Increasing length of an estradiol and progesterone timed artificial insemination protocol decreases pregnancy losses in lactating dairy cows

M. H. C. Pereira,* A. D. P. Rodrigues,* R. J. De Carvalho,† M. C. Wiltbank,‡ and J. L. M. Vasconcelos§¹

*Aluno do Programa de Pós-Graduação em Zootecnia, Faculdade de Medicina Veterinária e Zootecnia-Universidade Estadual Paulista, Botucatu 18618-000, Brazil

†Fazendas Reunidas ACP e Filhos, Minas Gerais, Carmo do Rio Claro 37150-000, Brazil

‡Department of Dairy Science, University of Wisconsin, Madison 53706

§Department of Animal Production, São Paulo State University, Botucatu 18168-000, Brazil

ABSTRACT

Our hypothesis was that increasing the length of an estradiol and progesterone (P4) timed artificial insemination (TAI) protocol would improve pregnancy per artificial insemination (P/AI). Lactating Holstein cows ($n = 759$) yielding 31 ± 0.30 kg of milk/d with a detectable corpus luteum (CL) at d -11 were randomly assigned to receive TAI (d 0) following 1 of 2 treatments: (8d) d $-10 =$ controlled internal drug release (CIDR) and 2.0 mg of estradiol benzoate, d $-3 =$ PGF_{2 α} (25 mg of dinoprost tromethamine), d $-2 =$ CIDR removal and 1.0 mg of estradiol cypionate, d 0 = TAI; or (9d) d $-11 =$ CIDR and estradiol benzoate, d $-4 =$ PGF_{2 α} , d $-2 =$ CIDR removal and estradiol cypionate, d 0 TAI. Cows were considered to have their estrous cycle synchronized in response to the protocol by the absence of a CL at artificial insemination (d 0) and presence of a CL on d 7. Pregnancy diagnoses were performed on d 32 and 60. The ovulatory follicle diameter at TAI (d 0) did not differ between treatments (14.7 ± 0.39 vs. 15.0 ± 0.40 mm for 8 and 9 d, respectively). The 9d cows tended to have greater P4 concentrations on d 7 in synchronized cows (3.14 ± 0.18 ng/mL) than the 8d cows (3.05 ± 0.18 ng/mL). Although the P/AI at d 32 [45 (175/385) vs. 43.9% (166/374) for 8d and 9d, respectively] and 60 [38.1 (150/385) vs. 40.4% (154/374) for 8d and 9d, respectively] was not different, the 9d cows had lower pregnancy losses [7.6% (12/166)] than 8d cows [14.7% (25/175)]. The cows in the 9d program were more likely to be detected in estrus [72.0% (269/374)] compared with 8d cows [62% (240/385)]. Expression of estrus improved synchronization [97.4 (489/501) vs. 81% (202/248)], P4 concentrations at d 7 (3.22 ± 0.16 vs. 2.77 ± 0.17 ng/mL), P/AI at d 32 [51.2 (252/489) vs. 39.4% (81/202)], P/AI at d 60 [46.3 (230/489) vs. 31.1% (66/202)], and decreased pregnancy loss [9.3

(22/252) vs. 19.8% (15/81)] compared with cows that did not show estrus, respectively. Cows not detected in estrus with small (<11 mm) or large follicles (>17 mm) had greater pregnancy loss; however, in cows detected in estrus, no effect of follicle diameter on pregnancy loss was observed. In conclusion, increasing the length of the protocol for TAI increased the percentage of cows detected in estrus and decreased pregnancy loss.

Key words: length of the protocol, estrus, pregnancy loss

INTRODUCTION

Programs for estrous cycle synchronization have been used for reproductive management in many parts of the world. These programs involve a series of hormonal treatments designed to synchronize a follicular wave, synchronize corpus luteum (CL) function, and, finally, synchronize ovulation, allowing timed AI (TAI) without the need for detection of estrus (Pursley et al., 1997). Various hormones and timing of protocols have been used, with the most widely used programs using either estradiol (E2) or GnRH at the beginning of the protocol to synchronize emergence of a new follicular wave and E2 or GnRH at the end of the protocol to synchronize ovulation near TAI (Vasconcelos et al., 2011a; Wiltbank et al., 2011a).

Several studies have evaluated the effect of altering the time from initiation of CL regression until TAI on fertility during estrous cycle synchronization programs in beef (Meneghetti et al., 2009; Peres et al., 2009; Bridges et al., 2010) and dairy cattle (Peters and Pursley, 2003; Ribeiro et al., 2012; Pereira et al., 2013a). For example, in dairy cows, a significant ($P < 0.01$) linear trend of an increasing percentage of cows pregnant per AI (P/AI) with increasing time from treatment with prostaglandin F_{2 α} (PGF), to induce CL regression, until treatment with GnRH, to induce synchronized ovulation (8.8% at 0 h; 13.2% at 12 h; 21.4% at 24 h; 28.0% at 36 h) was observed. In *Bos taurus* beef cattle, a summary of results from various studies done in the laboratory of Michael Day (Bridges et al., 2010) sug-

