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

R. S. Bisinotto,* 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*¹

*Department of Animal Sciences, University of Florida, Gainesville 32611

†College of Veterinary Medicine, University of Florida, Gainesville 32610

ABSTRACT

Objectives were to investigate 2 intervals from induction of ovulation to artificial insemination (AI) and the effect of supplemental progesterone for resynchronization on fertility of lactating dairy cows subjected to a 5-d timed AI program. In experiment 1, 1,227 Holstein cows had their estrous cycles presynchronized with 2 injections of PGF_{2α} at 46 and 60 d in milk (DIM). The timed AI protocols were initiated with GnRH at 72 DIM, followed by 2 injections of PGF_{2α} at 77 and 78 DIM and a second injection of GnRH at either 56 (OVS56) or 72 h (COS72) after the first PGF_{2α} of the timed AI protocols. All cows were time-inseminated at 72 h after the first PGF_{2α} injection. Pregnancy was diagnosed on d 32 and 60 after AI. In experiment 2, 675 nonpregnant Holstein cows had their estrous cycles resynchronized starting at 34 d after the first AI. Cows received the OVS56 with (RCIDR) or without (RCON) supplemental progesterone, as an intravaginal insert, from the first GnRH to the first PGF_{2α}. Pregnancy diagnoses were performed on d 32 and 60 after AI. During experiment 2, subsets of cows had their ovaries scanned by ultrasonography at the first GnRH, the first PGF_{2α}, and second GnRH injections of the protocol. Blood was sampled on the day of AI and 7 d later, and concentrations of progesterone were determined in plasma. Cows were considered to have a synchronized ovulation if they had progesterone <1 and >2.26 ng/mL on the day of AI and 7 d later, respectively, and if no ovulation was detected between the first PGF_{2α} and second GnRH injections during resynchronization. In experiment 1, the proportion of cows detected in estrus at AI was greater for COS72 than OVS56 (40.6 vs. 32.4%). Pregnancy per AI (P/AI) did not differ between OVS56 (46.4%) and COS72 (45.5%). In experiment 2,

cows supplemented with progesterone had greater P/AI compared with unsupplemented cows (51.3 vs. 43.1%). Premature ovulation tended to be greater for RCON than RCIDR cows (7.5 vs. 3.6%), although synchronization of the estrous cycle after timed AI was similar between treatments. Timing of induction of ovulation with GnRH relative to insemination did not affect P/AI of dairy cows enrolled in a 5-d timed AI program. Furthermore, during resynchronization starting on d 34 after the first AI, supplementation with progesterone improved P/AI in cows subjected to the 5-d timed AI protocol.

Key words: 5-d timed artificial insemination, dairy cow, progesterone, reproduction

INTRODUCTION

Reproductive programs based on GnRH and PGF_{2α} for timed AI maximize the proportion of cows inseminated early after the end of the voluntary waiting period and typically result in pregnancy per AI (P/AI) similar to that observed in breeding programs based on estrous detection (Chebel et al., 2006). From the first report of the Ovsynch protocol (d 0 GnRH, d 7 PGF_{2α}, d 9 GnRH, d 10 timed AI; Pursley et al., 1995), synchronization programs have been modified to improve fertility responses of inseminated lactating dairy cows. Recently, the reduction of the interval between the initial GnRH and the PGF_{2α} injections from 7 to 5 d (5-d Cosynch program) was shown to improve P/AI in lactating dairy cows (Santos et al., 2010). Nevertheless, all cows in that particular experiment were inseminated simultaneously with the final GnRH, which was administered 72 h after the injection of PGF_{2α}. Pursley et al. (1998) suggested that maximum P/AI in the conventional Ovsynch protocol was achieved when the interval between the final GnRH and AI was 16 h. In fact, when the interval between synchronization of follicular wave with GnRH and injection of PGF_{2α} to promote luteolysis was 7 d, induction of ovulation with the final GnRH 16 h before AI improved fertility of

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¹Corresponding author: jepsantos@ufl.edu

dairy cows compared with GnRH concurrent with AI (Brusveen et al., 2008).

