J. Dairy Sci. 98:3826–3839
http://dx.doi.org/10.3168/jds.2014-9040

Increasing estradiol benzoate, pretreatment with gonadotropin-releasing hormone, and impediments for successful estradiol-based fixed-time artificial insemination protocols in dairy cattle

P. L. J. Monteiro Jr.,* M. Borsato,* F. L. M. Silva,* A. B. Prata,* M. C. Wiltbank,† and R. Sartori*[1]
*Department of Animal Science, University of São Paulo, Piracicaba, SP 13418-900, Brazil
†Department of Dairy Science, University of Wisconsin-Madison, Madison 53706

ABSTRACT

With the objective to optimize fixed-time artificial insemination (FTAI) protocols based on estradiol benzoate (EB) and progesterone (P4), we performed 2 experiments (Exp.) in dairy cows. In Exp. 1 (n = 44), we hypothesized that increased EB (EB3 = 3 mg vs. EB2 = 2 mg) on d 0 would improve synchronization of ovarian follicle wave emergence. Likewise, in Exp. 2 (n = 82), we hypothesized that a GnRH treatment on d −3 (early in a follicular wave on d 0) versus d −7 (presence of a dominant follicle on d 0) would better synchronize wave emergence. Moreover, results from both experiments were combined to identify reasons for the lack of synchronization. All cows were treated with EB at the time of introduction of a P4 implant (d 0). On d 7, cows were given 25 mg of prostaglandin $F_{2\alpha}$; on d 8, the implant was removed and cows were given 1 mg of estradiol cypionate. All cows received FTAI on d 10. In both experiments, daily ultrasound evaluations were performed and, in Exp. 2, circulating P4 was evaluated during the protocol. Pregnancy per artificial insemination (P/AI) was determined on d 31 and 59 after FTAI. In Exp. 1, EB dose did not change time to wave emergence, but EB3 compared with EB2 decreased the percentage of cows with a corpus luteum on d 7 (19.8 vs. 55.3%) and time to ovulation (10.4 vs. 10.9 d). In Exp. 2, although we detected a tendency for delayed follicle wave emergence after the start of the FTAI protocol in cows ovulating to GnRH given on d −7, there was no difference in percentage of cows with a synchronized wave emergence (~80%). Regardless of treatment, more cows with P4 <0.1 ng/mL, compared with P4 ≥0.1 and <0.22 ng/mL at the time of AI, ovulated to the protocol (81.2 vs. 58.0%) and had increased P/AI (47.4 vs. 21.4%). An analysis of data from both experiments showed that only 73.8% (93/126) of cows had synchronized wave emergence, and only 77.8% (98/126) of cows ovulated at the end of the protocol. Fertility was much greater in cows that had emergence of a new wave synchronized and ovulated to end of the protocol [P/AI 61.3% (46/75)] compared with cows that failed to present one or both of the outcomes above [15.7% (8/51)]. Thus, although current FTAI protocols using EB and P4 produce P/AI between 30 and 40% for lactating dairy cows, there remains room for improvement because less than 60% (75/126) of the cows were correctly synchronized. Starting the FTAI protocol without the dominant follicle or increasing the dose of EB to 3 mg was not effective in increasing synchronization rate.
Key words: fixed-time AI, estradiol benzoate, progesterone, synchronization

INTRODUCTION

Reproductive efficiency in dairy herds around the world has declined for several decades (Lucy, 2001; Mee, 2012), although recent data on daughter pregnancy rate in the United States has indicated stabilization in genotypic values and a dramatic increase in phenotypic values for reproduction (Norman et al., 2009; Wiltbank and Pursley, 2014). The improvement in reproductive performance can be partially explained by the use of systematic reproductive management programs in the United States, including programs for synchronization of ovulation and fixed-time AI (**FTAI**). These programs allow cows to be inseminated at a designated time without the need for detection of estrus, and thus they increase the AI submission rate (Wiltbank and Pursley, 2014). Two types of hormonal programs have been implemented for synchronization of ovulation. Programs such as Ovsynch use GnRH and prostaglandin $F_{2\alpha}$ (**PGF**) to synchronize ovulation (Pursley et al., 1995, 1997), whereas programs in many parts of the world combine estradiol (**E2**), progesterone (**P4**), and

Received October 29, 2014.
Accepted February 23, 2015.
[1]Corresponding author: robertosartori@usp.br

PGF to synchronize ovulation (Bo et al., 1995b; Souza et al., 2009; Baruselli et al., 2012). Both types of FTAI programs attempt to synchronize follicular waves, corpus luteum (**CL**) function, and circulating reproductive hormones to allow ovulation of an optimal-sized follicle in an optimal hormonal environment at a designated time.

In GnRH-based programs, it is well known that many cows are not correctly synchronized by the program. For example, Giordano et al. (2012) reported that only about 50% of cows treated with Ovsynch were correctly synchronized, whereas Double-Ovsynch resulted in about 70% synchronization. To date, synchronization rates during E2-P4–based programs in lactating dairy cows have not been adequately evaluated. Early studies that established the physiological basis for these programs utilized beef heifers and found that treatment with various forms of E2 led to suppression of gonadotropins, regression of growing follicles, and emergence of a new follicular wave about 4 d after E2 treatment (Bo et al., 1993, 1994, 1995a). Souza et al. (2009) evaluated 45 lactating dairy cows using daily ultrasound evaluations and reported that 84.4% (38/45) had synchronized emergence of a new ovarian follicle wave after treatment with 2 mg of estradiol benzoate (**EB**), combined with insertion of a controlled internal drug release device containing P4. In addition, they found that 83.3% of cows had synchronized ovulation at the end of the protocol (Souza et al., 2009). Thus, similar to GnRH-based programs, protocols using E2 and P4 may have problems with correct synchronization of the new ovarian follicle wave at the beginning of the program and synchronized ovulation at the end of the program. Critical features of FTAI protocols that need to be optimized include follicular wave emergence and growth (Wiltbank et al., 2011), concentration of P4 during follicular growth (Bisinotto et al., 2013; Wiltbank et al., 2014), complete lysis of the CL at the time of AI (Souza et al., 2007; Pereira et al., 2013b; Wiltbank et al., 2014), and size of the ovulatory follicle (Wiltbank et al., 2011; Pereira et al., 2013a, 2014).

Programs that use E2 and P4 to synchronize ovulation have generally used 2 mg of EB at the start to synchronize the new ovarian follicular wave, although the early studies used 5 mg of various E2 derivatives, sometimes combined with P4 (Bo et al., 1993, 1994, 1995a). The studies of Souza et al. (2009) reported that higher-producing lactating dairy cows had earlier follicular wave emergence than did lower-producing dairy cows after treatment with 2 mg of EB. They postulated that the high E2 metabolism that has been found in lactating dairy cows (Sangsritavong et al., 2002; Wiltbank et al., 2006) might be responsible for a more rapid decrease in circulating E2 concentrations after EB treatment. At 24 h after treatment with 1.0, 2.0, or 4.0 mg of EB, nonlactating Holstein cows had plasma E2 concentrations of 26.0, 40.6, and 68.9 pg/mL, respectively, about half the concentration found in zebu beef cattle under the same conditions (Bastos et al., 2011). Thus, high-producing lactating dairy cows may require a greater dose of EB to achieve follicular wave regulation than is required in lower-producing dairy cows or beef cattle in which 2 mg is the standard treatment dose.

The GnRH-based FTAI programs have better fertility when initiated on certain days of the estrous cycle (Vasconcelos et al., 1999; Moreira et al., 2000), such as d 6 or 7 when there is a high ovulation rate in response to the first GnRH treatment and high circulating P4 during the protocol. Presynchronization programs have been developed to increase the percentage of cows at these optimal stages of the estrous cycle such as Presynch-12-Ovsynch (Moreira et al., 2001; Portaluppi and Stevenson, 2005), Double-Ovsynch (Souza et al., 2008; Herlihy et al., 2012), or G-6-G (PGF–2 d–GnRH–6 d–Ovsynch; Bello et al., 2006; Wiltbank and Pursley, 2014). Optimal times of the estrous cycle to initiate E2-P4 protocols or effects of presynchronization strategies on the efficacy of E2-P4 protocols have not yet been reported. It is possible that, in contrast with GnRH-based FTAI programs (Vasconcelos et al., 1999), the best time for initiation of the E2/P4-based FTAI protocols is in the absence of a dominant follicle.

Thus, these studies were based on 3 hypotheses related to E2-P4 protocols. First, we hypothesized that an increased dose of EB would increase synchronization of a new ovarian follicle wave emergence and thereby increase synchronization during the protocol. Second, we hypothesized that ovulation to a GnRH treatment on d −3 would synchronize the stage of the follicular wave to prevent presence of a dominant follicle at the start of the E2-P4 protocol and result in greater synchronization rate to the E2-P4 protocol than cows ovulating to GnRH on d −7 or not ovulating to GnRH. Finally, we hypothesized that complete characterization of the physiological response to E2-P4 protocols would demonstrate that the synchronization rate following the protocol was not optimal and was due to specific physiological responses to the protocol. Two experiments were performed with the following objectives. The first experiment evaluated whether 3.0 mg of EB produced better synchronization of a new follicle wave emergence than 2.0 mg. The second experiment evaluated whether ovulation to GnRH 3 d before starting the protocol would improve the overall synchronization of the protocol. Finally, the results from both experiments were combined to identify reasons for lack of synchronization to the protocol.

