. There was no difference (P = 0.9) in the interval from PGF to peak circulating E2 between CIDR_TAI and Ovsynch. Both CIDR_TAI (P = 0.008) and Ovsynch (P = 0.002) had shorter intervals from PGF to peak circulating E2 concentrations compared with CIDR_OBS.

Timing of Ovulation

All animals treated with CIDR_TAI and Ovsynch successfully ovulated in response to GnRH2. Three animals treated with CIDR_OBS failed to ovulate on the presumptive day of estrus or within 3 d after the presumptive day of estrus (not included in the analysis). The effect of synchronization treatment on the cumulative proportion of cows with follicles ovulating at different intervals following PGF is summarized in Figure 4. No difference (P = 0.12) was observed in the proportion of cows with follicles ovulating by 76 h after PGF. By 84 to 92 h after PGF, all animals treated with CIDR_TAI and Ovsynch had ovulated. In contrast, at 92 h after PGF, 0.53 of animals treated with CIDR_OBS had ovulated. By 116 h after PGF, treatment differences were no longer significant (P = 0.6). By 140 h after PGF, all animals treated with CIDR_OBS that would ultimately ovulate the dominant follicle had done so. Synchronization treatment had an effect (P < 0.001) on the interval from PGF to ovulation. The mean interval from PGF to ovulation was longer for CIDR_OBS (100.0 h) compared with CIDR_TAI and Ovsynch (84.4 and 83.2 h, respectively; both P < 0.001).

Postovulatory Circulating Progesterone and Luteal Volume

The effect of synchronization treatment on postovulatory P4 concentration profiles and luteal volume profiles is summarized in Figure 3. No differences between treatments in mean circulating P4 (P = 0.4) from the day of ovulation until d 14 after ovulation or in mean luteal volume (P = 0.5) from d 4 after ovulation until d 15 after ovulation were detected. No treatment by time interactions were observed for postovulatory P4 (P = 0.4) or luteal volume (P = 0.2).

Multiple Linear Regression Analysis

Peak Circulating Concentrations of E2. The multiple linear regression model containing independent variables selected for predicting peak circulating concentrations of E2 is summarized in Table 4. Dominant follicle diameter pre-ovulation, the non-linear (quadratic) term for dominant follicle diameter pre-ovulation, and DIM at the start of treatment were associated with peak circulating concentrations of E2 (model $R^2 = 0.54$).

Preovulatory Follicle Diameter. The multiple linear regression model containing independent variables selected for predicting preovulatory follicle diameter is summarized in Table 4. Peak circulating concentrations of E2, cumulative milk yield from week two to five of lactation, and the non-linear (quadratic) term for cumulative milk yield from week two to five of lactation were associated with preovulatory follicle diameter (model $R^2 = 0.58$).

DISCUSSION

This study provides evidence for differences in periovulatory estradiol concentrations between treatments to synchronize estrus and ovulation. Periovulatory E2 concentrations were greater for CIDR_OBS compared with Ovsynch. Administration of GnRH2 caused an abrupt decline in circulating E2 concentrations, and resulted in a narrow range in time to ovulation for CIDR_TAI and Ovsynch. In contrast, more variability in the time of ovulation, greater preovulatory follicle size, and greater periovulatory circulating concentrations of E2 were observed for CIDR_OBS.

In the present study, synchronization treatments were initiated at random stages of the estrous cycle with no presynchronization. Differences between treatments existed in dominant follicle diameter at GnRH1 but this did not affect the ovulatory response. Vasconcelos et al. (1999) reported that cows initiating the Ovsynch treatment during early