When cows are subjected to the 5-d program, the period of ovulatory follicle development is reduced by approximately 2 d compared with conventional programs (Santos et al., 2010). This reduction results in ovulatory follicles of smaller diameter, reduced concentration of estradiol in plasma, and a smaller proportion of cows in estrus at AI compared with cows in the conventional 7-d program (Santos et al., 2010). Although insemination of dairy cows 4 to 16 h after the onset of estrus (Dransfield et al., 1998) or 16 h after induction of ovulation (Pursley et al., 1998; Brusveen et al., 2008) improved P/AI, this response might not be the same in cows subjected to a shorter period of ovulatory follicle maturation in the 5-d program. The additional 16 h of follicle development might result in increased concentrations of estradiol in plasma and proportion of cows in estrus at AI, which has been linked with fertility of cows in timed AI programs (Santos et al., 2010). In a series of experiments with beef cows subjected to the 5-d program, extending proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). Therefore, the improved synchrony between ovulation/oocyte competence and numbers of viable sperm available for fertilization (Saacke, 2008) might be offset by the shortened proestrus and reduced proportion of cows in estrus when GnRH is given 56 compared with 72 h after luteolysis. The hypothesis for experiment 1 was that induction of ovulation concurrent with insemination would not compromise P/AI of lactating dairy cows subjected to a 5-d synchronization protocol compared with the administration of the final GnRH 16 h before AI.

After the first postpartum AI, 55 to 65% of the cows remain nonpregnant. These cows should receive a new insemination soon after the nonpregnancy diagnosis; however, the stage of the estrous cycle at which the resynchronization protocol is initiated affects ovarian responses to hormonal treatments, synchrony of ovulation and, consequently, P/AI (Vasconcelos et al., 1999). During resynchronized inseminations, the stage of the estrous cycle at the beginning of the protocol is generally unknown. This can influence synchrony of ovulation when cows are inseminated. The use of a controlled internal drug-release (CIDR) containing progesterone improved synchrony of estrous cycle when used in a timed AI protocol (Lima et al., 2009) and increased fertility of dairy cows in the first postpartum AI in some (Chebel et al., 2010) but not all (Lima et al., 2009) studies. The hypothesis for experiment 2 was that supplementation with progesterone during the resynchronization with the 5-d protocol would enhance

synchrony of the estrous cycle and improve fertility responses in lactating dairy cows.

The objectives of this study were to investigate 2 intervals between induction of ovulation relative to AI and supplemental progesterone during resynchronization on fertility when lactating dairy cows are subjected to a 5-d timed AI program.

MATERIALS AND METHODS

Experiment 1

Cows, Housing, and Diets. Lactating Holstein cows, 498 primiparous and 769 multiparous, from a single dairy herd in Central Florida were assigned to the study. Primiparous and multiparous cows were housed separately in freestall barns equipped with sprinklers and fans. Inseminations were performed from January 1 to April 9, 2009. Primiparous and multiparous cows received the same TMR to meet or exceed the nutrient requirements for a lactating Holstein cow producing 45 kg of milk per day with 3.5% fat and 3.2% true protein (NRC, 2001). Cows were fed twice daily and milked thrice daily. Diet consisted of rye grass silage, corn silage, alfalfa hay, ground corn, citrus pulp, soybean meal, molasses, minerals, and vitamins.

Treatments. Cow enrollment lasted 15 wk, and in a given week all cows at 46 ± 3 DIM were blocked by parity such that each block had either 2 primiparous or 2 multiparous cows. Within each block, a cow was randomly assigned to 1 of 2 treatments (Figure 1). Cows had their estrous cycles presynchronized with 2 i.m. injections of 25 mg of PGF_{2 α} (Lutalyse, 5 mg/mL dinoprost tromethamine sterile solution, Pfizer Animal Health, New York, NY) given 14 d apart, at 46 ± 3 and 60 ± 3 DIM. At 72 ± 3 DIM, cows received an i.m. injection of 100 μ g of GnRH (Cystorelin, 50 μ g/mL of gonadorelin diacetate tetrahydrate, Merial Ltd., Duluth, GA) followed by injections of PGF_{2 α} 5 and 6 d later (Santos et al., 2010). Cows then received either 1) a second injection of GnRH 56 h after the first PGF_{2 α} of the synchronization protocol and timed AI 16 h later (**OVS56**, n = 634; 241 primiparous and 393 multiparous) or 2) a second injection of GnRH and timed AI at 72 h after the first PGF_{2 α} of the synchronization protocol (**COS72**, n = 593; 236 primiparous and 357 multiparous). Cows were painted with chalk on their tailheads daily after the first GnRH of the timed AI protocol, and removal of chalk was used as an indication of estrus. Forty of the original 1,267 cows were observed in estrus and inseminated before the scheduled timed AI; therefore, they did not receive any of the treatments evaluated in the present experiment. These

Figure 1. Diagram of activities during experiment 1. COS72 = Cosynch-72, induction of ovulation with GnRH concurrent with AI; OVS56 = Ovsynch-56, induction of ovulation with GnRH 16 h before AI.

cows were not included in the statistical analyses and a total of 1,227 cows remained in the study.