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¹Corresponding author: vasconcelos@fmvz.unesp.br

gested that fertility increased as time from PGF_{2α} until GnRH increased. Similarly, in *Bos indicus* beef cattle synchronized with E2 and progesterone (P4), treatment with PGF_{2α} 4 d before TAI produced an increase in P/AI compared with PGF_{2α} treatment 2 d before TAI [time of controlled internal drug release (CIDR) removal and estradiol cypionate (ECP) treatment]. In suckled *Bos indicus* beef cattle with a CL 4 d before TAI, Meneghetti et al. (2009) reported an increase in P/AI (50.3 vs. 36.1%) when the PGF_{2α} was given 4 d before TAI rather than 2 d before TAI (time of CIDR removal and ECP treatment) of the protocol. In non-lactating *Bos indicus* beef cattle (Peres et al., 2009), treatment with PGF_{2α} 4 d before TAI resulted in lower P4 concentrations on the day of CIDR removal, a larger follicle at TAI, an increased percentage of cows that ovulated to the protocol (85.4% vs. 77.0%), and increased P/AI (52.0 vs. 36.4% for all cows; 60.9 vs. 47.2% for cows that ovulated) compared with cows treated with PGF_{2α} 2 d before TAI. In grazing dairy cows that were presynchronized with 2 PGF_{2α} treatments, increasing the interval from PGF_{2α} until GnRH and TAI from 58 to 72 h increased P/AI in a 5 d CoSynch TAI protocol (Ribeiro et al., 2012).

Recent studies from our laboratory evaluated the interval from PGF_{2α} until TAI in lactating dairy cattle synchronized with an E2 and P4-TAI protocol (Pereira et al., 2013a). Consistent with the results of previous studies using beef cattle, an increased interval between PGF_{2α} and TAI (2 vs. 3 d) increased P/AI in cows that received TAI (19.2 vs. 30.0% at d 60 pregnancy diagnosis), with a more subtle effect in cows that received timed embryo transfer (33.5 vs. 37.9%). The earlier PGF_{2α} treatment (3 d) resulted in a greater proportion of cows with low P4 concentrations (≤ 0.09 ng/mL) at TAI. Reduced P4 concentrations at TAI were associated with increased fertility, as has been observed in previous studies using GnRH-based TAI protocols (Souza et al., 2007; Martins et al., 2011; Giordano et al., 2012). In addition, reducing time from PGF_{2α} until induced ovulation decreased E2 concentrations near TAI, increased short luteal phases, and, at times, decreased P4 concentrations after AI (Vasconcelos et al., 2001; Bridges et al., 2010). Thus, the improved fertility with a longer time period from induction of luteolysis with PGF_{2α} until induction of synchronized ovulation could be related to greater time for CL regression and, thus, lower P4 at TAI or, alternatively, greater time for follicle growth, increased ovulatory follicle size, and increased E2 before synchronized ovulation.

The main hypothesis for the current study was that increasing the length of an estrous cycle synchronization protocol by 1 d would result in ovulation of a larger follicle and increased fertility in lactating dairy

cows. The extra day of protocol length was added in the presence of lower P4 by advancing the time when the P4-releasing vaginal implant was removed after PGF_{2α} treatment. Thus, the experimental design allowed greater time for follicle growth in a lower P4 environment, as well as a longer time for complete CL regression before removal of the P4 implant. In addition to the main hypothesis, we also hypothesized that cows ovulating small follicles would have reduced fertility in either protocol. This study used the E2 and P4-based TAI program that is commonly used in Brazil and many other parts of the world.

MATERIALS AND METHODS

This study used only cycling cows in the cooler time of the year in Brazil to optimize the opportunity to observe any effects of protocol length or follicle size on fertility. This experiment was conducted at 3 commercial dairy farms in Minas Gerais, Brazil, from June to November 2011. All animal procedures followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999). During the experimental period, cows were housed in freestall barns with access to an adjoining sod-based paddock. Throughout the experiment, cows were milked 3 times daily. All procedures, including injections, ovarian ultrasonography, pregnancy diagnosis, blood collection, and TAI, were performed while cows were restrained in self-locking head gates at the feedline. Cows were fed a TMR based on corn silage and Tifton hay as forages ad libitum with a corn-soybean meal-based concentrate and minerals and vitamins, which were balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

Animals and Treatments

This study used a total of 759 lactating Holstein cows that were synchronized with an E2 or P4-based TAI program that is commonly used in Brazil and many other parts of the world. At the beginning of the experiment (d -11), cows averaged 153 ± 3.8 DIM, yielding 31.0 ± 0.30 kg of milk/d, with a BCS of 2.84 ± 0.02 [1 (emaciated) to 5 (obese) scale; Wildman et al., 1982], a lactation number of 1.82 ± 0.05 [primiparous (1), $n = 473$; multiparous (≥ 2), $n = 286$], and had been bred 2.16 ± 0.09 times. Within each farm, cows were blocked by parity (primiparous and multiparous); all cows that were past the voluntary waiting period and not pregnant were used and randomized into the study, without regard to whether they had been previously used in the study. Within each block, 1,101 cows were scanned to

Figure 1. The ovaries were evaluated by ultrasound (US) in all cows on d -11 and cows with a detected corpus luteum were randomly assigned to begin the protocol on d -11 (9d) or the next day (8d). The 8-d protocol included a controlled internal drug release (CIDR) and 2.0 mg of estradiol benzoate (EB); 7 d later cows received PGF_{2α}; 24 h later the CIDR was removed and cows received 1.0 mg of estradiol cypionate (ECP); finally, 48 h after CIDR removal, timed AI (TAI) was performed. The 9-d protocol included CIDR and 2.0 mg of EB; 7 d later cows received PGF_{2α} and 48 h later the CIDR was removed and cows received 1.0 mg of ECP; 48 h after CIDR removal, TAI was performed. P4 = blood sampling and analysis of progesterone concentration (sample on d -11 and d 7).