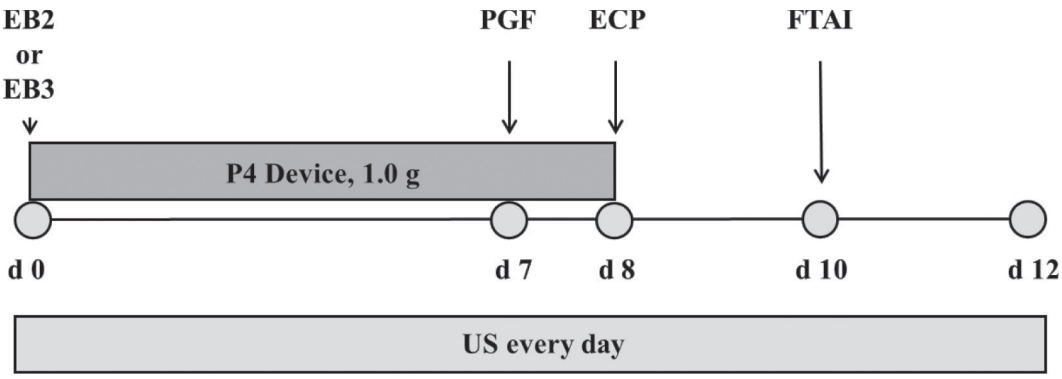

Figure 1. Diagram of activities for experiment 1. Study d 0 is the day the protocol began with insertion of intravaginal progesterone (P4) implant and estradiol benzoate (EB) treatment with either 2 mg of EB (EB2; n = 23) or 3 of mg EB (EB3; n = 21). On d 7, 33.5 mg of dinoprost tromethamine (PGF) was administered and on d 8, the P4 implant was removed and 1.0 mg of estradiol cypionate (ECP) was administered. On d 10, cows received fixed-time AI (FTAI). Transrectal ultrasonography (US) was performed daily from d 0 to ovulation (or d 12).

MATERIALS AND METHODS

The Animal Research Ethics Committee of Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ)/ University of São Paulo (São Paulo, Brazil) approved all procedures involving cows in this study.

Experiment 1

Cows, Housing, and Diets. This experiment was conducted in the dairy farm of ESALQ (São Paulo State, Brazil) and used 20 primiparous and 24 multiparous lactating Holstein cows. At the beginning of the study, cows averaged (±SEM) 23.8 ± 1.5 (range: 9.5 to 41.5) kg of milk/d, 350.8 ± 36.0 (range: 63 to 791) DIM, with BCS of 2.8 ± 0.1 [range: 2.25 to 3.25, using a 1 (emaciated) to 5 (obese) scale; Elanco Animal Health, 2009] and 3.9 ± 0.1 yr of age (range: 3.0 to 4.8). During the experimental period, cows were housed in a tiestall barn equipped with sprinklers and fans. Cows were milked 2 times daily, and milk yield for each cow was recorded. Feed was provided 2 times daily, concurrent with milking. Cows were fed ad libitum a TMR-based diet of corn silage, with a corn-soybean meal-based concentrate, and minerals and vitamins, and was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Protocols and Treatments. The cows were randomized to receive either 2.0 mg (**EB2**; n = 23) or 3.0 mg (**EB3**; n = 21) of EB (Sincrodiol, Ourofino, Ribeirão Preto, SP, Brazil) at the start of a FTAI protocol (Figure 1) that included insertion of an intravaginal implant containing 1.0 g of P4 (Sincrogest, Ourofino) at the same time as the EB treatment (d 0), PGF treatment on d 7 (33.5 mg of dinoprost tromethamine; Lutalyse, Zoetis, São Paulo, SP, Brazil), and removal of the P4 implant on d 8 together with treatment with 1.0 mg of estradiol cypionate (Zoetis). Two days after estradiol cypionate treatment (d 10), all cows were inseminated by the same technician with commercial semen from bulls with proven fertility (Figure 1).

Ultrasonography Evaluation. During the protocol, ovaries of all cows were examined by transrectal ultrasonography (DP-2200, Mindray, Shenzhen, China), using a 7.5-MHz linear-array transducer, every 24 h from d 0 until ovulation at the end of the protocol (or d 12) and on d 17. All CL and follicles ≥4 mm were measured and mapped. These daily ovarian maps were used to determine day of emergence of follicle wave (Ginther et al., 1997), day of follicle deviation (Ginther et al., 1997), size of follicle at time of deviation of the future ovulatory follicle, day of ovulation (day when the ovulatory follicle disappeared from the ovary), number of ovulations, size of ovulatory follicle, and presence and size of CL on each day (from d 0 until ovulation or d 12). On d 17, ovulation was confirmed by detection of a CL using ultrasonography. In this paper, follicle data are shown as average cross-sectional diameter, and CL data are shown as volume. For this, ultrasound measurements of follicle and CL were used to calculate average diameter, average of length (L) and width (W), and volume (V). Volume was calculated with the formula $V = 4/3 \times \pi \times R^3$, with radius (R) calculated using the formula $V = (L/2 + W/2)/2$. For a CL with a fluid-filled cavity, the volume of the cavity was calculated and subtracted from the total volume of the CL (Sartori et al., 2004). Pregnancy diagnoses were performed at 31 and 59 d after FTAI using ultrasonography.

Experiment 2

Cows, Housing, and Diets. This experiment was conducted on a dairy farm in São Paulo State, Brazil,

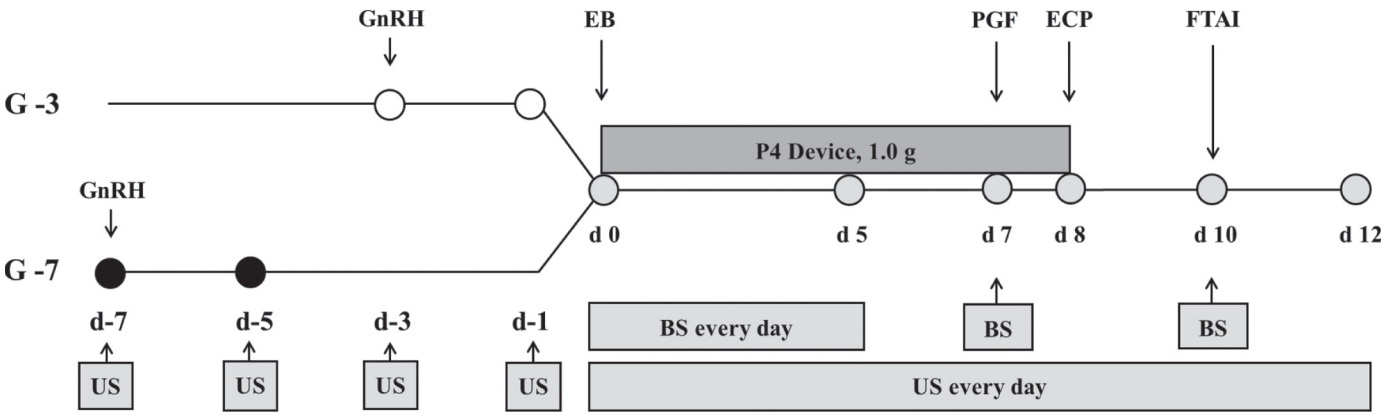

Figure 2. Diagram of activities for experiment 2. Prior to d 0 (initiation of protocol), cows received GnRH (10.5 mg of buserelin acetate) on either d −7 (G −7; n = 40) or d −3 (G −3; n = 42). All cows received the same synchronization protocol [d 0 = progesterone (P4) implant + 2 mg of estradiol benzoate (EB); d 7 = 33.5 mg of dinoprost tromethamine (PGF); d 8 = P4 implant removed + 1.0 mg of estradiol cypionate (ECP); d 10 = fixed-time AI (FTAI)]. Transrectal ultrasonography (US) was performed on d −7 and −5, for cows of G −7 group, and on d −3 and −1, for cows of G −3 group and subsequently on a daily basis in all cows from d 0 to ovulation (or d 12). Blood samples (BS) were collected daily from d 0 to 5 and on d 7 and d 10.

using 82 lactating dairy cows (Holstein, n = 70; Holstein × Jersey crossbred, n = 12), both primiparous (n = 37) and multiparous (n = 45). At the beginning of the study, cows averaged (±SEM) 33.8 ± 1.0 (range: 19.0 to 53.8) kg of milk/d, 161.3 ± 18.4 (range: 39 to 359) DIM, with BCS of 2.7 ± 0.1 (range: 2.25 to 3.50) and 4.7 ± 0.2 yr of age (range: 2.2 to 10.2). During the experimental period, cows were housed in a freestall barn equipped with sprinklers and fans. Cows were milked 3 times daily, and milk yield for each cow was recorded automatically. Feed was provided 3 times daily, concurrent with milking. Cows were fed ad libitum a TMR-based diet of corn silage, barley, and corn and soybean meal-based concentrate with minerals and vitamins, which was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Protocols and Treatments. The cows were subjected to the same FTAI protocol as used in experiment 1, except that all cows received 2 mg of EB on d 0 (Figure 2). Before the FTAI protocol, cows were randomly assigned to receive GnRH (10.5 μg of buserelin acetate; Sincroforte, Ourofino) either 7 d before the start of the FTAI protocol (n = 40) or 3 d before the start of the FTAI protocol (n = 42). According to the ovulatory response to the GnRH treatment, cows were assigned into 3 groups: cows that ovulated to the d −7 GnRH treatment (**G −7**), and which were expected to have a dominant follicle at the onset of the FTAI protocol; cows that ovulated to the d −3 GnRH treatment (**G −3**), and which were expected to have a new follicular wave but not yet have a dominant follicle at the onset of the FTAI protocol; and cows that did not ovulate following GnRH treatment (**NoOv**) and were not at a synchronized stage of follicle development on d 0.