Pregnancy Diagnosis and Calculation of Reproductive Outcomes. Pregnancy was diagnosed by transrectal ultrasonography (Easi-Scan equipped with 7.5-MHz linear transducer, BCF Technology, Livingston, UK) on d 32 after the timed AI. Presence of an amniotic vesicle with a live embryo (i.e., with heart beat) was used as determinant of pregnancy. Pregnant cows on d 32 were re-examined for pregnancy by transrectal palpation 4 wk later, on d 60 of gestation.

Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 32 or 60 d after AI by the number of cows receiving AI. Proportion of pregnancy loss was calculated as the number of cows that lost a pregnancy between d 32 and 60 after AI divided by the number of cows diagnosed pregnant on d 32 after AI.

Milk Yield and BCS. Body condition of all cows was scored (Ferguson et al., 1994) on the day of the first PGF_{2α} of the presynchronization protocol at 46 ± 3 DIM. For analysis of effect of BCS on reproductive outcomes, BCS was categorized as low (≤2.50) or moderate (≥2.75). Yields of milk were recorded for individual cows once monthly, using an on-farm milk meter (Tru-Test Ltd., Manukau, New Zealand). The

average for the first 3 mo postpartum was used in the statistical analysis. Cows were categorized according to milk production above or below the mean value within parity.

Experiment 2

Cows, Housing, and Diets. Lactating Holstein cows, 285 primiparous and 390 multiparous, from a single site in Central Florida were assigned to the study. Cows were housed and fed as described in experiment 1. Inseminations were performed from February 2 to June 4, 2009.

Treatments. Cows diagnosed as nonpregnant by transrectal ultrasonography on d 32 after the first AI postpartum (112 ± 3 DIM) were enrolled in experiment 2. Cows were blocked by parity and method of synchronization at first AI (from experiment 1: AI at estrus, OVS56, or COS72) at 112 ± 3 DIM. Within each block of 2 cows, they were assigned randomly to 1 of 2 treatments (Figure 2). All cows received an injection of GnRH 2 d after the nonpregnancy diagnosis (d 34 after the previous AI), followed by an injection of PGF_{2α} 5 and 6 d after the first GnRH. A second injection of GnRH was administered 56 h after the first PGF_{2α} and insemination was performed

Figure 2. Diagram of activities during experiment 2. BS = blood samples for analysis of progesterone concentration in plasma; CIDR = controlled internal drug-release containing 1.38 g of progesterone; RCIDR = treatment with CIDR; RCON = no treatment with CIDR; US = ultrasonography of ovaries.

16 h later. Cows assigned to control (**RCON**, $n = 334$) did not receive any further treatment, whereas cows assigned to supplemental progesterone (**RCIDR**, $n = 341$) received an intravaginal insert containing 1.38 g of progesterone (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health) from the first GnRH to the first PGF_{2α} of the resynchronization protocol. Cows were painted with chalk on their tailheads daily after the first GnRH of the resynchronization protocol, and removal of chalk was used as indication of estrus. Thirty-nine of the 675 cows were observed in estrus after the first PGF_{2α} of the resynchronization and before the day of timed AI. Because they received the treatments and display of estrus might have been influenced by supplemental progesterone, these cows remained in the statistical analyses.

Ultrasonography of Ovaries. A subset of 576 cows had their ovaries examined on the day of the first GnRH of the resynchronization by transrectal ultrasonography, and the presence of a corpus luteum (**CL**) was recorded to determine if responses to resynchronization treatments differed according to ovarian status of cows. Another subset of cows from both RCON ($n = 173$) and RCIDR ($n = 167$) treatments were scanned during the resynchronization protocol on the days of the first PGF_{2α} and second GnRH injections (Figure 2). A map of each ovary was drawn and the position of follicles

≥ 5 mm in diameter and CL were recorded. Ovulation in response to the first GnRH of the resynchronization protocol was determined as the presence of a newly formed CL on the day of the first PGF_{2α} injection; therefore, it was considered only for cows evaluated at both injections. Premature ovulation was characterized by the disappearance of one or more follicles ≥ 8 mm in diameter present at the first PGF_{2α} and absent at the second GnRH injection.

Blood Sampling and Analysis of Progesterone in Plasma. Blood was sampled from 398 cows by puncture of the coccygeal vein or artery into evacuated tubes containing K₂ EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood was sampled on the day of AI and 7 d later. Samples were immediately cooled by placing them on ice and they arrived at the laboratory within 6 h of collection. Blood tubes were centrifuged at $3,000 \times g$ for 15 min in a refrigerated centrifuge at 4°C for plasma separation. Plasma samples were frozen at -20°C for later analyses.

Concentration of progesterone was analyzed in all plasma samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.1 ng/mL calculated at 2 SD below the mean counts per minute at maximum binding. Samples were analyzed in 3 assays. Moderate (1.5 ng/mL) and high (2.5 ng/mL) concen-

tration samples were incorporated several times in each assay and used to calculate the intraassay CV. The intraassay CV were 5.3, 5.2, and 5.7% for the first, second, and third assays, respectively. The interassay CV was 7.1%.