detect the presence of a detectable CL (d -11); cows with a CL (759) were randomly assigned to receive TAI (d 0) with 1 of 2 treatments (Figure 1). The 8-d treatment (**8d**) consisted of an intravaginal CIDR P4 insert containing 1.9 g of P4 (Zoetis, São Paulo, Brazil) that was new, second use (previously used for 8 d), or third use (previously used for 16 d), and 2.0 mg (i.m.) of estradiol benzoate (**EB**; 2.0 mL of Estrogin, Farmavet, São Paulo, SP, Brazil) on d -10; 25 mg (i.m.) of dinoprost tromethamine (PGF_{2α}; 5.0 mL of Lutalyse, Zoetis) on d -3; CIDR withdrawal and 1.0 mg (i.m.) of estradiol cypionate (**ECP**; 0.5 mL, Zoetis) on d -2; and TAI on d 0. The 9-d treatment (**9d**) was a CIDR insert and 2.0 mg (i.m.) of EB on d -11, Lutalyse on d -4, CIDR withdrawal and 1.0 mg of ECP on d -2, and TAI on d 0. The protocols started on different days; 9d started on the day of ultrasonography (**US**; d -11) and 8d started 1 d later (d -10). The CIDR removal and ECP treatment were performed at d -2 in both treatments (Figure 1). The TAI was performed on the same day in both treatments (d 0) by experienced technicians using commercial frozen-thawed semen from 23 different bulls. All cows were painted with chalk on their tail heads daily after the CIDR removal. Cows were considered in estrus when they were observed to stand after mounting or when chalk was completely removed.

US

In both groups, ovaries were evaluated by transrectal US (Aloka SSD-500 with a 7.5-MHz linear-array trans-

ducer, Aloka, Tokyo, Japan) at d -11 to determine presence of a CL, at d 0 to measure the diameter of the largest follicle present, and 7 d after TAI to determine the presence of CL. Pregnancy diagnoses were performed by detection (transrectal US) of an embryo on d 32 or a fetus on d 60. Cows were considered to have their estrous cycle synchronized in response to the protocol by the absence of a CL at AI (d 0) and presence of a CL on d 7. Pregnancy per AI was calculated dividing the number of pregnant cows on d 32 or 60 by the number of cows that were inseminated. Pregnancy loss from 32 to 60 d was calculated dividing the number of cows that lost their pregnancy by the number of pregnant cows at d 32.

Sample Collection

Rectal temperature was measured in all cows using a digital thermometer (Jumbo Display Lab Thermometer, Delta Trak, Pleasanton, CA) in the morning at d 0 and 7. Heat stress in cows was defined by rectal temperature ($<39.1^{\circ}\text{C}$ = no heat stress; $\geq 39.1^{\circ}\text{C}$ = heat stress), given that 39.1°C is considered a threshold for heat stress in dairy cattle (Berman et al., 1985; West, 2003).

Milk production was measured daily between d 0 and 7, and average daily production through this interval was used in the analysis. Blood samples were collected on d -11 ($n = 753$) and 7 ($n = 742$) by coccygeal venipuncture into commercial, 10-mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). After bleeding, tubes were placed on ice immediately,

maintained at 4°C for 12 h, and centrifuged at $1,500 \times g$ for 15 min at room temperature for serum collection. Serum was stored at -20°C for subsequent P4 analysis. Serum concentrations of P4 were analyzed using the Coat-A-Count solid phase ^{125}I radioimmunoassay kit (DPC Diagnostic Products Inc., Los Angeles, CA) that had been previously validated in our laboratory (Santos and Vasconcelos, 2006). The intra-assay coefficient of variation was 3.98%, the interassay coefficient of variation was 11.34%, and the assay sensitivity was 0.02 ng/mL.

Statistical Analysis

The experiment was analyzed as a complete randomized design. The binomial variables (estrus expression, estrous cycle synchronization, P/AI on d 32 and 60, and pregnancy losses from 32 to 60 d) were analyzed using the GLIMMIX procedures of SAS (SAS Institute Inc., Cary, NC) with farm as a random effect and other variables included in the models as appropriate, including effects of treatment, parity, and their interactions, as well as DIM, BCS, CIDR use, and milk yield as covariates. The continuous dependent variables (i.e., lactation number, DIM, BCS, milk production, previous AI number, temperature at AI, temperature at d 7, P4 concentrations at d -11 and 7, and follicular diameter at d 0) were analyzed using the MIXED procedures of SAS; other variables included in the models were the effects of treatment, parity, and their interactions, as well as DIM, BCS, CIDR use, and milk yield as covariables. The GLM procedure of SAS was used to determine if each individual measurement influenced pregnancy rates linearly, quadratically, cubically, or all. The LOGISTIC procedure was used to determine the intercept and slope(s) values according to maximum likelihood estimates from each significant continuous

order effect, and the probability of pregnancy was determined according $\text{Probability} = (e^{\text{logistic equation}}) / (1 + e^{\text{logistic equation}})$. Logistic curves were constructed according to the minimum and maximum values detected for each individual measurement.

Satterthwaite approximation was used to determine the denominator degrees of freedom for the tests of fixed effects with the random statement containing the effect of group. Covariables found to be not significant ($P > 0.10$) were removed from the statistical model. The results are expressed as $\text{LSM} \pm \text{SEM}$ for continuous variables. In all analyses, differences were considered significant when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a statistical tendency.