Ultrasonography Evaluation. During the protocol, ovaries of all cows were examined by transrectal ultrasonography (DP-2200, Mindray), using a 7.5-MHz linear-array transducer, on d −7 to check for the presence of follicles ≥10 mm and on d −5 to confirm ovulation (G −7 cows); on d −3 to check for the presence of follicles ≥10 mm and on d −1 to confirm ovulation (G −3 cows); and in all cows daily from d 0 until ovulation after the end of the protocol or d 12, and on d 17, to confirm ovulation. All CL and follicles ≥4 mm were measured and mapped, and maps were used for the same determinations described for experiment 1. Pregnancy diagnoses were performed at 31 and 59 d after FTAI.

Cows were considered positive for emergence of a new wave when emergence occurred between d 1 and d 6. Cows that had the ovulatory follicular wave emerge before d 1 were classified as lacking emergence of a new wave and, if ovulation occurred from this follicular wave, were classified as ovulating a persistent follicle.

Blood Sampling and Analysis of Progesterone in Plasma. Blood was sampled from all 82 cows every 24 h from d 0 until 5, and on d 7 and 10, by puncture of the coccygeal vein or artery into evacuated tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, tubes with blood were placed on ice and kept refrigerated until transported to the laboratory within 4 to 5 h after collection. Blood tubes were centrifuged at 1,700 × *g* for 15 min at 4°C for plasma separation. Aliquots of plasma were frozen at −20°C until assayed. Concentra-

tions of P4 were analyzed by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the kit was 0.02 ng/mL. The intraassay coefficients of variation (CV) were 5.4% in assay 1 and 4.3% in assay 2. The interassay CV was 4.1%.

Analysis of Synchronization to the Protocol Using Cows from both Experiments

Cows in both studies were treated with a similar EB-P4–based FTAI protocol and had daily ultrasound evaluations during the protocol. Therefore, an analysis was performed to determine the reasons that cows failed to synchronize the emergence of a new ovarian follicle wave or to ovulate to the end of the protocol. In this analysis, data from all 126 cows from both experiments were evaluated (n = 44 from experiment 1; n = 82 from experiment 2). Cows that had emergence of a new ovarian follicle wave synchronized between d 1 and 6 of protocol (after EB treatment) and ovulated at the end of the protocol were considered synchronized to the protocol.

Statistical Analysis

For both experiments, sample size was calculated using the POWER procedure of SAS (version 9.3; SAS/STAT, SAS Institute Inc., Cary, NC) using a one-sided test to provide sufficient experimental units to detect statistical significance ($\alpha = 0.05$; $\beta = 0.20$), considering a standard deviation of 1 d to detect a difference in the day of wave emergence. For this variable, the number of experimental units needed per treatment was 14.

Continuous data with repeated measures over time were analyzed by ANOVA using the MIXED procedure of SAS (version 9.3) with models fitting a Gaussian distribution. Data were tested for normality of residuals, and data with residuals not normally distributed were transformed before analysis. The models included the fixed effects of treatment, day of measurement, parity, number of services, breed, BCS, DIM, milk yield categorized within parity during the week of d 0 as above or below the mean value, interactions between treatment and day and treatment and parity, and the random effects of cows nested within treatment. The covariance structure that resulted in the smallest Akaike's information criterion was selected for the model. Model fitting was evaluated using the fit statistics.

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS fitting a binary distribution. The models included the fixed effects of treatment, parity, breed, BCS category, and milk yield categorized within parity on the week of d 0 as above or below the mean value. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the *F*-tests in the mixed models. Model fitting was evaluated using the fit statistics. The estimates were back-transformed using the ILINK function of SAS to generate the adjusted percentages. For analyses of the combined data from experiment 1 and 2 or for analyses in experiment 2 when a treatment outcome was 0 or 100%, we used the Fisher Exact Test using the FREQ procedure of SAS. In all analyses, only variables with $P < 0.20$ were kept in the final model, unless the variable was essential. Differences were considered significant when $P < 0.05$, whereas tendencies were considered when $0.15 > P \geq 0.05$.

RESULTS

Experiment 1

Day of emergence, day of deviation, and size of future ovulatory follicle at time of deviation were not affected by EB dose (Table 1). The percentage of cows that synchronized the emergence of a new ovarian follicle wave was similar between treatments. The percentage of cows with a CL on the day of PGF treatment was reduced ($P < 0.05$) for cows in EB3 compared with EB2. The percentage of cows ovulating at the end of the protocol was high (~90%) and not altered by EB treatment. However, time of ovulation was later ($P < 0.01$) for cows in EB2 compared with EB3. In addition, multiple ovulation was surprisingly high (33%) but not affected by treatment. We observed no effect of treatment on pregnancy per AI (**P/AI**; Table 1).

Absence of CL at the time of protocol initiation affected ($P < 0.01$) the number of follicles ovulating per cow at the end of the protocol, with 2.1 ± 0.21 ovulations for cows without a CL and 1.2 ± 0.08 for cows with a CL. Similarly, percentage of cows with multiple ovulation after AI tended ($P = 0.06$) to be greater in cows without a CL [80.2% ± 19.0 (4/5)] than cows with a CL [26.9% ± 8.6 (9/34)] at the time of the FTAI protocol initiation. In contrast, we observed no effect of absence of a CL on the day of PGF treatment on multiple ovulation rate [29.0% ± 9.5 (7/24) vs. 39.9% ± 13.0 (6/15)].

Experiment 2

Days of Events and Size of Ovarian Structures. Day of emergence and day of deviation were delayed in cows in G −7 compared with G −3 and NoOv (Table 2). We found no difference among groups for percentage of cows with synchronized follicle wave emergence and size of the future ovulatory follicle at

Table 1. Results (LSM ± SEM; n/n) for experiment 1, comparing 2.0 (EB2; n = 23) versus 3.0 (EB3; n = 21) mg of estradiol benzoate at the beginning of an estradiol/progesterone-based fixed-time AI protocol (d 0)

Item	EB2	EB3	*P*-value
Day of emergence,[1] d	3.4 ± 0.17	3.6 ± 0.19	0.36
Synchronized follicle wave emergence,[2] %	82.6 ± 7.9 (19/23)	71.4 ± 9.9 (15/21)	0.39
Size of future ovulatory follicle at deviation,[3] mm	8.0 ± 0.29	8.0 ± 0.30	0.84
Presence of corpus luteum on d 7, %	55.3 ± 13.3 (13/21)	19.8 ± 9.8 (4/17)	<0.05
Day of deviation,[3] d	5.9 ± 0.21	6.1 ± 0.23	0.63
Maximum size of ovulatory follicle,[3] mm	14.1 ± 0.68	13.9 ± 0.62	0.80
Day of ovulation, d	10.9 ± 0.14	10.4 ± 0.13	<0.01
Ovulation rate,[4] %	89.1 ± 6.9 (20/23)	92.2 ± 5.9 (19/21)	0.71
Multiple ovulation rate,[5] %	26.5 ± 11.0 (5/20)	42.1 ± 12.1 (8/19)	0.35
Synchronized wave emergence and single ovulation, %	56.6 ± 11.0 (13/23)	33.4 ± 10.9 (7/21)	0.16
Pregnancies per AI, %			
31 d	47.7 ± 17.2 (11/23)	40.9 ± 15.0 (9/21)	0.71
59 d	42.6 ± 16.5 (10/23)	36.7 ± 14.2 (8/21)	0.73

[1]Only cows that had emergence of a new wave between d 1 and 6 were included.

[2]Number of cows that had emergence a new ovarian follicle wave between d 1 and 6 divided by the total number of cows treated.

[3]Only cows that had new ovarian follicle wave emergence (d 1 to 6) and a single ovulation were included.

[4]Number of cows that ovulated divided by number of cows treated.

[5]Number of cows that ovulated 2 or more follicles divided by the number of cows treated.

deviation. A greater percentage of NoOv cows had no CL present at the time of protocol initiation compared with the other 2 groups. In addition, a greater percentage of these cows had CL regression between d 0 and 3 compared with G −7 or G −3. However, there were no differences between groups in CL regression between d 3 and 7 of the protocol (Table 2). Overall, a greater ($P < 0.05$) percentage of cows regressed the CL between the beginning of the protocol and the day of PGF in the NoOv group compared with the G −3 and G −7 groups. This may have influenced size of the ovulatory follicle, which was greater for cows in the NoOv group compared with G −3 and G −7 groups (Table 2). Independent of treatment group, cows that had a CL on the day of PGF ovulated a smaller ($P = 0.05$) follicle (14.9 ± 0.3 mm) than cows that did not have a CL on the day of PGF (16.5 ± 0.5 mm).

A comparison was made between milk production and various measures of timing and size of the future ovulatory follicles, which includes only those cows with emergence of a new follicular wave (d 1 to 6) and single ovulation (data not shown). Day of follicle wave emergence was delayed ($P = 0.03$) in cows with lower milk yield (3.4 ± 0.20 d) compared with cows with higher milk yield (2.8 ± 0.20 d). However, milk yield did not affect the day of deviation, day of ovulation, size of future ovulatory follicle at deviation, or maximum size of ovulatory follicle. In addition, the percentage of cows that had emergence of a new follicular wave (d 1 to 6) and percentage of cows with multiple ovulation were not affected by milk yield.