Evaluation of Protocol Synchronization. Synchronization of the estrous cycle after treatment was evaluated in 409 cows based on 2 criteria. In both cases, cows that had premature ovulation (between PGF_{2α} and final GnRH) were considered to not have a synchronized estrous cycle. First, the standard threshold concentration of 1 ng/mL of progesterone was used to determine if progesterone was low on the day of AI (<1 ng/mL) and high 7 d later (≥ 1 ng/mL). Cows with low and high progesterone on the day of AI and 7 d later, respectively, were considered to have a synchronized estrous cycle.

A second criterion was used based on the cut-point for concentrations of progesterone in plasma that best predicted pregnancy on d 32 after AI. Receiver operator characteristic (ROC) curves were used to determine the concentrations of progesterone at AI (≤ 0.41 ng/mL) and on d 7 after AI (> 2.26 ng/mL) that resulted in highest accuracy for prediction of pregnancy on d 32 after insemination. Cows with progesterone ≤ 0.41 ng/mL on the day of AI and progesterone > 2.26 on d 7 after AI were considered to have a synchronized estrous cycle.

Pregnancy Diagnosis and Calculation of Reproductive Outcomes. Pregnancy diagnosis and calculation of reproductive outcomes were performed as described in experiment 1.

Milk Yield. Milk production during the first 3 mo postpartum considered for experiment 1 were used in experiment 2. Cows were categorized according to milk production as described for experiment 1.

Experimental Design and Statistical Analyses

Experiment 1. Cows were assigned to treatments in a randomized block design. Weekly, cows in the range of 43 to 49 DIM were blocked by parity (primiparous or multiparous) and randomly assigned to 1 of 2 treatments within each block. A sample size calculation was performed (Minitab version 15, Minitab Inc., State College, PA) to allow for sufficient experimental units to detect a difference of 6 percentage units between treatments when P/AI range from 35 to 45% ($\alpha = 0.05$; $\beta = 0.20$). Under these assumptions, we calculated that 518 to 534 experimental units per treatment were needed to detect a statistical effect under the range of expected P/AI. More cows were added to ensure that potential attrition during the study would not limit the number of experimental units initially planned.

Binary responses were analyzed by logistic regression using the LOGISTIC procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC). A backward stepwise regression model was used and variables were continuously removed from the model by the Wald statistic criterion if the significance was > 0.10 . Initial models included the effects of treatment, parity, BCS, milk yield, and interactions of treatment and parity, treatment and BCS, and treatment and milk yield. Treatment was forced into the final model in all analyses. Effects of inseminator and sire and their interactions with treatment were included in the models for P/AI and pregnancy loss. Additional covariates and their interactions with treatment were added to the models if univariate analyses identified them as significant ($P < 0.10$). Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as a tendency.

Experiment 2. Cows were assigned to treatments in a randomized block design. Weekly, cows diagnosed as nonpregnant after the first AI were blocked by method of insemination at first insemination (from experiment 1: AI on estrus, OVS56, or COS72) and parity (primiparous or multiparous) and, within each block, they were randomly assigned to 1 of 2 resynchronization treatments.

Binary responses were analyzed as described in experiment 1. Initial models included the effects of treatment, parity, milk yield, and interactions between treatment and parity, and treatment and milk yield. Effects of inseminator and sire and their interactions with treatment were included in the models for P/AI and pregnancy loss.

Concentrations of progesterone at AI and on d 7 after AI were analyzed by ANOVA using the GLIMMIX procedure of SAS for normal distribution. The models included the effects of treatment, parity, milk yield, and interactions between treatment and parity.

Receiver operating characteristic curves were generated using the ROC curve analysis option of MedCalc version 11.2.1 (MedCalc Software, Mariakerke, Belgium) to determine the optimal concentration of progesterone at AI and on d 7 after AI that results in greatest sensitivity and specificity to predict pregnancy in dairy cows. Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as a tendency.

RESULTS

Experiment 1

Average BCS and milk yield did not differ between OVS56 (2.81 ± 0.01 and 44.1 ± 0.43 kg/d) and COS72 (2.79 ± 0.01 and 43.6 ± 0.43 kg/d). Cows in COS72

Table 1. Effect of moment of administration of the final GnRH relative to timed AI on fertility responses of dairy cows (experiment 1)

Fertility Response, % (n/n)	Treatment ¹		AOR ² (95% CI)	P-value
	OVS56	COS72		
Estrus at timed AI ³	32.4 (199/615)	40.6 (235/579)	1.44 (1.13–1.83)	<0.001
Pregnant				
Day 32	46.4 (294/634)	45.5 (270/593)	1.00 (0.78–1.26)	0.99
Day 60	40.7 (256/629)	38.6 (228/591)	0.94 (0.75–1.18)	0.60
Pregnancy loss ⁴	11.4 (33/289)	14.9 (40/268)	1.37 (0.84–2.25)	0.21

¹All cows had their estrous cycle presynchronized with 2 injections of PGF_{2α} given 14 d apart and the timed AI protocols initiated 12 d later. COS72 = d 0 GnRH, d 5 and 6 PGF_{2α}, d 8 GnRH + timed AI; OVS56 = d 0 GnRH, d 5 and 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI.