RESULTS

As summarized in Table 1, no differences ($P > 0.10$) were found between treatment groups on lactation number, DIM, milk production, BCS, and previous AI number. All cows included in the study had a visible CL at the beginning of the study (d -11) and circulating P4 was not different ($P = 0.20$) between treatment groups (Table 1). No difference ($P > 0.10$) was observed in temperature at AI or on d 7 after AI between treatment groups (Table 1). No difference ($P = 0.30$) was noted between treatment groups in average ovulatory follicle size in synchronized cows. However, a tendency was observed for greater circulating P4 on d 7 after AI in 9d cows as opposed to 8d cows, either when all cows ($P = 0.09$) were included or when only synchronized cows ($P = 0.06$) were included in the analysis (Table 1).

The 9d cows were more likely ($P < 0.01$) to be detected in estrus compared with 8d cows (Table 2). A high percentage of cows had their estrous cycle synchronized to either protocol (>90%), with only 3 cows with a visible CL at the time of AI and 55 cows with no CL on d 7 after AI. No difference was observed between groups ($P = 0.52$) in the percentage of cows that had their estrous cycle synchronized (Table 2). The overall double ovulation rate was 13.6% with no treatment effects ($P = 0.78$) on the percentage of cows that had double ovulation (Table 2). Both protocols resulted in a high P/AI for d 32 pregnancy diagnosis in cows with their estrous cycle synchronized (~48%) and at d 60 (>40%), with no difference ($P > 0.10$) between protocols (Table 2). Nevertheless, pregnancy losses between 32 and 60 d of pregnancy were decreased ($P = 0.03$) for 9d cows (7.8%) compared with 8d cows (15.2%; Table 2).

Only cows with a CL detected by US at the beginning of the protocol were used in this study. The P4 concentration at the start of the protocol (d -11) had

Figure 2. Treatment effects by progesterone concentration at the beginning of the protocol (d -11) on pregnancy per AI (P/AI) rate at the d 60 pregnancy diagnosis in dairy cows that had their estrous cycle synchronized in response to the protocol (corpus luteum at d 7). An effect of progesterone concentration on P/AI was observed in cows receiving the 9-d ($P = 0.03$) but not the 8-d ($P = 0.88$) protocol.

Table 1. Comparison of various measures for cows treated with protocols of either 8 or 9 d in length (LSM $\pm$ SEM with n in parentheses)

Item	Protocol length		P-value
	8 d	9 d	
Lactation number ¹	1.76 $\pm$ 0.68 (385)	1.88 $\pm$ 0.70 (374)	0.21
DIM ¹	152.0 $\pm$ 9.05 (385)	150.1 $\pm$ 9.09 (374)	0.92
Milk production ¹ (kg/d)	30.7 $\pm$ 0.65 (384)	30.7 $\pm$ 0.66 (374)	0.90
BCS ¹	2.85 $\pm$ 0.04 (385)	2.85 $\pm$ 0.04 (374)	0.83
Previous AI number ¹	2.06 $\pm$ 0.32 (385)	2.06 $\pm$ 0.32 (374)	0.99
Progesterone on d -11 ¹ (ng/mL; protocol start)	3.92 $\pm$ 0.35 (382)	4.14 $\pm$ 0.35 (371)	0.20
Temperature (°C) at AI ¹ (d 0)	38.6 $\pm$ 0.12 (384)	38.6 $\pm$ 0.12 (374)	0.56
Temperature (°C) 7 d post-AI ¹ (d 7)	38.6 $\pm$ 0.07 (364)	38.6 $\pm$ 0.07 (360)	0.94
Ovulatory follicle ² (mm)	14.7 $\pm$ 0.39 (312)	15.0 $\pm$ 0.40 (280)	0.30
Progesterone at d 7 ¹ (ng/mL)	2.88 $\pm$ 0.20 (377)	3.07 $\pm$ 0.20 (364)	0.09
Progesterone at d 7 ² (ng/mL)	3.05 $\pm$ 0.18 (351)	3.14 $\pm$ 0.18 (336)	0.06

¹All inseminated cows.²Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.

no effect ($P = 0.88$) on P/AI at d 60 pregnancy diagnosis for 8d cows; however, an effect ($P = 0.03$) of P4 concentration at d -11 was found for P/AI at 60 d in cows with their estrous cycle synchronized by the 9d protocol (Figure 2). In cows with P4 < 4.0 ng/mL at d -11, the 9d protocol tended ($P = 0.10$) to have greater P/AI at 60 d [46.8 (89/187) vs. 38.6% (77/192) for 9d and 8d, respectively]. In cows with P4 $\geq$ 4.0 ng/L at d -11, the P/AI did not differ ($P = 0.75$) between treatments [41.2 (62/150) vs. 42.7% (68/159) for 9d and 8d, respectively].