to mid diestrus (d 5 to 12 of the estrous cycle) when they had an active CL and serum P4 concentrations were high, had smaller ovulatory follicles, improved ovulatory responses to GnRH1, and greater conception rates than cows initiating Ovsynch during metestrus, late diestrus, or proestrus. Ovulatory responses to GnRH1 in the present study were consistent with previous studies where estrous cycles were not presynchronized (Pursley et al., 1995; Vasconcelos et al., 1999; Bello et al., 2006). In the current study, the presence of a CL was used as a proxy for cycling status at onset of synchronization treatments. Stevenson et al. (2008) reported an overall accuracy of 84% with this technique for estimating prior luteal activity. The incidence of anovulation in the present study was less than the incidence of 23.3% reported by Bamber et al. (2009) for high producing lactating dairy cows treated with treatments to synchronize estrus or ovulation in the US. Anovulation has been associated with a decreased probability of conception and an increased incidence of embryo loss associated with Ovsynch treatments (Gumen et al., 2003). Thus, anovulation at the end of the voluntary waiting period amongst high producing lactating dairy cows in the US contributes to the impaired reproductive performance which is observed when cows are inseminated after TAI (Gumen et al., 2003).

Supplementation with P4 increased circulating P4 concentrations within 24 h in animals treated with CIDR_OBS and CIDR_TAI compared with animals treated with Ovsynch. Importantly, by 24 h after PGF (immediately before CIDR removal), circulating P4 concentrations were greater for CIDR_OBS and CIDR_TAI compared with Ovsynch. Thus, animals treated with CIDR_OBS and CIDR_TAI were exposed to greater circulating P4 concentrations for a longer time frame during the period of ovulatory follicle growth and development compared with animals treated with Ovsynch. Revah and Butler (1996) reported that lesser P4 concentrations in the cycle preceding ovulation may allow increased LH pulse frequency, thereby increasing the risk of excessive stimulation

of the follicle and premature activation of the oocyte, and thus increased risk of inferior oocyte quality before ovulation and poor embryo quality after fertilization (Ahmad et al., 1995).

In the present study, diameter of the dominant follicle pre-ovulation was greater for CIDR_OBS compared with Ovsynch. Bello et al. (2006) reported that an ovulatory follicle diameter at the time of GnRH2 of Ovsynch of approximately 16 mm was most likely to lead to a successful pregnancy. Other studies have reported that larger (Lopes et al., 2007) or smaller (Vasconcelos et al., 1999) preovulatory follicle diameter resulted in improved pregnancy outcomes following Ovsynch. Excessively small ovulatory follicle size was also associated with reduced fertility, reduced E2 concentrations before AI, and reduced P4 concentrations after AI (Vasconcelos et al., 2001). The superior reproductive performance observed by Herlihy et al. (2011) with CIDR_OBS may be partly explained by the findings of the present study which suggest that treatment of animals with CIDR_OBS resulted in increased preovulatory follicle diameter.

After P4 withdrawal, an increase in LH pulse frequency and mean LH concentrations promote follicle growth and increased estradiol production. Following CIDR removal, circulating E2 concentrations increased rapidly for CIDR_OBS and CIDR_TAI. Although the onset of proestrus (i.e., the interval from luteal regression to the LH surge) was delayed by 24 h for animals treated with CIDR_OBS and CIDR_TAI, proestrus concentrations of E2 were greater for CIDR_OBS compared with Ovsynch. At 60 h after PGF circulating concentrations of E2 remained greater for CIDR_OBS compared with Ovsynch. With TAI treatments, GnRH2 is given to precisely control the timing of ovulation. In the current study, GnRH2 caused a rapid reduction in circulating E2 concentrations in both TAI treatments. Consequently, Ovsynch had lesser peak preovulatory E2 and both CIDR_TAI and Ovsynch had peak preovulatory E2 earlier

compared with CIDR_OBS. Typically, during TAI treatments, GnRH2 induces an endogenous LH surge before circulating E2 concentrations have reached peak concentrations, as evidenced by a lack of estrous behavior in most cows treated with Ovsynch (Souza et al., 2007). Greater preovulatory E2 concentrations have been linked to improved likelihood of successful pregnancy establishment (Lopes et al., 2007). The greater conception rates reported by Herlihy et al. (2011) for CIDR based treatments may be at least partially attributable to higher periovulatory concentrations of E2. Hawk (1983) reported that reductions in periovulatory E2 might compromise spermatozoa transport in the female reproductive tract, and give rise to a suboptimal oviductal/uterine environment, and impaired oocyte fertilization.