Figure 3 shows the dynamics of ovulatory follicle growth for cows that ovulated a persistent follicle (ovulated a single follicle that was present on d 0; n = 17) compared with cows that ovulated a single follicle that emerged from a new follicular wave (d 1 to 6 of protocol; n = 42). On all days during the protocol, the future ovulatory follicle was larger for cows that ovulated a persistent follicle than for cows that ovulated a follicle that emerged during the protocol ($P < 0.05$). Cows ovulating a new follicle tended ($P \leq 0.07$) to have higher P/AI (Figure 3).

Concentration of Progesterone. At the time of protocol initiation (d 0), circulating P4 concentrations were greater in G −7 and NoOv cows compared with G −3 cows (Figure 4). As the protocol progressed, the P4 concentrations progressively decreased in NoOv as CL continued to regress, and increased in G −3 with growth of the new CL. Therefore, concentrations of P4 were greater in G −7 and G −3 than in NoOv cows on d 4, 5, and 7 of the protocol. By d 7, circulating P4 concentrations had decreased ($P < 0.05$) in G −7 and NoOv cows compared with d 0 values but had not changed in G −3 cows. A comparison of overall circulating P4 concentrations during the entire period indicated a tendency for a treatment effect ($P = 0.06$), an effect of day ($P < 0.001$), and a treatment by day interaction ($P = 0.01$). Concentrations of P4 were 0.5 ng/mL greater in G −7 (3.2 ± 0.3) compared with NoOv (2.7 ± 0.2) cows, with G −3 (2.7 ± 0.3) cows not significantly different from the other 2 groups.

Independent of treatment group, cows that were pregnant on d 31 or 59 after FTAI had greater circulating P4 concentrations during the protocol than cows that were not pregnant ($P = 0.01$; Figure 5). There was also an effect of day ($P < 0.001$) but no day by pregnancy status interaction. For all days that were analyzed during the protocol except d 7, cows that were

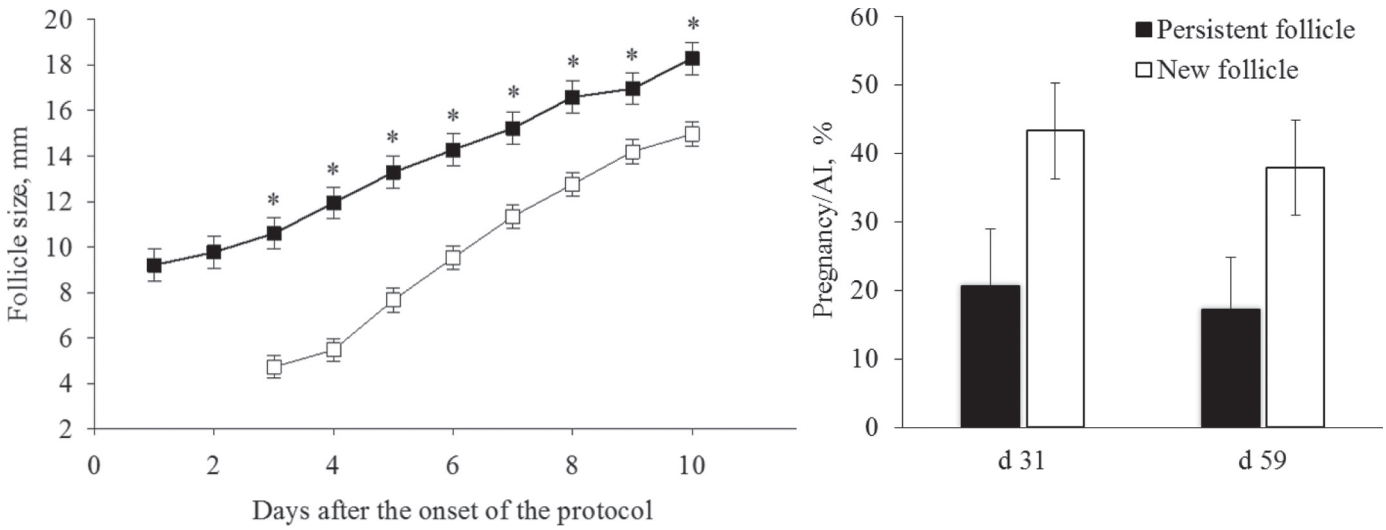

Figure 3. Future ovulatory follicle size between d 1 and 10 of protocol (left) and pregnancy/AI of single-ovulating cows that ovulated a persistent or a new follicle (right) independent of treatment. Cows were considered to have ovulated a persistent follicle (n = 17) when they ovulated a follicle that was present on d 0. Cows were considered to have ovulated a new follicle (n = 42) when they had emergence of a new ovarian follicle wave between d 1 and 6. There was an interaction between follicle type and day ($P < 0.001$). Cows that ovulated a persistent follicle tended to have a lower P/AI on d 31 ($P = 0.06$) and d 59 ($P = 0.07$) than those that ovulated a new follicle. *Within day, effect of follicle age ($P < 0.05$). Error bars represent SEM.

later found to be pregnant had greater circulating P4 compared with cows that were subsequently found to be nonpregnant.

On the day of FTAI, the circulating P4 concentrations were categorized in cows with P4 <0.10 ng/mL or cows with P4 = 0.10 to 0.22 ng/mL. The percentage of cows that ovulated at the end of protocol was greater ($P = 0.04$) for cows with lower circulating P4 (<0.10 ng/mL; Figure 6). In addition, we found an effect of P4 on P/AI at either d 31 ($P = 0.03$) or d 59 ($P =$

Table 2. Results (LSM ± SEM; n/n) for experiment 2, in which cows ovulated following treatment with 10.5 μg of buserelin acetate (GnRH) 3 d (G −3; n = 24) or 7 d (G −7; n = 20) before the start of the protocol (d 0); cows that did not ovulate to GnRH were allocated to another group (NoOv; n = 38)

Item	G −3	G −7	NoOv	*P*-value
Day of emergence,[1] d	2.9 ± 0.25^{y}	3.6 ± 0.27^{x}	2.8 ± 0.21^{y}	0.08
Synchronized follicle wave emergence,[2] %	78.7 ± 8.9 (17/24)	82.3 ± 8.7 (15/20)	78.3 ± 7.5 (27/38)	0.92
Size of future ovulatory follicle at deviation,[3] mm	8.2 ± 0.29	9.1 ± 0.38	8.6 ± 0.25	0.19
Day of deviation,[3] d	5.2 ± 0.38^{b}	6.4 ± 0.36^{a}	5.2 ± 0.25^{b}	<0.01
Corpus luteum (CL)				
No CL on d 0, %	0.0^{b} (0/24)	0.0^{b} (0/20)	36.8^{a} (14/38)	<0.01
Early regression (d 0 to 3),[4] %	0.0^{b} (0/24)	10.0^{b} (2/20)	45.8^{a} (11/24)	0.01
Later regression (d 3 to 7),[4] %	34.2 ± 10.7 (8/24)	21.1 ± 9.7 (4/20)	26.3 ± 9.8 (6/24)	0.63
Regression (d 0 to 7),[4] %	35.6 ± 10.7^{b} (8/24)	30.5 ± 11.0^{b} (6/20)	72.5 ± 9.9^{a} (17/24)	0.02
Maximum size of ovulatory follicle,[3] mm	15.1 ± 0.75^{b}	14.6 ± 0.83^{b}	16.6 ± 0.57^{a}	0.04
Day of ovulation, d	10.6 ± 0.10	10.7 ± 0.13	10.6 ± 0.08	0.75
Ovulation rate,[5] %	71.2 ± 10.2^{ab} (18/24)	53.8 ± 11.8^{b} (11/20)	81.7 ± 6.4^{a} (30/38)	0.12
Multiple ovulation rate, %	19.7 ± 14.7 (3/18)	12.3 ± 14.0 (1/11)	8.5 ± 6.0 (3/30)	0.70
Synchronized wave emergence and single ovulation, %	42.6 ± 12.6 (11/24)	32.9 ± 12.8 (7/20)	44.7 ± 8.8 (17/38)	0.70
Pregnancies per AI, %				
31 d	41.2 ± 11.5 (11/24)	35.6 ± 12.0 (7/20)	42.6 ± 9.1 (15/38)	0.89
59 d	38.2 ± 11.3 (10/24)	36.8 ± 12.1 (7/20)	34.1 ± 8.6 (12/38)	0.95

[a,b]Means within a row with different superscripts differ at $P \leq 0.05$.

[x,y]Means within a row with different superscripts differ at $P > 0.05$ and ≤ 0.10.

[1]Only cows that had emergence of a new wave between d 1 and 6 were included.

[2]Number of cows that had emergence of a new ovarian follicle wave between d 1 and 6 divided by the total of cows treated.

[3]Only cows that had emergence of a new ovarian follicle wave (d 1 to 6) and single ovulation were included.

[4]Only cows that had a CL on d 0 were included in the analysis.

[5]Number of cows that ovulated divided by the number of cows treated.