Treatment did not have an effect ($P = 0.30$) on average follicle diameter (Table 1); however, the distribution of follicle diameters was altered by treatment (Table 3). Although a similar percentage of small (≤ 11 mm) and large (> 17 mm) ovulatory follicles were observed, a greater percentage ($P = 0.01$) of 8d cows had

a medium follicle diameter (11.1–17 mm) compared with 9d cows. Conversely, more ($P = 0.02$) 9d cows had premature ovulation (ovulation before TAI) than 8d cows. Circulating P4 on d 7 after TAI (Table 3) increased ($P < 0.01$) with increasing follicle diameter at TAI. No differences ($P > 0.10$) in circulating P4 were observed between treatment groups in any of the follicle classes, except for greater ($P < 0.01$) P4 in prematurely-ovulating cows for 9d versus 8d cows. Follicle diameter at TAI affected ($P \leq 0.05$) P/AI at 32 and 60 d in both treatments for cows that had their estrous cycle synchronized (Table 3; Figure 3). Lower fertility was noted in cows that ovulated too small (≤ 11 mm) or too large (> 17 mm) follicles (Figure 3). The cows with premature ovulation had similar fertility as the cows that ovulated large (> 17 mm) follicles. The pregnancy losses did not differ ($P = 0.96$) between

Table 2. Effects of protocol length on expression of estrus, estrous cycle synchronization, double ovulation, and pregnancy per AI (P/AI) for all cows and only for cows that had their estrous cycle synchronized (LSM with n/n in parentheses)

Item	Protocol length		P-value
	8 d	9 d	
Expression of estrus ¹	63.4 (240/385)	73.0 (269/374)	<0.01
Estrous cycle synchronization ²	92.8 (352/379)	91.5 (339/370)	0.52
Double ovulation ¹	12.3 (43/352)	15.1 (51/337)	0.26
P/AI ¹			
At 32 d	45.0 (175/385)	43.9 (166/374)	0.77
At 60 d	38.1 (150/385)	40.4 (154/374)	0.52
Pregnancy loss	14.7 (25/175)	7.6 (12/166)	0.04
P/AI ²			
At 32 d	48.1 (170/352)	47.9 (163/339)	0.96
At 60 d	40.5 (145/352)	43.9 (151/339)	0.37
Pregnancy loss	15.2 (25/170)	7.8 (12/163)	0.03

¹All inseminated cows.²Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.

treatments in cows ovulating small follicles (<11 mm). However, in cows ovulating follicles between 11.1 and 17 mm, the 9d protocol tended to have lower ($P = 0.08$) pregnancy loss. When cows that ovulated larger follicles were grouped with cows that had premature ovulation, the 9d protocol tended ($P = 0.08$) to have lower pregnancy loss [9.6 (5/52) vs. 22.9% (8/35) for 9d and 8d, respectively].

Expression of estrus (Table 4) increased ($P < 0.01$) the percentage of cows with CL on d 7, increased ($P < 0.01$) circulating P4 concentrations on d 7, and increased ($P < 0.01$) P/AI at the d 32 and 60 pregnancy diagnoses in synchronized cows in both treatments. No interaction was detected between expression of estrus and treatment for any of these variables. A greater pregnancy loss from 32 to 60 d ($P < 0.01$) was observed in cows that did not show estrus.

Follicle diameter at TAI affected ($P < 0.01$) P/AI at the d 32 and 60 pregnancy diagnoses in synchronized cows, independent of whether cows were detected in estrus (Figure 4). In cows not detected in estrus, the follicle diameter had an effect ($P = 0.01$) on pregnancy loss (Figure 5); however, in cows that were detected in

Figure 3. Effect of follicle diameter at time of AI (d 0) on pregnancy per AI (P/AI) rate at 32 and 60 d and pregnancy loss (P. Loss) in dairy cows that had their estrous cycle synchronized in response to the protocol (corpus luteum at d 7). Significance was set at $P < 0.01$ for 32 d, $P < 0.01$ for 60 d, and $P = 0.15$ for P. Loss.

estrus, the follicle diameter did not have effect ($P = 0.97$) on pregnancy loss from 32 to 60 d.

Heat stress was observed in 27% (196/723) of cows during the experiment (at least one measurement $\geq 39.1^\circ\text{C}$). Cows with heat stress had a similar ($P = 0.80$)

Table 3. Effects of protocol length (8 or 9 d) and follicle diameter at timed AI on progesterone (ng/mL) at d 7, pregnancy per AI (P/AI) at d 32, d 60, and pregnancy losses (32–60 d) for synchronized dairy cows (LSM with n/n in parentheses)

Item	Follicle diameter (mm) at d 0				P-value
	8–11	11.1–17	>17	Ovulated	
Distribution ¹					
8 d	9 (33/352)	62 (217/352)	17 (60/352)	12 (42/352)	—
9 d	9 (33/339)	53 (178/339)	20 (68/339)	18 (60/339)	—
P-value	0.87	0.01	0.29	0.02	
Estrus expression ¹					
8 d	66.7 (22/33)	63.8 (137/218)	72.9 (43/60)	—	0.44
9 d	84.9 (28/33)	74.9 (132/178)	72.9 (49/68)	—	0.35
P-value	0.08	0.02	0.99		
Progesterone at d 7 ¹ (ng/mL)	2.67 $\pm$ 0.22	2.97 $\pm$ 0.17	3.40 $\pm$ 0.19	3.50 $\pm$ 0.20	<0.01
8 d	2.57 $\pm$ 0.27	2.96 $\pm$ 0.18	3.43 $\pm$ 0.23	3.08 $\pm$ 0.25	0.01
9 d	2.78 $\pm$ 0.26	2.99 $\pm$ 0.16	3.37 $\pm$ 0.21	3.78 $\pm$ 0.21	<0.01
P-value	0.51	0.91	0.76	<0.01	
P/AI at d 32 ¹	40.8 ^b	55.3 ^a	36.5 ^b	39.1 ^b	<0.01
8 d	42.4 (14/33) ^{ab}	55.6 (121/217) ^a	34.7 (21/60) ^b	33.0 (14/42) ^b	<0.01
9 d	39.4 (13/33) ^y	55.1 (98/178) ^{a,x}	38.2 (26/68) ^b	43.3 (26/60) ^{ab}	0.05
P-value	0.53	0.89	0.71	0.32	
P/AI at d 60 ¹	37.2 ^y	49.1 ^{a,x}	29.6 ^b	33.8 ^b	<0.01
8 d	38.8 (13/33) ^{ab}	47.5 (105/217) ^a	25.6 (16/60) ^b	25.5 (11/42) ^b	<0.01
9 d	36.4 (12/33) ^y	51.7 (92/178) ^{a,x}	33.8 (23/68) ^b	40.0 (24/60) ^{ab}	0.04
P-value	0.56	0.51	0.38	0.15	
Pregnancy loss ¹	7.9	10.4	17.3	12.7	0.52
8 d	7.7 (1/14)	14.4 (16/121)	22.8 (5/21)	22.0 (3/14)	0.54
9 d	8.9 (1/13)	6.2 (6/98)	11.8 (3/26)	7.9 (2/26)	0.81
P-value	0.96	0.08	0.28	0.22	