In agreement with previous findings (Vasconcelos et al., 2001; Lopes et al., 2007), the present study demonstrated that peak circulating concentrations of E2 and preovulatory dominant follicle diameter were positively associated. Cumulative milk yield from wk 2 to 5 of lactation was negatively associated with preovulatory follicle diameter in the present study. This is in contrast to Lopez et al (2004), who reported that milk production was positively correlated with preovulatory follicle diameter. Those authors also reported that milk production was negatively correlated with circulating concentrations of E2. Lower circulating E2 was suggested as a reason for the greater preovulatory follicle size by delaying the time to estradiol induced LH surge and ovulation (Lopez et al., 2004). In the current study, DIM at the start of synchronization treatment was positively associated with peak circulating concentrations of E2. This reflects improved follicle steroidogenic capacity with increasing postpartum interval, likely due to increased LH pulse frequency associated with improved energy status (Canfield and Butler, 1990).

Treatments to synchronize ovulation allow the time of AI to be optimized, whereas increased variability in the timing of ovulation is observed when treatments to synchronize estrus are utilized. A decline in pregnancy rate occurs as the duration of preovulatory follicle dominance increases (Mihm et al., 1994; Austin et al., 1999). Prolonged dominance of the ovulatory follicle reduces embryo quality both in cows submitted to TAI treatments (Cerri et al., 2009) and cows inseminated following spontaneous estrus (Bleach et al., 2004). Saumande and Humblot (2005) reported that 80.6% of the variation in the interval between the onset of estrus and ovulation is explained by variation in the interval between the onset of estrus and the occurrence of the LH surge. Animals treated with CIDR_TAI and Ovsynch were administered GnRH, which resulted in a narrow range in time to ovulation whereby all animals had ovulated by 96 h following PGF. In contrast, more variability was observed in the interval from PGF to ovulation for animals treated with CIDR_OBS as these animals relied on an endogenous LH surge. Despite greater variation in the interval from PGF to ovulation observed with CIDR_OBS, the incidence of prolonged dominance of the ovulatory follicle was low (i.e., one animal with an interval from PGF to ovulation > 116 h). Furthermore, based on the fertility performance reported by Herlihy et al. (2011) using similar treatments, it is unlikely that prolonged dominance is prevalent in Irish dairy cattle treated with CIDR_OBS. Larger preovulatory follicle size and greater circulating concentrations of E2 were due to a longer period of preovulatory follicle growth, but there was no evidence that animals treated with CIDR_OBS developed persistent follicles.

The later occurrence of ovulation for CIDR_OBS compared with CIDR_TAI suggests that the timing of GnRH2 may have been suboptimal for animals treated with CIDR_TAI. Where GnRH2 was not administered (i.e., CIDR_OBS) the duration of proestrus was increased and greater periovulatory concentrations of E2 were observed.

Thus, we hypothesize that a modest increase in the interval between CIDR removal and GnRH2 may be beneficial for preovulatory follicle growth and E2 production.

In the present study, differences between treatments in ovulatory follicle size, and periovulatory circulating concentrations of P4 and E2 were observed. Despite this, no treatment differences in postovulatory P4 concentrations or luteal volume were observed. This is perhaps surprising, and was not consistent with Vasconcelos et al. (2001) who suggested that reducing the size of the ovulatory follicle had a negative impact on fertility due to development of smaller corpora lutea giving rise to lesser P4 concentrations. Importantly, size of the ovulatory follicle reported by Vasconcelos et al. (2001) was smaller than size of the ovulatory follicle reported in the present study. While the timing of GnRH2 for CIDR_TAI may have been suboptimal for final follicle development, the results suggest no adverse effects on subsequent CL development or steroidogenic capacity of the CL.

CONCLUSIONS

This study has demonstrated differences in periovulatory E2 concentrations between treatments to synchronize estrus and ovulation. Increased periovulatory E2 concentrations were observed for CIDR_OBS compared with Ovsynch. Administration of GnRH2 caused an abrupt decline in circulating E2 concentrations and resulted in a narrow range in time to ovulation for CIDR_TAI and Ovsynch. In contrast, a larger variation in the time of ovulation was observed for CIDR_OBS due to variation in time to estrus and the LH surge. While differences between treatments in ovulatory follicle size, and periovulatory P4 and E2 concentrations were observed, no differences in postovulatory P4 or luteal volume profiles were observed.