²AOR = adjusted odds ratio; OVS56 is the reference for comparison.

³Evaluated based on removal of tail chalk at timed AI.

⁴Number of pregnant cows on d 32 that were not pregnant on d 60 divided by the number of pregnant cows on d 32; 5 OVS56 and 2 COS72 cows left the study before reconfirmation of pregnancy on d 60 after AI.

were more likely ($P < 0.001$) to be detected in estrus on the day of AI than cows in OVS56 (Table 1). A tendency ($P = 0.08$) for an interaction between treatment and milk yield was detected, and most of the effect was observed in cows with milk yield below the mean (45.1 vs. 31.7% for COS72 and OVS56, respectively), and less so for cows with milk above the mean (36.2 vs. 32.9% for COS72 and OVS56, respectively). Primiparous cows were more likely ($P = 0.03$) to be detected in estrus at AI than multiparous cows [40.3 vs. 33.8%; adjusted odds ratio (AOR) = 1.32; 95% CI = 1.03 to 1.68], but no interaction between treatment and parity was observed. Expression of estrus did not differ between cows classified as low or moderate BCS.

Treatment did not affect P/AI on either d 32 or 60 after AI (Table 1). Expression of estrus at AI was not associated with P/AI on either d 32 (estrus = 45.6 vs. no estrus = 46.2%) or d 60 after AI (estrus = 39.6 vs. no estrus = 39.9%). Parity and milk yield did not influence P/AI, but cows with moderate BCS had greater ($P < 0.001$) P/AI than those with low BCS (43.1 vs. 31.0%; AOR = 1.68; 95% CI = 1.29 to 2.19). The risk of pregnancy loss between d 32 and 60 of gestation did not differ between treatments (Table 1). Pregnancy loss was not influenced by expression of estrus at AI, milk yield, and BCS, or by their interactions with treatment. Although pregnancy loss did not differ between multiparous (14.9%) and primiparous (10.0%) cows, an interaction ($P = 0.02$) between treatment and parity was observed. For multiparous cows, pregnancy loss tended ($P = 0.06$) to increase when receiving COS72 compared with OVS56 (19.5 vs. 10.7%; AOR = 1.82; 95% CI = 0.99 to 3.34); however, for primiparous cows, no difference was observed for cows receiving COS72 and OVS56 (7.1 vs. 12.8%; AOR = 0.52, 95% CI = 0.20 to 1.37).

Experiment 2

The average milk yield in the first 3 mo postpartum did not differ between RCON (42.8 ± 0.56 kg/d) and RCIDR cows (42.8 ± 0.59 kg/d). On the day of the first GnRH at 34 d after previous AI, 72.2% of the cows had a visible CL present in the ovaries and the proportion was not different between treatments (RCON = 71.6 vs. RCIDR = 72.8%).

Ovarian Responses and Progesterone Concentrations in Plasma. Ovulation to the first GnRH of the resynchronization on d 34 after the previous AI was low, and it did not differ between treatments (Table 2). Cows without a CL had greater ($P < 0.001$) ovulation in response to GnRH than those with a CL (77.4 vs. 20.8%; AOR = 16.40; 95% CI = 8.62 to 31.25), but no interaction between method of resynchronization and presence of CL was observed for ovulation rate. The proportion of cows with a visible CL at the first PGF_{2α} of the resynchronization treatments did not differ between RCON and RCIDR. Similarly, the diameter of the largest follicle on the day before AI was not affected by treatment.