^{a,b}Values within a row with different superscript letters differ ($P < 0.05$).

^{x,y}Values within a row with different superscript letters tended to differ ($0.05 < P \leq 0.1$).

¹Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.

Table 4. Effects of expression of estrus on estrous cycle synchronization, progesterone on d 7, pregnancy per AI (P/AI) at d 32 and 60, and pregnancy loss from 32 to 60 d in synchronized cows (LSM $\pm$ SEM with n/n in parentheses)

Item	Estrus		P-value
	No	Yes	
Ovulatory follicle diameter ¹ (mm)	14.6 $\pm$ 0.37	14.8 $\pm$ 0.33	0.69
Estrous cycle synchronization ¹	81.0 (202/248)	97.4 (489/501)	<0.01
Progesterone on d 7 ¹	2.77 $\pm$ 0.17	3.22 $\pm$ 0.16	<0.01
P/AI at d 32 ¹	39.4 (81/202)	51.2 (252/489)	<0.01
P/AI at d 60 ¹	31.1 (66/202)	46.3 (230/489)	<0.01
Pregnancy loss ¹	19.8 (15/81)	9.3 (22/252)	<0.01
Pregnancy loss ¹			
No heat stress	10.7 (6/56)	7.6 (14/185)	0.45
With heat stress	36.0 (9/25)	14.3 (8/57)	0.02
P-value	<0.01	0.14	

¹Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.

percentage of cows with a CL on d 7 [91.3 (180/196) vs. 91.4% (487/527)] and similar P/AI in synchronized cows at 32 d [44.6 (82/180)] vs. 49.2% (241/487); $P = 0.38$] compared with cows without detected heat stress (all measurements $\leq 39.0^\circ\text{C}$), respectively. However, at the d 60 pregnancy diagnosis, lower P/AI ($P < 0.01$) was observed in cows with at least one temperature measurement $> 39.0^\circ\text{C}$ [33.2 (65/180) vs. 44.6% (221/487)]. This was due to greater ($P < 0.01$) pregnancy loss in cows with detected heat stress [23.8 (17/82) vs. 9.4% (20/241)]. An interaction of estrus and heat stress was detected on pregnancy loss from 32 to 60 d ($P = 0.04$), in which heat-stressed cows that did not show estrus had the greatest pregnancy loss (Table 4).

A tendency ($P = 0.06$) was observed for the P4 concentration 7 d after TAI to have an effect on P/AI. Increasing P4 concentrations on d 7 after AI was associated with increasing P/AI (Figure 6).

Figure 4. Effect of expression of estrus and follicle diameter at time of AI (d 0) on pregnancy per AI (P/AI) rate at 32 (panel A) and 60 d (panel B) pregnancy diagnosis in dairy cows that had their estrous cycle synchronized (corpus luteum on d 7). Significance was set at $P = 0.09$ for 32 d with no estrus, $P < 0.01$ for 32 d with estrus, $P = 0.03$ for 60 with no estrus, and $P < 0.01$ for 60 with estrus.

DISCUSSION

The current study evaluated whether increasing the length of time with reduced circulating P4 would increase fertility in a TAI program that used E2 and P4 to synchronize the estrous cycle of cycling lactating dairy cows. The use of only lactating cows with a CL at the beginning of the protocol during the cooler months of the year produced a high percentage of cows that responded to the protocols with ovulation ($>90\%$) and excellent fertility ($>40\%$). We postulated that increasing the length of time from PGF_{2α} treatment until TAI by 1 d would allow more time for follicle development and thereby increase expression of estrus and follicle diameter at the time of AI. Consistent with this hypothesis, an increase was observed in the percentage of cows detected in estrus in the longer compared with the shorter protocol. Expression of estrus was associated with better synchronization and reduced pregnancy losses from 32 to 60 d. Nevertheless, no detectable increase in follicle diameter was noted, although the

Figure 5. Effect of expression of estrus and follicle diameter at time of AI (d 0) on pregnancy loss between 32 and 60 d in dairy cows that had a synchronized estrous cycle (corpus luteum on d 7). Significance was set at $P = 0.01$ for no estrus and $P = 0.97$ for estrus.