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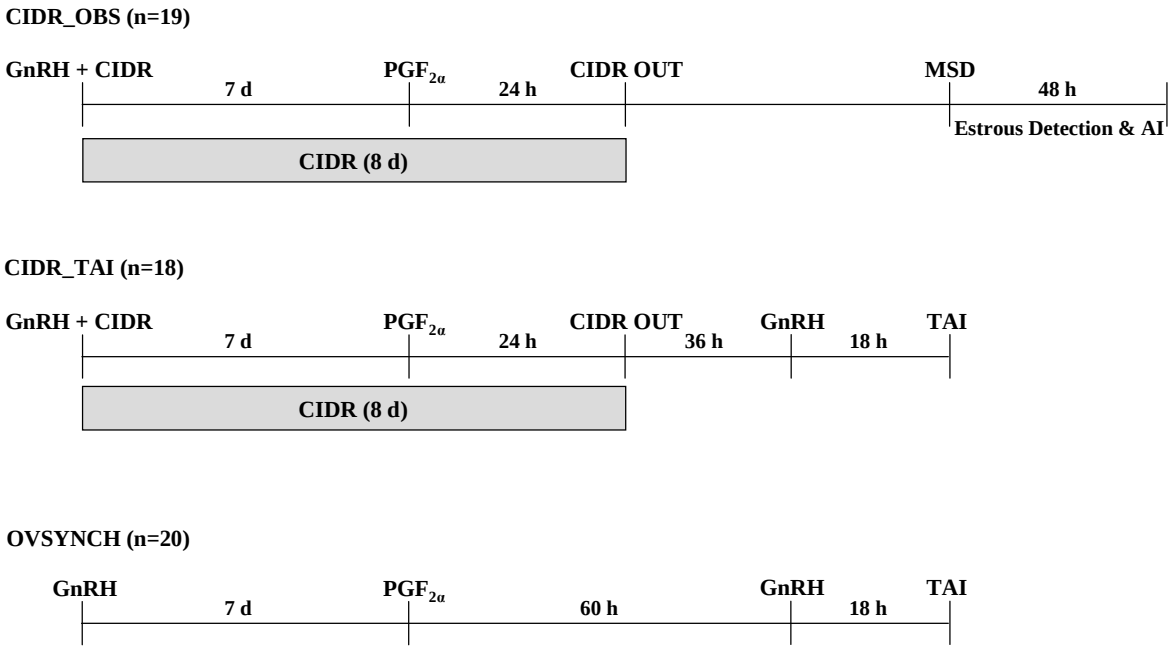

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669 **Figure 1.** Schematic diagram of treatments to synchronize estrus and ovulation**.** CIDR =
670 controlled internal drug release; OBS = observed estrus; TAI = timed AI. Insemination
671 commenced on a fixed date referred to as the mating start date (MSD). Synchronization
672 treatments were initiated at a random stage of the estrous cycle and applied to lactating
673 dairy cows before the first service. CIDR_OBS = 10 µg GnRH and CIDR insert at d 0, 25
674 mg $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule,
675 following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 µg GnRH and CIDR
676 insert at d 0, 25 mg $PGF_{2\alpha}$ at d 7, CIDR removed d 8, 10 µg GnRH 60 h after $PGF_{2\alpha}$ or 36
677 h after CIDR removal; animals received TAI 18 h after GnRH2. Ovsynch = 10 µg GnRH
678 at d 0, 25 mg $PGF_{2\alpha}$ at d 7, 10 µg GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after
679 GnRH2.