A small proportion of cows ovulated between the first PGF_{2α} and second GnRH injections of the resynchronization protocol (5.6%; 19/340). The risk of premature ovulation tended ($P = 0.08$) to be less for cows supplemented with progesterone during the resynchronization protocol than for nonsupplemented cows (Table 2). Occurrence of premature ovulation was not affected by the presence of a CL at the beginning of the resynchronization protocol and no interaction between treatment and the presence of a CL was found to affect the occurrence of premature ovulation. The proportion of cows that ovulated between the first PGF_{2α} and second GnRH injections of the resynchronization protocol

Table 2. Effect of supplementation with progesterone during the resynchronization protocol on fertility responses of dairy cows (experiment 2)

Fertility response	Treatment ¹		AOR ² (95% CI)	P-value
	RCON	RCIDR		
Ovulation to 1st GnRH, % (n/n)	36.5 (58/159)	34.8 (56/161)	1.07 (0.62–1.86)	0.81
Corpus luteum at PGF _{2α} , % (n/n)	86.8 (138/159)	90.1 (145/161)	1.34 (0.66–2.71)	0.42
Diameter of largest follicle, ³ mm	16.4 ± 0.4	15.5 ± 0.4	—	0.13
Premature ovulation, ⁴ % (n/n)	7.5 (13/173)	3.6 (6/167)	0.38 (0.13–1.12)	0.08
Progesterone, ng/mL				
Day of AI	0.24 ± 0.04	0.31 ± 0.04	—	0.12
7 d after AI	2.88 ± 0.11	2.73 ± 0.11	—	0.35
Progesterone, ⁵ % (n/n)				
<1.00 ng/mL at AI	96.4 (186/193)	95.1 (195/205)	0.73 (0.27–1.97)	0.54
≥1.00 ng/mL 7 d after AI	94.3 (182/193)	90.2 (185/205)	0.56 (0.26–1.20)	0.14
≤0.41 ng/mL at AI	89.1 (172/193)	90.2 (185/205)	1.13 (0.59–2.16)	0.71
>2.26 ng/mL 7 d after AI	66.3 (128/193)	67.3 (138/205)	1.04 (0.68–1.58)	0.87
Synchronization, ⁶ % (n/n)				
Progesterone at 1 ng/mL	84.9 (169/199)	84.3 (177/210)	0.95 (0.56–1.63)	0.86
Greatest accuracy	52.8 (105/199)	59.1 (124/210)	1.29 (0.87–1.91)	0.20

¹Nonpregnant cows had their estrous cycle resynchronized 34 d after the first postpartum AI. RCON = d 0 GnRH, d 5 and 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI; RCIDR = d 0 GnRH + CIDR insertion, d 5 PGF_{2α} + CIDR removal, d 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI.

²AOR = adjusted odds ratio; RCON is the reference for comparison.

³Measured on the day before AI.

⁴Disappearance of one or more follicles ≥8 mm in diameter present at the first PGF_{2α} and absent at the second GnRH injection.

⁵Concentration of progesterone in plasma on the day of AI and 7 d later using conventional cut-off values (1 ng/mL) or optimized values for luteolysis (≤0.41 ng/mL) and luteal function on d 7 (>2.26 ng/mL) according to greatest accuracy from the receiver operator characteristic curves to predict pregnancy on d 32 after AI.

⁶Progesterone at 1 ng/mL = cows without premature ovulation and progesterone <1 ng/mL at AI and progesterone ≥1 ng/mL on d 7 after AI; Greatest accuracy = cows without premature ovulation and with progesterone ≤0.41 ng/mL at AI and progesterone >2.26 ng/mL on d 7 after AI.

tended ($P = 0.08$) to be greater for primiparous than for multiparous cows (7.7 vs. 3.5%; AOR = 2.65; 95% CI = 0.91 to 7.75).

Concentration of progesterone in plasma on the day of AI and 7 d later did not differ between treatments (Table 2). Parity did not affect plasma concentration of progesterone at AI. Primiparous cows, however, had a greater ($P = 0.02$) concentration of progesterone in plasma on d 7 after AI than multiparous cows (2.97 ± 0.11 vs. 2.64 ± 0.10 ng/mL). Concentration of progesterone in plasma was similar between cows with milk production above and below the average on both the day of AI and 7 d later.

Receiver operating characteristic curves were generated to determine the concentration of progesterone in plasma sampled on the day of AI and 7 d later with the greatest accuracy to predict pregnancy on d 32 after insemination. On the day of AI, the concentration that resulted in the greatest combined sensitivity (94.6%; 95% CI = 90.3 to 97.4%) and specificity (14.6%; 95% CI = 10.0 to 20.1%) was ≤0.41 ng/mL. On d 7 after AI, the best cut-off concentration was 2.26 ng/mL, resulting in a sensitivity of 83.2% (95% CI = 77.1 to 88.3) and specificity of 47.6% (95% CI = 40.6 to 54.6).

The proportions of cows with progesterone concentrations in plasma ≤0.41 or <1.0 ng/mL on the day of AI were not affected by treatment (Table 2). Similarly, the proportions of cows with progesterone concentrations in plasma ≥1.0 or >2.26 ng/mL 7 d after AI did not differ between treatments. Synchronization of estrous cycle after treatments using either progesterone concentration of 1 ng/mL at AI and 7 d later, or progesterone ≤0.41 at AI and >2.26 ng/mL 7 d after AI as cut-points was not influenced by supplementation with progesterone during resynchronization.