Figure 6. Effect of progesterone concentration on d 7 after AI on pregnancy per AI (P/AI) rate at 60 d in synchronized dairy cows with progesterone ≥ 1.0 ng/mL on d 7; $P = 0.06$.

distribution of follicle sizes was altered. Our main hypothesis was that the longer protocol would result in greater fertility. Although, no overall change in P/AI was detected, we observed a decrease in pregnancy loss from 32 to 60 d with the longer protocol. This result highlights the importance of pregnancy loss as a critical reproductive measure in lactating dairy cows, and the strong association we detected between pregnancy loss and lack of estrus, as well as greater follicle size, potentially facilitates a greater understanding of the underlying causes of pregnancy loss. Our final hypothesis, that follicle size at TAI would be associated with differences in fertility, was clearly supported by our results with ovulation of larger and smaller follicles associated with reduced fertility. Thus, increasing protocol length from 8 to 9 d seemed to have a positive effect, with increased expression of estrus and reduced pregnancy loss; however, variation in responses of individual cows to either protocol resulted in a substantial number of cows that did not express estrus and did not have optimal follicle size. Examination of more than 700 cows for some individual ovarian, hormonal, and reproductive measures provides some insight into potential future improvements that could be made in these protocols.

The majority of cows expressed estrus during the protocols (70%; 411/590), even cows that ovulated smaller follicles (≤ 11 mm; 76%; 50/66), probably due to the ECP treatment used to induce ovulation at the end of the protocol. Our previous research has demonstrated that cows induced to ovulate with ECP have increased expression of estrus compared with cows induced to ovulate with GnRH (Pereira et al., 2013b). In addition, increasing the protocol length increased expression of estrus (63 vs. 73% for 8d and 9d, respectively), possibly due to greater endogenous E2 production or reduced circulating P4 near TAI. The longer protocol would

allow greater time for follicle growth, possibly increasing E2, and greater time for CL regression, reducing circulating P4 (Pereira et al., 2013a).

Of particular importance, expression of estrus was associated with greater fertility in our study, consistent with previous observations of improved fertility in cows showing estrus during TAI protocols (Pancarci et al., 2002; Kasimanickam et al., 2005; Souza et al., 2007). For example, in the Heatsynch protocol (d -10 GnRH, d -3 PGF_{2α}, d -2 ECP, and d 0 TAI), cows that displayed estrus after the ECP treatment had greater P/AI [42.5% (306)] than cows that did not display estrus [21.1% (71)] at TAI (Cerri et al., 2004). In a different study using the Heatsynch protocol, expression of estrus improved P/AI at 27 (43.6 vs. 17.0%; $P < 0.01$) and 41 d (36.6 vs. 12.0%; $P < 0.01$) compared with cows not detected in estrus (Galvão et al., 2004). Additionally, cows displaying estrus in programs with Co-Synch at 48 or 72 h after the PGF_{2α} treatment had greater P/AI at 40 (54.7 vs. 31.5%) and 68 d (53.3 vs. 31.5%) after AI (Hillegass et al., 2008). In our study, the improvement in fertility at 32 (~30% more pregnancies; 51.2/39.4) and 60 d (~50% more pregnancies; 46.3/31.1) was substantial. Previously, Ribeiro et al., (2012) altered the length of proestrus by changing the interval to GnRH treatment and TAI in a 5-d Ovsynch protocol from 72 h after PGF_{2α} treatment (Cos 72) compared with 58 h (Cos 58). Greater length of proestrus increased E2 concentrations near TAI ($P = 0.04$), but did not affect P/AI at d 30; however, at the d 65 pregnancy diagnosis, Cos 72 had greater fertility than Cos 58 ($P < 0.05$). We also observed a major reduction (~50%; 19.8/9.3) of pregnancy loss in cows that showed estrus in our study. Similarly, Galvão et al. (2004) reported that cows detected in estrus had lower pregnancy loss than cows not in estrus [16.1 (224) vs. 29.6% (27) for estrus and no estrus, respectively]. In addition, in a previous study done during summer heat stress in Brazil, reduced pregnancy loss ($P = 0.05$) was observed for an E2 and P4 protocol that used ECP to synchronize ovulation [11% (16/135)] compared with the 5-d Cosynch protocol that uses GnRH to synchronize ovulation [19.6% (24/119)]. Our study did not allow us to determine the mechanism that results in lower fertility and greater pregnancy loss in cows that did not demonstrate estrus. It is intriguing to speculate that insufficient E2 near TAI may alter uterine gene and protein expression, producing an environment that is less compatible with maintenance of pregnancy, as previously described in various species (Miller et al., 1977; Bartol et al., 1981; Buhi, 2002). A recent study in beef cattle has shown an effect of E2 near TAI on P/AI at the d 27 pregnancy diagnosis, but did not observe an effect of E2 on subsequent pregnancy loss (Jinks et al.,

2013). However, a recent study in ovariectomized beef cattle reported that cows that did not receive E2 in the preovulatory period maintained pregnancy until d 19, based on expression of interferon tau-stimulated gene expression; by d 29, though, they had reduced pregnancies compared with cows that received either ECP or EB to increase circulating E2 during the preovulatory period (Roberts et al., 2012). It seems possible that the high pregnancy loss observed in dairy cows compared with beef cattle may be due to high E2 metabolism and reduced E2 concentrations near estrus or timed ovulation in lactating dairy cows (Sangsritavong et al., 2002; Vasconcelos et al., 2003; Wiltbank et al., 2006). Greater sensitivity of the uterine environment in a lactating dairy cow to the preovulatory effects of E2 might also explain the differences. In our study, ECP was given to increase circulating E2 in the preovulatory period; however, added E2 from the follicle may be required to produce estrus and an optimal uterine environment. Alternatively, a greater time for CL regression and reduced circulating P4 near TAI may increase expression of estrus and potentially improve pregnancy maintenance by enhancing the uterine environment. Development of more optimized protocols, potentially demonstrated by increased expression of estrus, should improve P/AI and reduce pregnancy losses.