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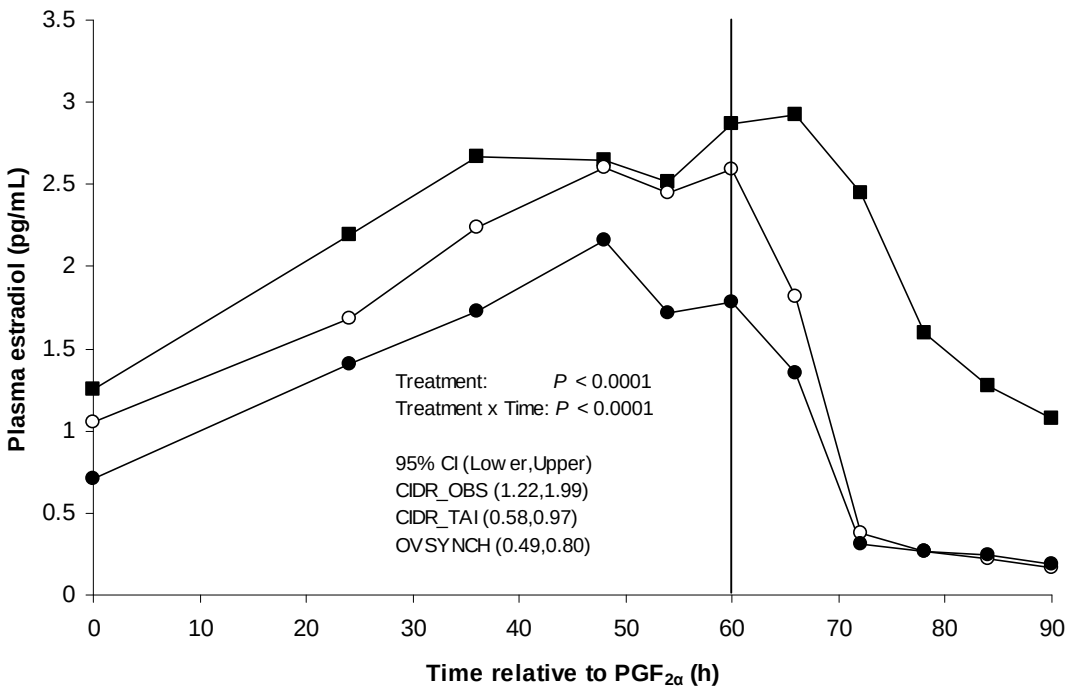

Figure 2. Effect of synchronization treatment on mean (95% confidence interval) estradiol concentrations (pg/mL) from the time of $PGF_{2\alpha}$ until 90 h following $PGF_{2\alpha}$ for CIDR_OBS (■), CIDR_TAI (○), and Ovsynch (●). The vertical line at 60 h represents the timing of GnRH2 for animals treated with CIDR_TAI and Ovsynch. Log transformed data was used to calculate *P*-values. Least squares means of the non-transformed data were used to generate Figure 2. CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the final GnRH injection.