Estrus at AI, P/AI, and Pregnancy Loss. The incorporation of a CIDR insert to the resynchronization protocol did not affect expression of estrus at AI (Table 3). The presence of a visible CL at the beginning of the resynchronization protocol did not influence expression of estrus at AI, and no interaction between presence of a CL and treatment was observed. Milk production was not associated with expression of estrus. Nonetheless, an interaction ($P = 0.04$) between treatment and milk yield was observed for detection of estrus because supplementation with progesterone tended ($P = 0.10$) to increase the proportion of cows in estrus at AI for those with production above the mean (39.1 vs. 29.8%;

Figure 3. Pregnancy per AI according to presence of corpus luteum (CL) at the first GnRH of the resynchronization protocol (experiment 2). Solid bars = RCON (d 0 GnRH, d 5 and 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI); Open bars = RCIDR (d 0 GnRH + CIDR insertion, d 5 PGF_{2α} + CIDR removal, d 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI). Number of cows: RCON with CL = 204; RCIDR with CL = 215; RCON without CL = 79; and RCIDR without CL = 78.

AOR = 1.52; 95% CI = 0.92 to 2.49), but not for cows with production below the mean (RCIDR = 26.8 vs. RCON = 33.1%; AOR = 0.74; 95% CI = 0.45 to 1.21). Expression of estrus at AI was not affected by parity or by the interaction between treatment and parity.

Supplementation with progesterone during the resynchronization protocol increased ($P \leq 0.05$) P/AI on d 32 and 60 after the second AI postpartum (Table 3). Interestingly, the benefit from the CIDR was observed only in cows that had a CL when the resynchronization protocol was initiated, and these differences were 8 and 9 percentage units greater on d 32 and 60 after AI,

respectively (Figure 3). Pregnancies per AI on d 32 and 60 after insemination were not affected by detection of estrus at AI, and no interaction between expression of estrus and supplementation with progesterone on P/AI was observed. Primiparous cows had greater ($P = 0.03$) P/AI than multiparous cows on d 60 after AI (46.8 vs. 37.9%; AOR = 1.43; 95% CI = 1.05 to 1.95). No interactions between treatment and parity were observed on P/AI. Cows with a CL on the day of the first PGF_{2α} of the resynchronization had greater ($P < 0.05$) P/AI on d 32 (47.7 vs. 29.7%; AOR = 2.25; 95% CI = 1.07 to 4.76) and on d 60 after AI (41.4 vs. 24.3%; AOR = 2.36; 95% CI = 1.06 to 5.24).

The risk of pregnancy loss was not affected by supplementation with progesterone during the resynchronization protocol (Table 3). Cows detected in estrus at AI tended ($P = 0.09$) to have a lower risk of pregnancy loss than those not in estrus (6.5 vs. 12.8%; AOR = 0.45; 95% CI = 0.17 to 1.15). Parity and milk yield were not associated with risk of pregnancy loss in the first 60 d of gestation.

DISCUSSION

The success of programs for synchronization of the estrous cycle and ovulation depends on an orchestrated manipulation of physiological processes such as recruitment of a new follicular wave, control of follicular dominance (Cerri et al., 2009), length of proestrus (Bridges et al., 2008), and the interval between induction of ovulation with GnRH and AI (Pursley et al., 1998). Reduction in the period of follicular dominance was shown to improve embryo quality (Cerri et al., 2009). Accordingly, shortening the interval between the initial GnRH and the injection of PGF_{2α} from 7 to 5 d increased P/AI in lactating dairy cows (Santos et al., 2010).

Table 3. Effect of supplementation with progesterone during the resynchronization protocol on fertility responses of dairy cows (experiment 2)

Fertility Response, % (n/n)	Treatment ¹		AOR ² (95% CI)	P-value
	RCON	RCIDR		
Estrus at timed AI ³	31.5 (90/286)	32.5 (96/295)	1.05 (0.74–1.48)	0.80
Pregnant				
Day 32	43.1 (144/334)	51.3 (175/341)	1.38 (1.02–1.88)	0.04
Day 60	37.8 (126/333)	45.5 (153/336)	1.37 (1.00–1.87)	0.05
Pregnancy loss ⁴	11.9 (17/143)	10.0 (17/170)	0.82 (0.40–1.68)	0.59

¹Nonpregnant cows had their estrous cycle resynchronized 34 d after the first postpartum AI. RCON = d 0 GnRH, d 5 and 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI; RCIDR = d 0 GnRH + CIDR insertion, d 5 PGF_{2α} + CIDR removal, d 6 PGF_{2α}, d 7.3 GnRH, d 8 timed AI.