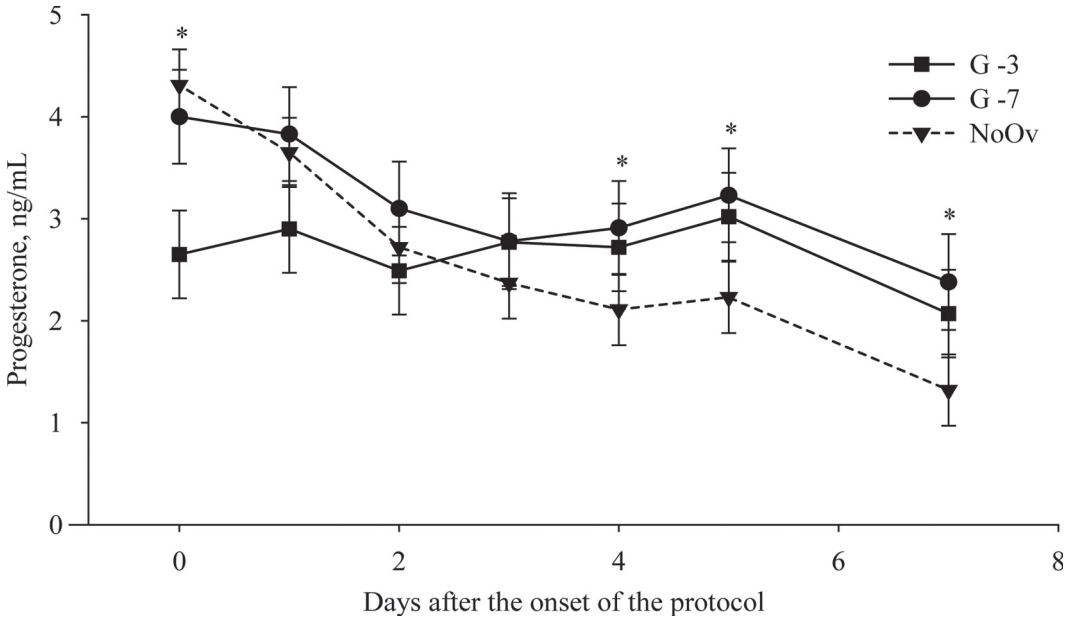

Figure 4. Plasma progesterone concentrations during the first 7 d after initiation of the fixed-time AI protocol. G −3 = cows that ovulated after receiving GnRH 3 d before protocol initiation (n = 24); G −7 = cows that ovulated after receiving GnRH 7 d before protocol initiation (n = 20); NoOv = cows that did not ovulate to GnRH given 3 d or 7 d before protocol initiation (n = 38). There was a tendency for a treatment effect ($P = 0.06$), effect of day ($P < 0.001$), and interaction between treatment × day ($P = 0.01$). *Treatment effect within day ($P < 0.05$). Error bars represent SEM.

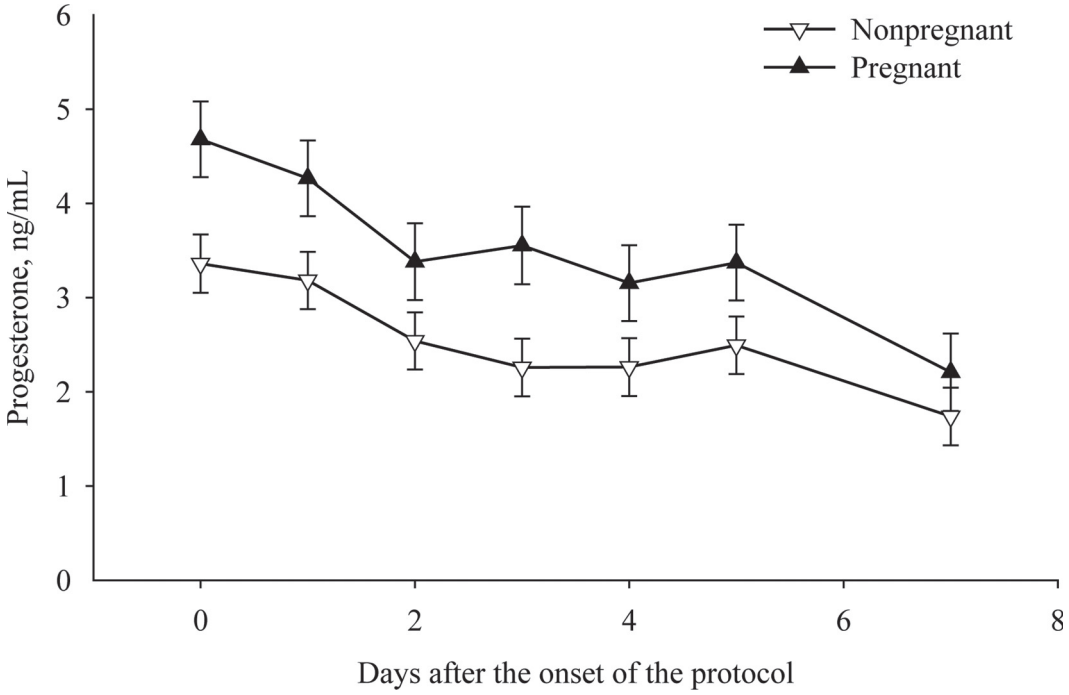

Figure 5. Plasma progesterone (P4) concentrations from protocol initiation (d 0) until d 7. Nonpregnant (n = 53) = cows not pregnant at 59 d after fixed-time AI (FTAI); pregnant (n = 29) = cows pregnant at 59 d after FTAI. Cows received FTAI on d 10 of the protocol. From d 0 to 5 and on d 7, concentrations of P4 averaged 2.5 ± 0.23 and 3.5 ± 0.29 ng/mL for nonpregnant and pregnant cows, respectively. There were effects of pregnancy status ($P = 0.01$) and day ($P < 0.001$) but no interaction between pregnancy status and day. Error bars represent SEM.

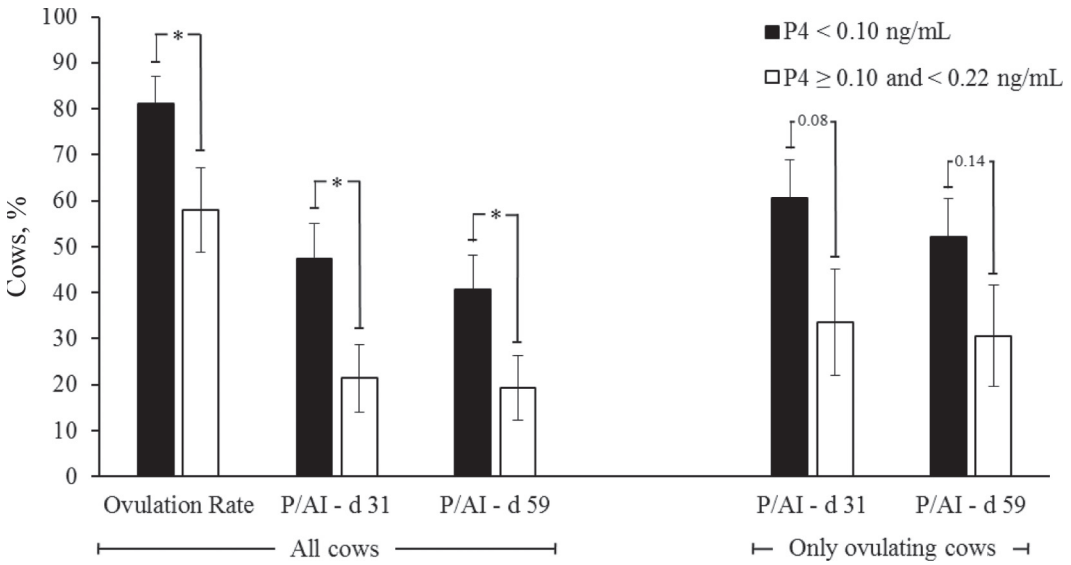

Figure 6. Ovulation rate (percentage of cows that ovulated at the end of the protocol) and pregnancy/AI (P/AI) at d 31 and d 59 after fixed-time AI (FTAI) for cows that had progesterone (P4) <0.10 ng/mL (n = 49) or P4 ≥0.10 and <0.22 ng/mL (n = 33) on day of FTAI. *Effect of P4 <0.10 ng/mL vs. P4 ≥0.10 and <0.22 ng/mL ($P \leq 0.05$). Error bars represent SEM.

0.05; Figure 6). When only cows that ovulated were considered in the analysis, circulating P4 tended ($P = 0.08$) to influence P/AI on d 31 and 59 ($P = 0.14$) after FTAI (Figure 6).

Ovulation Rate and P/AI. Day of ovulation and multiple ovulation rate were not different among groups, but ovulation rate tended to be higher in NoOv than in G −7, and G −3 did not differ from the other treatments (Table 2). However, the percentage of cows that ovulated at the end of the protocol did not differ ($P = 0.92$) for cows that had emergence of a new follicular wave [d 0 to d 6; 71.7 ± 6.1% (42/59)] compared with cows that did not have emergence of a new follicular wave [72.9 ± 9.7% (17/23)].

The P/AI on d 31 and 59 were not affected by treatment (Table 2). However, independent of treatment, cows that did not have synchronization of a new ovarian follicle wave emergence compared with cows with a new follicle tended to have a decrease in P/AI on d 31 [20.6% ± 8.5 (6/23) vs. 43.3% ± 7.0 (27/59); $P = 0.06$] and d 59 [17.1% ± 7.7 (5/23) vs. 37.9% ± 6.8 (24/59); $P = 0.07$] after FTAI.

Independent of treatment group, cows that had CL regression between d 0 and 3 compared with cows with no CL regression tended to have lower P/AI on d 31 [23.5% ± 9.7 (5/20) vs. 45.6% ± 8.2 (23/45); $P = 0.11$] and on d 59 [18.1% ± 8.7 (4/20) vs. 42.5% ± 8.3 (22/45); $P = 0.07$]. In contrast, when all cows that had CL regression between d 0 and 7 were compared with cows that did not have CL regression, there was no difference in P/AI based on either the d 31 [36.5% ± 9.0 (14/33) vs. 40.1% ± 9.2 (14/32)] or d 59 [32.6% ± 8.9 (13/33) vs. 36.2% ± 9.1 (13/32)] pregnancy diagnosis.