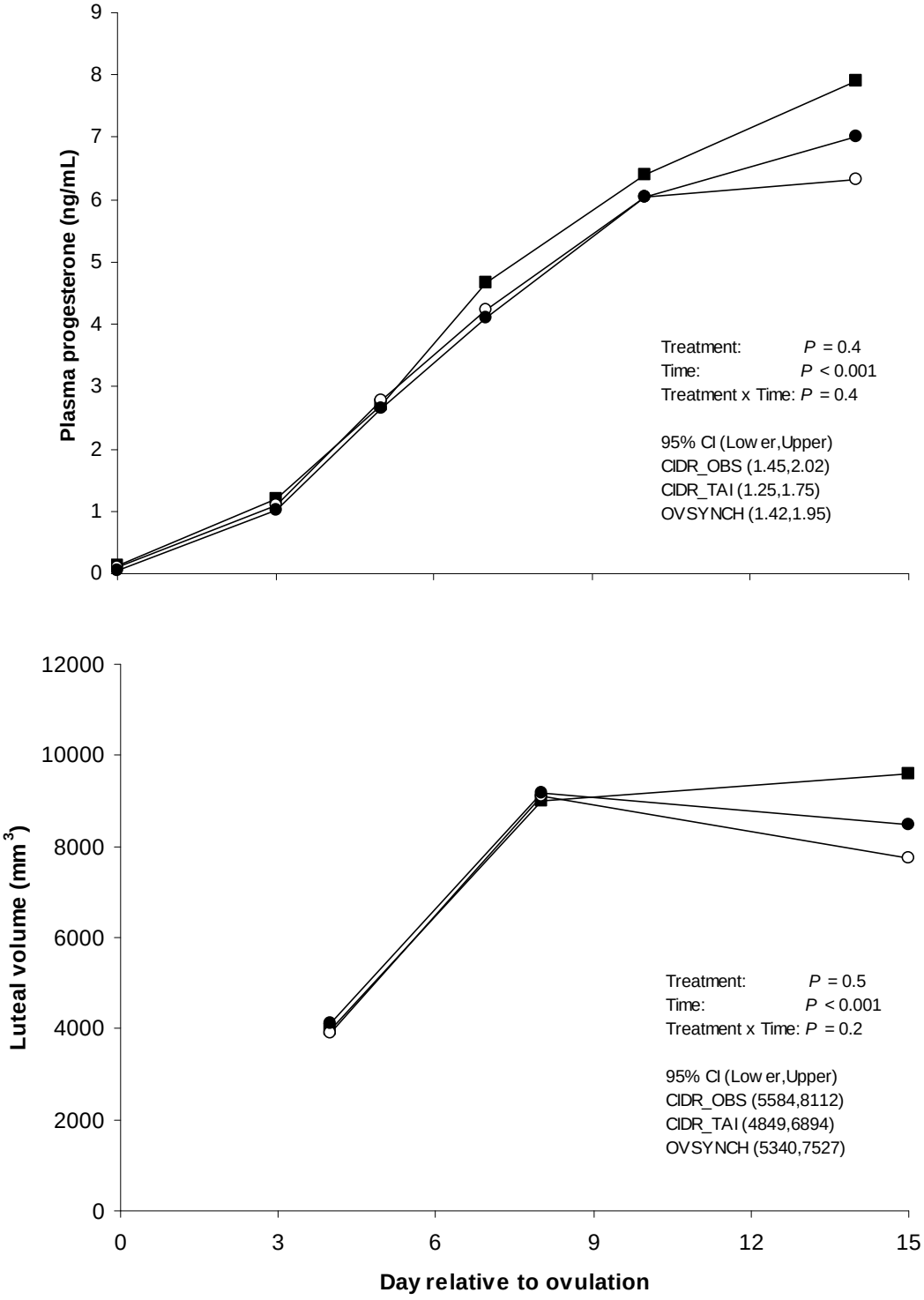

Figure 3. Effect of synchronization treatment on mean P4 concentrations (ng/mL) from the day of ovulation until d 14 after ovulation and mean luteal volume (mm^3) from d 4 after ovulation until d 15 after ovulation for CIDR_OBS (■), CIDR_TAI (○), and Ovsynch (●). Log transformed data was used to calculate *P*-values and least squares

means of the non-transformed data were used to generate Figure 3. CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the final GnRH injection.