Optimization of ovulatory follicle size has been an important objective in development of protocols that use either GnRH or E2 to synchronize ovulation near TAI (Perry et al., 2005; Souza et al., 2009; Pereira et al., 2013b). In the present study, an effect of follicle diameter on fertility was observed in either protocol. Consistent with previous studies (Vasconcelos et al., 2001; Souza et al., 2007; Wiltbank et al., 2011a), fertility was reduced for cows that ovulate smaller follicles (<11 mm) and larger follicles (>17 mm). This effect of follicle diameter was detected at both d 32 and 60 pregnancy diagnoses, in both treatment protocols, and in cows with or without expression of estrus.

Induced ovulation of small follicles using GnRH was associated with reduced fertility, reduced circulating E2 concentrations before AI, reduced P4 concentrations after AI, and an increased percentage of cows exhibiting short luteal phases following AI (Vasconcelos et al., 2001). In beef cattle, GnRH-induced ovulation of smaller follicles (<11.3 mm) resulted in reduced circulating E2 at AI, reduced P/AI, and greater pregnancy loss (Perry et al., 2005). In contrast to GnRH-induced ovulation, pregnancy outcome following spontaneous estrus and ovulation was not affected by follicle diameter (Perry et al., 2005). In our study, relatively few cows (<10%) ovulated small follicles (≤ 11 mm), and these cows tended to have lower P/AI. Recent research in which single embryos were collected and transferred

in beef cows induced to ovulate either smaller or larger follicles using GnRH (Atkins et al., 2013; Jinks et al., 2013) showed that the concentration of E2 at GnRH-induced ovulation (second GnRH) in the recipient cows was one of the most important factors that determined pregnancy outcome (Atkins et al., 2013). Similarly, circulating E2 at GnRH-induced ovulation (second GnRH) in the recipient cows, but not the donor cows, was predictive of pregnancy success at d 27 of gestation (Jinks et al., 2013). Additionally, administration of ECP 24 h before AI increased pregnancy success in cows induced to ovulate a smaller dominant follicle (<12.2 mm). Thus, the primary benefit of increased preovulatory E2 is mediated through alterations in the maternal environment of the recipient cows. Whether the inadequate E2 is responsible for reduced reproductive success in lactating dairy cows ovulating smaller follicles following an ECP-induced ovulation remains to be determined.

Alternatively, ovulation of larger follicles is also associated with reduced fertility in lactating dairy cows (Townson et al., 2002; Bleach et al., 2004; Cerri et al., 2009). An interesting paradox is that ovulation of a larger follicle is associated with lower fertility, but is also associated with greater circulating P4 after AI. Greater P4 after AI has, at times, been associated with greater fertility (Demetrio et al., 2007; Wiltbank et al., 2011b; Forro et al., 2012). Optimization of follicle size in TAI programs is clearly an intricate balance between oocyte quality, adequate circulating E2 near AI, and adequate circulating P4 after AI.

Surprisingly, pregnancy loss was not associated with ovulation of smaller follicles (<11 mm), but was more associated with ovulation of larger follicles, particularly in the absence of expression of estrus and reduced length of the protocol. Previous studies have shown reduced or a tendency for reduction in pregnancy loss in cows with expression of estrus (Pereira et al., 2013b), greater circulating E2 near TAI (Souza et al., 2007; Hillegass et al., 2008; Souza et al., 2011), greater circulating P4 before AI (Wiltbank et al., 2011b), and increased length of proestrus (Ribeiro et al., 2012). In the present study, cows that showed estrus had decreased pregnancy losses in cows ovulating all sizes of follicles, even though an effect of follicle size on P/AI was observed using either the d 32 or 60 pregnancy diagnosis. However, the high pregnancy loss in cows that did not show estrus was primarily due to excessive pregnancy loss in cows that ovulated large follicles. In contrast, in a recent study in our laboratory, high pregnancy loss in a GnRH-based protocol was clearly associated with ovulation of smaller follicles (Pereira et al., 2013b). One possible explanation for this unexpected result is that the high pregnancy loss may be due to an interaction of

less than optimal embryo (ovulation of larger follicle) with a less than optimal uterine environment (lack of estrus and reduced E2). This intriguing but speculative idea should be pursued in future research.

Cows with heat stress ($>39.1^{\circ}\text{C}$) had greater pregnancy losses [23.8% (17/82)] than cows without heat stress [9.4% (20/241)]. It is well-established that heat stress can reduce fertility in lactating cows (Vasconcelos et al., 2006; Vasconcelos et al., 2011a; Vasconcelos et al., 2011b; Vasconcelos et al., 2011c). In heat-stressed cows, expression of estrus was associated with reduced pregnancy loss, but expression of estrus did not affect pregnancy loss in non-heat-stressed cows. Our previous results were consistent with E2 near TAI having a positive effect on pregnancy maintenance in heat-stressed cows (Pereira et al., 2013b).

In conclusion, increasing the length of an E2 and P4-based TAI protocol increased expression of estrus and reduced pregnancy loss. Although no overall effect of protocol length on P/AI was observed, clear effects of size of the ovulatory follicle and expression of estrus on P/AI were noted. Our results indicate that improvements in fertility and reductions in pregnancy loss are likely in E2 and P4-based TAI protocols by optimizing the ovulatory follicle diameter, increasing expression of estrus, and optimization of proestrous hormonal environment.

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