Finally, independent of treatment group, ovulatory follicle size had no effect on P/AI (using linear regression analysis) based on either the d 31 ($P = 0.73$) or d 59 ($P = 0.88$) pregnancy diagnosis. The P/AI was not different between cows ovulating smaller (≤14 mm), medium (>14 and ≤17 mm) or larger (>17 mm) follicles based on d 31 [69.4% ± 12.4 (11/17), 51.3% ± 12.2; (15/29), and 58.7% ± 16.7 (7/13), respectively] or d 59 [65.6% ± 12.7 (10/17), 50.0% ± 12.3 (14/29), and 46.3% ± 17.1 (5/13)] pregnancy diagnosis.

Combination of Experiments 1 and 2

Results from experiment 1 and 2 were combined to evaluate the percentage of cows that were synchronized based on specific measures (Table 3). In total, 73.8% of cows (93/126) had emergence of a new ovarian follicle wave during the protocol, with greater ($P = 0.01$) P/AI in cows with new wave emergence compared with cows with a persistent follicle. Overall, 77.8% of cows ovulated at the end of the protocol (98/126) with no cow becoming pregnant that did not have ovulation at the end of the protocol (0/28). Therefore, overall synchronization rate to the protocol, based on follicle emergence and ovulation, was 59.5% (75/126) with much greater ($P < 0.01$) P/AI in synchronized compared with nonsynchronized cows (Table 3).

Table 3. Results of the analysis of synchronization to the protocol using all cows from both experiments[1]

Item	Cows with response,[2] % (n/n)	P/AI, % (n/n)		*P*-value
		Yes	No	
Emerged new wave[3]	73.8 (93/126)	49.5 (46/93)	24.2 (8/33)	0.01
Ovulated to protocol[4]	77.8 (98/126)	55.1 (54/98)	0 (0/28)	<0.01
Overall synchronization[5]	59.5 (75/126)	61.3 (46/75)	15.7 (8/51)	<0.01

[1]Cows were classified by whether they had emergence of the new follicular wave during the protocol (d 1 to 6) and whether they had ovulation at the end of the protocol to determine the percentage of cows that were synchronized to the fixed-time AI protocol and the pregnancies/AI (P/AI) for synchronized (yes) and nonsynchronized (no) cows.

[2]Percentage of cows that emerged a new ovarian follicle wave or ovulated to the end of the protocol or had an overall synchronization of the protocol.

[3]Cows that had emergence of a new ovarian follicle wave between d 1 and 6.

[4]Cows that ovulated between d 9.5 and 11.5 of the protocol.

[5]Cows were considered synchronized when they had new follicle wave emergence between d 1 and 6 and ovulated between d 9.5 and 11.5 of the protocol.

DISCUSSION

Reproductive management in many parts of the world use hormonal programs that synchronize ovulation to allow for FTAI of all eligible cows. This research evaluated the dynamic changes in ovarian structures and reproductive hormones during modifications of an E2-P4 FTAI synchronization protocol. Our first hypothesis—that increasing the dose of EB would increase synchronization of a new ovarian follicle wave emergence—was rejected because the higher dose of EB (3 mg vs. 2 mg) did not improve follicle wave synchronization but instead led to earlier regression of the CL and earlier ovulation at the end of the protocol. Although we have been unable to find other studies that have tested the specific hypothesis of dose of EB compared with synchronization of a new ovarian follicle wave emergence, our results on increased CL regression are consistent with other research results, as discussed below. Our second hypothesis, that synchronization of the protocol could be improved by initiating the E2-P4 protocol at a specific stage of the follicular wave, was proposed because of the success of presynchronization programs on fertility in GnRH-based protocols (Moreira et al., 2001; Herlihy et al., 2012; Wiltbank and Pursley, 2014). However, we also rejected this second hypothesis because we observed that, irrespective of the stage of the follicular wave at protocol initiation, many cows did not ovulate at the end of the protocol or ovulated a persistent follicle, due to lack of emergence of a new follicular wave following EB treatment. Probably the most interesting results from this study were found when we combined the results of both experiments to evaluate the specific physiological abnormalities that led to lack of synchronization during the protocols. Only about 60% of cows were synchronized by the E2-P4 protocol, based on emergence of a new follicular wave and ovulation at the end of the protocol. Cows that were synchronized to the protocol had excellent fertility to the FTAI (~60%) but cows that were not synchronized had low fertility (15.7%). Although the results of these experiments are consistent with previous research, as discussed below, they provide novel physiological insights into problems and possible resolutions associated with E2-P4–based protocols for synchronizing ovulation.

Treatments with P4 and various types of E2 have been shown to result in the emergence of a new follicular wave (Bo et al., 1995a,b); however, consistent with the results of a previous study in lactating dairy cows (Souza et al., 2009), more than 25% (33/126) of dairy cows in our study did not demonstrate emergence of a new follicular wave after EB treatment. Insufficient EB does not seem to explain this problem, because increasing the dose of EB from 2.0 to 3.0 mg did not reduce the percentage of cows that ovulated a persistent follicle (29% persistent follicles after 3 mg of EB; 6/21). Moreover, follicle wave stage does not seem to explain the results, considering that in either the presence or absence of a dominant follicle on d 0, about 20% of cows failed to have a new wave emergence synchronized. It is unclear why some cows did not have new wave emergence after EB treatment but one possibility, which was not tested in our study, is that circulating FSH or LH concentrations were insufficiently suppressed in response to EB and P4 treatment at protocol initiation and therefore follicular wave turnover did not occur. Lactating dairy cows have increased E2 and P4 metabolism due to elevated liver blood flow (Sangsritavong et al., 2002; Wiltbank et al., 2006); therefore, the P4 implant and EB may have produced an insufficient increase in P4 and E2 to suppress gonadotropins. In addition, similar circulating P4 concentrations seem to be less inhibitory to LH pulses in lactating compared with nonlactating cows (Vasconcelos et al., 2003).

Another important consideration is that treatment with EB, particularly the 3-mg dose of EB, was associated with regression of the CL. Increasing the EB dose from 2 to 3 mg clearly produced greater CL regression during the protocol because the number of cows with CL at the time of PGF treatment (d 7) decreased from about 60% with EB2 to less than 20% with EB3. We did not measure circulating P4 in this first study; however, CL regression would be followed by a substantial decrease in circulating P4 and probably increased frequency of GnRH or LH pulses and possibly FSH pulses. These gonadotropin changes could underlie the rescue of some of the persistent follicles after EB treatment. Several other studies have reported regression of the CL after treatment with EB (Araujo et al., 2009) or 17β-estradiol (Ford et al., 1975; Thatcher et al., 1986). It is unclear if EB2 caused CL regression because we did not have a control group that received no EB treatment. However, it is clear that most CL regress in response to EB3. Treatment with E2 can increase PGF production by the uterus (Ford et al., 1975; Thatcher et al., 1986). This effect appears to be mediated by binding of E2 to estrogen receptor α, subsequent upregulation of oxytocin receptor expression, binding of oxytocin to the oxytocin receptor, and subsequent production of PGF pulses due to the pulsatile pattern of oxytocin pulses (McCracken et al., 1999; Fleming et al., 2006; Spencer et al., 2007). Thus, although greater doses of EB might be expected to produce greater suppression of gonadotropins, this effect might be more than counteracted by the decrease in circulating P4 due to increased CL regression with a greater dose of EB. A better way to produce a more consistent regression of all follicles might be to increase the amount of P4 simultaneously to EB treatment, such as by using high doses of injectable P4 or by including 2 P4 implants during the protocol, but these approaches also need to be tested.

Our observations of low fertility in cows that ovulated a persistent follicle are similar to results from numerous other studies (Ahmad et al., 1996; Bleach et al., 2004; Cerri et al., 2009; Santos et al., 2010) and are consistent with the idea that increasing the dominance period of follicles reduces fertility in the oocyte that is ovulated from the persistent follicle. This effect may be due to premature germinal vesicle breakdown due to excessive LH stimulation of the dominant follicle (Ahmad et al., 1994; Revah and Butler, 1996). Oocytes from persistent follicles are fertilized but generally undergo cessation of cellular division and embryo degeneration during the first few days of embryo development (Ahmad et al., 1994; Cerri et al., 2009). For example, ovulatory follicles that were 12 d compared with 9 d old had a lower percentage of freezable embryos and a greater percentage of degenerate embryos (Cerri et al., 2009). In addition, lower circulating P4 concentrations during follicle growth can result in reduced fertility of the follicle, independent of follicle age (Rivera et al., 2011; Wiltbank et al., 2014). This idea was supported by our data because circulating P4 concentrations were consistently greater in cows that became pregnant compared with cows that were nonpregnant, independent of treatment group (Figure 5). Lower fertility and increased double ovulation have consistently been observed in cows with lower circulating P4 during ovulatory follicle growth in GnRH-based synchronization protocols (Lopez et al., 2005; Bisinotto et al., 2013; Wiltbank et al., 2014). Thus, an important area of opportunity to improve E2-P4–based synchronization protocols in lactating dairy cows is to increase the percentage of cows with complete follicle suppression, emergence of a new follicular wave, and elevated circulating P4 during the protocol.