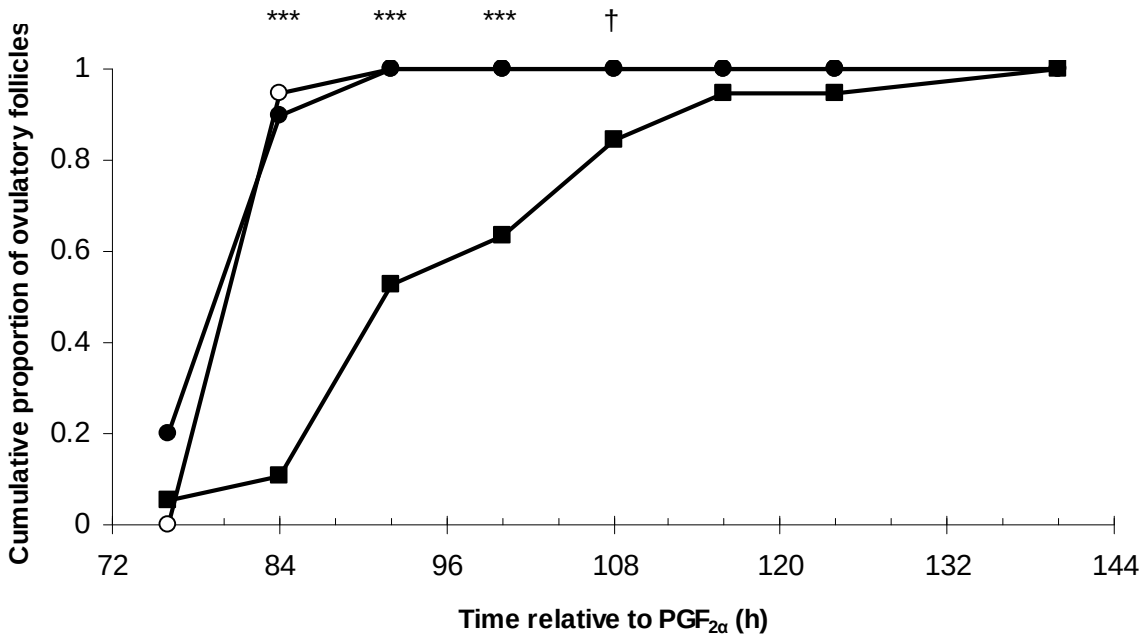

Figure 4. Effect of synchronization treatment on the cumulative proportion of cows with follicles ovulating at different intervals following $PGF_{2\alpha}$ for CIDR_OBS (■), CIDR_TAI (○), and Ovsynch (●). CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. *** indicates treatment effect at $P < 0.001$. † indicates treatment effect at $P = 0.06$. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the final GnRH injection.

Table 1. Ovarian characteristics at treatment initiation

	CIDR_OBS	CIDR_TAI	Ovsynch	SEM	*P*-value
Across all cows on the study (n=57)					
DF diameter at GnRH1 (mm)	14.7[a]	12.6[ab]	12.5[b]	0.63	0.03
% ovulating to GnRH1	63.2	66.7	85.0	-	0.3
% cows with a CL at GnRH1	89.5	72.2	90.0	-	0.3
CL diameter at GnRH1 (mm)	22.3	19.5	22.4	1.29	0.3
Cows that ovulated to GnRH1 (n=41)					
DF diameter at GnRH1 (mm)	13.9	12.5	12.3	0.76	0.3
% cows with a CL at GnRH1	91.7	66.7	88.2	-	0.3
CL diameter at GnRH1 (mm)	24.0[a]	16.6[b]	23.4[a]	1.49	0.009
Cows that failed to ovulate to GnRH1 (n=16)					
DF diameter at GnRH1 (mm)	15.4	13.9	13.2	1.26	0.5
% cows with a CL at GnRH1	85.7	83.3	100.0	-	1.0
CL diameter at GnRH1 (mm)	20.2	22.1	20.8	2.65	0.8

Least squares means within a row with different superscripts differ ($P < 0.05$).

CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals

779 received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25
780 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the
781 final GnRH injection.
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802 **Table 2.** Effect of synchronization treatment on mean (pooled SEM) dominant follicle (DF) diameter during synchronization

	CIDR_OBS	CIDR_TAI	Ovsynch	SEM	*P*-value
Across all cows on the study (n=57)					
DF diameter 24 h after PGF (mm)	14.9	14.6	14.5	0.58	0.9
DF diameter 80 h after PGF (mm)[1]	16.7[ab]	17.7[a]	15.2[b]	0.66	0.05
DF diameter immediately pre-ovulation (mm)	18.5[a]	17.1[ab]	16.0[b]	0.62	0.02
Cows that ovulated to GnRH1 (n=41)					
DF diameter 24 h after PGF (mm)	14.1	14.2	14.6	0.65	0.8
DF diameter 80 h after PGF (mm)	16.9	16.9	15.9	0.68	0.5
DF diameter immediately pre-ovulation (mm)	18.0[a]	16.8[ab]	15.8[b]	0.65	0.04
Cows that failed to ovulate to GnRH1 (n=16)					
DF diameter 24 h after PGF (mm)	16.4	14.8	14.2	1.56	0.6
DF diameter 80 h after PGF (mm)	18.6	16.9	16.1	1.91	0.6
DF diameter immediately pre-ovulation (mm)	19.3	16.9	15.8	1.98	0.4

803 [1] 22 h after GnRH2 for CIDR_TAI and Ovsynch.

804 Least squares means within a row with different superscripts differ ($P < 0.05$).

805 CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of

806 the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of

807 $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12.

808 CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h

809 after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, 10 μg

810 of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the final GnRH injection.

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Table 3. Effect of synchronization treatment on mean (95% confidence interval) preovulatory concentrations of estradiol (pg/mL)

	CIDR_OBS	CIDR_TAI	Ovsynch	SEM	*P*-value
Time of PGF	0.72	0.60	0.49	-	0.6
	(0.43,1.22)	(0.35,1.03)	(0.29,0.81)		
24 h after PGF	1.84	1.42	1.26	-	0.17
	(1.38,2.45)	(1.06,1.91)	(0.95,1.67)		
36 h after PGF[1]	2.46[a]	1.87[ab]	1.48[b]	-	0.03
	(1.87,3.23)	(1.41,2.47)	(1.13,1.93)		
60 h after PGF[2]	2.47[a]	2.04[ab]	1.37[b]	-	0.03
	(1.78,3.42)	(1.45,2.87)	(1.00,1.89)		
72 h after PGF[3]	1.88[a]	0.40[b]	0.30[b]	-	< 0.001
	(1.48,2.39)	(0.30,0.52)	(0.24,0.38)		
Peak preovulatory E2	3.85[a]	2.82[ab]	2.39[b]	-	0.006
	(3.10,4.78)	(2.26,3.52)	(1.93,2.95)		
Time of peak E2 relative to PGF (h)	67.8[a]	52.1[b]	49.8[b]	3.52	0.001

[1] 12 h after CIDR removal for CIDR_OBS and CIDR_TAI.

[2] Time of GnRH2 for CIDR_TAI and Ovsynch.

[3] 12 h after GnRH2 for CIDR_TAI and Ovsynch.

Least squares means within a row with different superscripts differ ($P < 0.05$).

CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25

827 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the
828 final GnRH injection.
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852 **Table 4.** Multiple linear regression models for peak circulating concentrations of estradiol and preovulatory follicle diameter

	Peak Circulating Concentrations of Estradiol			Preovulatory Follicle Diameter		
Item	Parameter Estimate	SE	*P*-value	Parameter Estimate	SE	*P*-value
Intercept	-4.37	1.11	0.0003	17.91	5.45	0.002
Preovulatory dominant follicle diameter (mm)	0.47	0.12	0.0005			
(Preovulatory dominant follicle diameter)^{2†} (mm)	-0.01	0.003	0.004			
DIM at start of treatment (days)	0.009	0.003	0.01			
$^{\#}$Peak estradiol (pg/mL)				3.64	0.60	< 0.001
Cumulative milk yield wk 2 to 5 of lactation (kg)				-0.02	0.02	0.15
(Cumulative milk yield wk 2 to 5 of lactation)^{2‡} (kg)				0.00003	0.00001	0.05
Model R^2		0.54			0.58	

853 †Non-linear association between peak circulating concentrations of E2 and preovulatory follicle diameter.

854 ‡Non-linear association between preovulatory follicle diameter and cumulative milk yield wk 2 to 5 of lactation.

855 $^{\#}$Peak E2 = log transformed data.

CIDR = controlled internal drug release; OBS = observed estrus; TAI = timed AI. Synchronization treatments were initiated at a random stage of the estrous cycle and applied to lactating dairy cows before the first service. CIDR_OBS = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8; animals were inseminated by the a.m./p.m. rule, following detection of estrus on d 10, 11, and 12. CIDR_TAI = 10 μg of GnRH and CIDR insert at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, CIDR removed at d 8, 10 μg of GnRH 60 h after $PGF_{2\alpha}$ or 36 h after CIDR removal; animals received TAI 18 h after the final GnRH injection. Ovsynch = 10 μg of GnRH at d 0, 25 mg of $PGF_{2\alpha}$ at d 7, 10 μg of GnRH 60 h after $PGF_{2\alpha}$; animals received TAI 18 h after the final GnRH injection.