Another important hormonal problem that we observed during the protocol was that some cows had higher circulating P4 (>0.1 ng/mL) near the time of AI than others. This discrete difference was associated with a decrease in P/AI at the d 31 and 59 pregnancy diagnoses. One important reason for the decrease in fertility was a decrease in ovulation at the end of the protocol in cows with small P4 elevations. Another study reported that an elevation in circulating P4 to >0.1 ng/mL resulted in a reduced P/AI in lactating dairy cows treated with an E2-P4–based FTAI protocol (Pereira et al., 2013b). This appears to be a lower threshold for P4 than has been reported in studies using GnRH-based protocols in which the threshold for reduced fertility was between 0.3 and 0.5 ng/mL for P4 (Souza et al., 2007; Brusveen et al., 2009; Bisinotto et al., 2010; Giordano et al., 2012, 2013). It seems likely that any difference in P4 threshold could relate to inhibition of an estradiol cypionate-induced LH surge by low concentrations of P4, because the action of E2 is blocked by P4 at the hypothalamic GnRH level (Robinson et al., 2000; Richter et al., 2002). Thus, we observed a decrease in percentage of cows that ovulated, probably due to lack of a GnRH or LH surge in some cows with P4 >0.1 ng/mL. In contrast, induction of ovulation by using GnRH is likely to occur, even in the presence of low concentrations of P4; however, the threshold that inhibits gamete transport, a second mechanism that reduces fertility (Day and Polge, 1968; Hunter, 1968), may be reached when circulating P4 exceeds 0.3 to 0.5 ng/mL. In our study, when we evaluated only those cows that ovulated at the end of the protocol, a tendency remained for reduced fertility (>20% reduction) in cows with a small elevation in circulating P4. Thus, other mechanisms in addition to inhibition of ovulation

are likely to underlie part of the reduction in fertility in cows with slightly higher P4 near AI. In these experiments, it is likely that if we had treated cows with a second dose of PGF, as has been done in GnRH-based FTAI protocols (Brusveen et al., 2009; Santos et al., 2010; Ribeiro et al., 2012), we could have diminished the problem. However, other factors such as stress may also explain increases in circulating P4 at the time of AI and cannot be ruled out. For example, Maziero et al. (2011) have shown an increase in circulating P4 associated with increased cortisol in cows submitted to acute stress (road transportation) during proestrus. Future experiments need to be designed to specifically evaluate whether there is a difference in P4 threshold near AI in GnRH versus estradiol cypionate-induced ovulation protocols and to differentiate the mechanisms of fertility inhibition as well as effective treatments for this problem in FTAI protocols.

A major problem that we and others (Souza et al., 2009; Pereira et al., 2013a,b) have observed in E2-P4–based FTAI protocols is that some cows do not ovulate at the end of the protocol. In our study, more than 20% of cows did not ovulate at the end of the protocol and none of these cows became pregnant to FTAI. As discussed above, more than half of the cows that did not ovulate at the end of the protocol (13/23 in experiment 2) had elevated P4 that probably inhibited the estradiol cypionate-induced GnRH/LH surge. In addition, some cows (9/28, both experiments) appeared to have late emergence or no emergence of a follicular wave during the protocol and, therefore, the dominant follicle was still small at the time of estradiol cypionate treatment. A previous study in lactating Holstein dairy cows reported that the dominant follicle did not acquire ovulatory capacity until it reached a diameter of >10.0 mm (Sartori et al., 2001). One focus in future studies to improve E2-P4–based FTAI protocols should be to increase ovulation at the end of the protocol.

One surprising observation was that the proportion of cows with synchronized new ovarian follicle wave emergence and ovulation of a single follicle was <50% in both of our experiments. One reason was that approximately 20% of cows did not have a new follicular wave and another 20% did not ovulate at the end of the protocol, as discussed above. However, the multiple ovulation rate was also unexpectedly high in these studies, particularly in experiment 1. Multiple ovulation rate is responsible for the high twinning rate in lactating dairy cows (Wiltbank et al., 2000; del Rio et al., 2006). Although high milk production (Fricke and Wiltbank, 1999; Lopez et al., 2005) and low circulating P4 before AI (Cerri et al., 2011; Wiltbank et al., 2012) are clearly risk factors for increased double ovulation, numerous other risk factors could produce high double ovulation and twinning rates (Kinsel et al., 1998; del Rio et al., 2007).

CONCLUSIONS

Although FTAI protocols using EB and P4 yield P/AI as high as 40% (considered satisfactory for lactating dairy cows), they need improvements to provide a more consistent synchronization of ovarian follicle wave emergence, higher circulating P4 during the protocol, successful induction of complete luteolysis, as well as higher ovulation rates. Absence of a dominant follicle or increasing the dose of EB to 3 mg at the beginning of the FTAI protocol was not effective in providing those improvements.

ACKNOWLEDGMENTS

The authors thank the forage quality and conservation group from the Luiz de Queiroz College of Agriculture (ESALQ/USP, Piracicaba, Brazil), and the owner and staff of Massaranduva Farm (Itai, SP, Brazil) for the use of their cows and facilities. Our appreciation is extended to veterinarian Leonardo Silva for assistance during experiment 2, and to Eunice Oba from the University of São Paulo State (UNESP, Botucatu, Brazil). P. L. J. Monteiro Jr. was supported by a scholarship from São Paulo Research Foundation (FAPESP, grant # 2011/11344-7, São Paulo, Brazil). This project was funded by a grant from FAPESP (# 2011/11395-0) and from the Brazilian National Council for Scientific and Technological Development (CNPq, Brasília, Brazil).

REFERENCES

Ahmad, N., S. W. Beam, W. R. Butler, D. R. Deaver, R. T. Duby, D. R. Elder, J. E. Fortune, L. C. Griel, L. S. Jones, R. A. Milvae, J. L. Pate, I. Revah, D. T. Schreiber, D. H. Townson, P. C. W. Tsang, and E. K. Inskeep. 1996. Relationship of fertility to patterns of ovarian follicular development and associated hormonal profiles in dairy cows and heifers. J. Anim. Sci. 74:1943–1952.

Ahmad, N., F. N. Schrick, R. L. Butcher, and E. K. Inskeep. 1994. Fertilization rate and embryo development in relation to persistent follicles and peripheral estradiol-17-beta in beef cows. Biol. Reprod. 50(Suppl. 1):65.

Araujo, R. R., O. J. Ginther, J. C. Ferreira, M. M. Palhao, M. A. Beg, and M. C. Wiltbank. 2009. Role of follicular estradiol-17beta in timing of luteolysis in heifers. Biol. Reprod. 81:426–437.

Baruselli, P. S., J. N. S. Sales, R. V. Sala, L. M. Viera, and M. F. Sa Filho. 2012. History, evolution and perspectives of timed artificial insemination programs in Brazil. Anim. Reprod. 9:139–152.

Bastos, M. R., R. S. Surjus, A. B. Prata, M. A. P. Meschiatti, M. Borsato, G. B. Mourão, A. M. Pedroso, A. V. Pires, and R. Sartori. 2011. Dose effect of estradiol benzoate associated with progesterone on the synchronization of follicular wave emergence in *Bos indicus* and *Bos taurus* cows. Page 382 in Proc. Brazilian Embryo Technology Society (SBTE) Annual Meeting. ASV Faculdade de Veterinária, Porto Alegre, RS, Brazil.

Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. J. Dairy Sci. 89:3413–3424.

Bisinotto, R. S., E. S. Ribeiro, F. S. Lima, N. Martinez, L. F. Greco, L. Barbosa, P. P. Bueno, L. F. S. Scagion, W. W. Thatcher, and J. E. P. Santos. 2013. Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol. J. Dairy Sci. 96:2214–2225.

Bisinotto, R. S., E. S. Ribeiro, L. T. Martins, R. S. Marsola, L. F. Greco, M. G. Favoreto, C. A. Risco, W. W. Thatcher, and J. E. P. Santos. 2010. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. J. Dairy Sci. 93:5798–5808.

Bleach, E. C. L., R. G. Glencross, and P. G. Knight. 2004. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. Reproduction 127:621–629.

Bo, G. A., G. P. Adams, M. Caccia, M. Martinez, R. A. Pierson, and R. J. Mapletoft. 1995a. Ovarian follicular wave emergence after treatment with progestogen and estradiol in cattle. Anim. Reprod. Sci. 39:193–204.

Bo, G. A., G. P. Adams, L. F. Nasser, R. A. Pierson, and R. J. Mapletoft. 1993. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 40:225–239.

Bo, G. A., G. P. Adams, R. A. Pierson, and R. J. Mapletoft. 1995b. Exogenous control of follicular wave emergence in cattle. Theriogenology 43:31–40.

Bo, G. A., G. P. Adams, R. A. Pierson, H. E. Tribulo, M. Caccia, and R. J. Mapletoft. 1994. Follicular wave dynamics after estradiol 17-beta treatment of heifers with or without a progestogen implant. Theriogenology 41:1555–1569.

Brusveen, D. J., A. H. Souza, and M. C. Wiltbank. 2009. Effects of additional prostaglandin $F_{2\alpha}$ and estradiol-17 beta during Ovsynch in lactating dairy cows. J. Dairy Sci. 92:1412–1422.

Cerri, R. L., R. C. Chebel, F. Rivera, C. D. Narciso, R. A. Oliveira, M. Amstalden, G. M. Baez-Sandoval, L. J. Oliveira, W. W. Thatcher, and J. E. Santos. 2011. Concentration of progesterone during the development of the ovulatory follicle: II. Ovarian and uterine responses. J. Dairy Sci. 94:3352–3365.

Cerri, R. L. A., H. M. Rutigliano, R. C. Chebel, and J. E. P. Santos. 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction 137:813–823.

Day, B. N., and C. Polge. 1968. Effects of progesterone on fertilization and egg transport in pig. J. Reprod. Fertil. 17:227–230.

del Rio, N. S., B. W. Kirkpatrick, and P. M. Fricke. 2006. Observed frequency of monozygotic twinning in Holstein dairy cattle. Theriogenology 66:1292–1299.

del Rio, N. S., S. Stewart, P. Rapnicki, Y. M. Chang, and P. M. Fricke. 2007. An observational analysis of twin births, calf sex ratio, and calf mortality in Holstein dairy cattle. J. Dairy Sci. 90:1255–1264.

Elanco Animal Health. 2009. The 5-point body condition scoring system. Elanco Animal Health, Greenfield, IN.

Fleming, J. G. W., T. E. Spencer, S. H. Safe, and F. W. Bazer. 2006. Estrogen regulates transcription of the ovine oxytocin receptor gene through GC-rich SP1 promoter elements. Endocrinology 147:899–911.

Ford, S. P., C. W. Weems, R. E. Pitts, J. E. Pexton, R. L. Butcher, and E. K. Inskeep. 1975. Effects of estradiol-17 beta and progesterone on prostaglandins F in sheep uteri and uterine venous plasma. J. Anim. Sci. 41:1407–1413.

Fricke, P. M., and M. C. Wiltbank. 1999. Effect of milk production on the incidence of double ovulation in dairy cows. Theriogenology 52:1133–1143.

Ginther, O. J., K. Kot, L. J. Kulick, and M. C. Wiltbank. 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. Theriogenology 48:75–87.

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

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

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

Hunter, R. H. F. 1968. Effect of progesterone on fertilization in golden hamster. J. Reprod. Fertil. 16:499–502.

Kinsel, M. L., W. E. Marsh, P. L. Ruegg, and W. G. Etherington. 1998. Risk factors for twinning in dairy cows. J. Dairy Sci. 81:989–993.

Lopez, H., D. Z. Caraviello, L. D. Satter, P. M. Fricke, and M. C. Wiltbank. 2005. Relationship between level of milk production and multiple ovulations in lactating dairy cows. J. Dairy Sci. 88:2783–2793.

Lucy, M. C. 2001. ADSA Foundation Scholar Award—Reproductive loss in high-producing dairy cattle: Where will it end? J. Dairy Sci. 84:1277–1293.

Maziero, R. R. D., A. C. Martins, M. R. Mollo, I. Martin, M. R. Bastos, J. C. P. Ferreira, R. Rumpf, and R. Sartori. 2011. Ovarian function in cows submitted to acute stress during proestrus. Livest. Sci. 138:105–108.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: A neuroendocrine-mediated event. Physiol. Rev. 79:263–323.

Mee, J. F. 2012. Reproductive issues arising from different management systems in the dairy industry. Reprod. Domest. Anim. 47:42–50.

Moreira, F., R. L. de la Sota, T. Diaz, and W. W. Thatcher. 2000. Effect of day of the estrous cycle at the initiation of a timed artificial insemination protocol on reproductive responses in dairy heifers. J. Anim. Sci. 78:1568–1576.

Moreira, F., C. Orlandi, C. A. Risco, R. Mattos, F. Lopes, and W. W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J. Dairy Sci. 84:1646–1659.

Norman, H. D., J. R. Wright, S. M. Hubbard, R. H. Miller, and J. L. Hutchison. 2009. Reproductive status of Holstein and Jersey cows in the United States. J. Dairy Sci. 92:3517–3528.

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

Pereira, M. H. C., A. D. P. Rodrigues, R. J. De Carvalho, M. C. Wiltbank, and J. L. M. Vasconcelos. 2014. Increasing length of an estradiol and progesterone timed artificial insemination protocol decreases pregnancy losses in lactating dairy cows. J. Dairy Sci. 97:1454–1464.

Pereira, M. H. C., A. D. P. Rodrigues, T. Martins, W. V. C. Oliveira, P. S. A. Silveira, M. C. Wiltbank, and J. L. M. Vasconcelos. 2013a. Timed artificial insemination programs during the summer in lactating dairy cows: Comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol. J. Dairy Sci. 96:6904–6914.

Pereira, M. H. C., C. P. Sanches, T. G. Guida, A. D. R. Rodrigues, F. L. Aragon, M. B. Veras, P. T. Borges, M. C. Wiltbank, and J. L. M. Vasconcelos. 2013b. Timing of prostaglandin F-2 alpha treatment in an estrogen-based protocol for timed artificial insemination or timed embryo transfer in lactating dairy cows. J. Dairy Sci. 96:2837–2846.

Portaluppi, M. A., and J. S. Stevenson. 2005. Pregnancy rates in lactating dairy cows after presynchronization of estrous cycles and variations of the Ovsynch protocol. J. Dairy Sci. 88:914–921.

Pursley, J. R., M. R. Kosorok, and M. C. Wiltbank. 1997. Reproductive management of lactating dairy cows using synchronization of ovulation. J. Dairy Sci. 80:301–306.

Pursley, J. R., M. O. Mee, and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology 44:915–923.

Revah, I., and W. R. Butler. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. J. Reprod. Fertil. 106:39–47.

Ribeiro, E. S., R. S. Bisinotto, M. G. Favoreto, L. T. Martins, R. L. A. Cerri, F. T. Silvestre, L. F. Greco, W. W. Thatcher, and J. E. P. Santos. 2012. Fertility in dairy cows following presynchronization and administering twice the luteolytic dose of prostaglandin $F_{2\alpha}$ as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78:273–284.

Richter, T. A., J. E. Robinson, and N. P. Evans. 2002. Progesterone blocks the estradiol-stimulated luteinizing hormone surge by disrupting activation in response to a stimulatory estradiol signal in the ewe. Biol. Reprod. 67:119–125.

Rivera, F. A., L. G. D. Mendonca, G. Lopes, J. E. P. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderon, and R. C. Chebel. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. Reproduction 141:333–342.

Robinson, J. E., A. E. Healey, T. G. Harris, E. A. Messent, D. C. Skinner, J. A. Taylor, and N. P. Evans. 2000. The negative feedback action of progesterone on luteinizing hormone release is not associated with changes in GnRH mRNA expression in the ewe. J. Neuroendocrinol. 12:121–129.

Sangsritavong, S., D. K. Combs, R. Sartori, L. E. Armentano, and M. C. Wiltbank. 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 beta in dairy cattle. J. Dairy Sci. 85:2831–2842.

Santos, J. E. P., C. D. Narciso, F. Rivera, W. W. Thatcher, and R. C. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. J. Dairy Sci. 93:2976–2988.

Sartori, R., P. M. Fricke, J. C. P. Ferreira, O. J. Ginther, and M. C. Wiltbank. 2001. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol. Reprod. 65:1403–1409.

Sartori, R., J. M. Haughian, R. D. Shaver, G. J. M. Rosa, and M. C. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J. Dairy Sci. 87:905–920.

Souza, A. H., H. Ayres, R. M. Ferreira, and M. C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208–215.

Souza, A. H., A. Gumen, E. P. B. Silva, A. P. Cunha, J. N. Guenther, C. M. Peto, D. Z. Caraviello, and M. C. Wiltbank. 2007. Supplementation with estradiol-17 beta before the last gonadotropin-releasing hormone injection of the Ovsynch protocol in lactating dairy cows. J. Dairy Sci. 90:4623–4634.

Souza, A. H., S. Viechnieski, F. A. Lima, F. F. Silva, R. Araujo, G. A. Bo, M. C. Wiltbank, and P. S. Baruselli. 2009. Effects of equine chorionic gonadotropin and type of ovulatory stimulus in a timed-AI protocol on reproductive responses in dairy cows. Theriogenology 72:10–21.

Spencer, T. E., G. A. Johnson, F. W. Bazer, R. C. Burghardt, and M. Palmarini. 2007. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod. Fertil. Dev. 19:65–78.

Thatcher, W. W., M. Terqui, J. Thimonier, and P. Mauleon. 1986. Effect of estradiol-17beta on peripheral plasma concentration of 15-keto-13,14-dihydro PGF2alpha and luteolysis in cyclic cattle. Prostaglandins 31:745–756.

Vasconcelos, J. L. M., S. Sangsritavong, S. J. Tsai, and M. C. Wiltbank. 2003. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. Theriogenology 60:795–807.

Vasconcelos, J. L. M., R. W. Silcox, G. J. M. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067–1078.

Wiltbank, M., H. Lopez, R. Sartori, S. Sangsritavong, and A. Gumen. 2006. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology 65:17–29.

Wiltbank, M. C., P. M. Fricke, S. Sangsritavong, R. Sartori, and O. J. Ginther. 2000. Mechanisms that prevent and produce double ovulations in dairy cattle. J. Dairy Sci. 83:2998–3007.

Wiltbank, M. C., and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170–185.

Wiltbank, M. C., R. Sartori, M. M. Herlihy, J. L. M. Vasconcelos, A. B. Nascimento, A. H. Souza, H. Ayres, A. P. Cunha, A. Keskin, J. N. Guenther, and A. Gumen. 2011. Managing the dominant follicle in lactating dairy cows. Theriogenology 76:1568–1582.

Wiltbank, M. C., A. H. Souza, P. A. Carvahlo, A. P. Cunha, J. O. Giordano, P. M. Fricke, G. M. Baez, and M. G. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8:70–81.

Wiltbank, M. C., A. H. Souza, P. D. Carvalho, R. W. Bender, and A. B. Nascimento. 2012. Improving fertility to timed artificial insemination by manipulation of circulating progesterone concentrations in lactating dairy cattle. Reprod. Fertil. Dev. 24:238–243.