²AOR = adjusted odds ratio; RCON is the reference for comparison.

³Evaluated based on removal of tail chalk at timed AI.

⁴Number of pregnant cows on d 32 that were not pregnant on d 60 divided by the number of pregnant cows on d 32. One RCON and 5 RCIDR cows left the study before reconfirmation of pregnancy on d 60 after AI.

No differences in P/AI were observed when induction of ovulation using GnRH was performed either 16 h before or concurrent with timed AI at 72 h after the first PGF_{2α} injection in the 5-d program. This is contrary to previous research with cows subjected to a standard 7-d (from GnRH to PGF_{2α} injections) Ovsynch program in which optimal P/AI was achieved when AI was performed 16 h after the GnRH injection for induction of ovulation and was significantly reduced when cows were inseminated simultaneously with induction of ovulation (Pursley et al., 1998; Brusveen et al., 2008). In the present experiment, delaying the final GnRH to 72 h allowed for a longer period of proestrus for COS72 than for OVS56, which resulted in a greater proportion of cows detected in estrus on the day of AI. The longer proestrus period for the COS72 treatment occurred with a comparable period of low progesterone from luteolysis to AI in both treatments.

The advantage of performing AI 16 h after the final GnRH has been attributed to the synchrony between oocyte and numbers of viable spermatozoa available for fertilization (Saacke, 2008). Cows subjected to the 7-d program are expected to ovulate approximately 28 h after the final GnRH (Pursley et al., 1995), which is similar to the time of ovulation relative to the onset of estrus described for lactating dairy cows (27.6 h; Walker et al., 1996). Insemination of dairy cows at the onset of estrus reduced fertilization rate (Dalton et al., 2001) and P/AI (Dransfield et al., 1998) compared with AI 12 h later. This reduction was associated with a decrease in the number of accessory spermatozoa per embryo/ovum, suggesting a continuous loss of functional sperm reservoirs in the additional 12 h preceding ovulation (Dalton et al., 2001). Therefore, insemination of cows 16 h after the final GnRH might increase the number of spermatozoa available for fertilization compared with insemination concurrent with the final GnRH.

Although administration of the final GnRH 16 h before AI has been shown to benefit fertility of dairy cows in the standard 7-d timed AI program, the additional 16 h of proestrus for cows in COS72 would allow for further growth of the ovulatory follicle (Peters and Pursley, 2003), greater concentrations of estradiol, and more cows in estrus at the time of AI (Hillegass et al., 2008). This might be particularly important in a program with a reduced period of follicle development. Fertility of dairy cows has been positively associated with the diameter of the ovulatory follicle (Vasconcelos et al., 2001) and concentrations of estradiol in plasma (Lopes et al., 2007). In fact, P/AI in response to timed AI programs are generally superior for cows detected in estrus on the day of insemination compared with cows that do not exhibit signs of estrus (Hillegass et al., 2008; Santos et al., 2010). In the 5-d program, the period of

follicular growth is shortened by 2 d compared with the 7-d programs, which results in smaller ovulatory follicles, reduced concentrations of estradiol, and a lesser proportion of cows in estrus at AI (Santos et al., 2010). Therefore, the additional 16 h of proestrus might benefit the fertility of dairy cows in the 5-d program and may offset the better synchrony between induction of ovulation and AI offered by the OVS56. Indeed, experiments conducted with beef cows have shown that reducing the interval from the initial GnRH to the PGF_{2α} increased P/AI only when the period of proestrus was extended from 60 to 72 h (Bridges et al., 2008). This might be even more important for high-producing dairy cows considering the extensive metabolism of steroids (Sangritavong et al., 2002) and the generally low concentrations of estradiol in plasma during proestrus (Lopes et al., 2007; Hillegass et al., 2008).

Intravaginal inserts for controlled release of progesterone have been used to improve synchrony of ovulation and P/AI in response to timed AI protocols (Chebel et al., 2006; Lima et al., 2009). After the first postpartum AI, approximately 55 to 65% of the cows are expected to be diagnosed as nonpregnant 28 to 35 d after insemination. The stage of the estrous cycle in which the first GnRH of the resynchronization protocol is administered influenced the subsequent P/AI in some (Fricke et al., 2003; Sterry et al., 2006), but not all (Silva et al., 2009) studies. In experiment 2, cows received the first GnRH of the resynchronization protocol 34 d after the previous AI. The length of the estrous cycle in lactating dairy cows averages 22.9 ± 0.7 d (Sartori et al., 2004); therefore, on average, it is expected that most of the cows would be on d 11 of the estrous cycle when the resynchronization protocol was initiated. This is important because approximately 50% of cows treated with GnRH between d 10 and 16 of the estrous cycle failed to ovulate (Vasconcelos et al., 1999), which, in association with spontaneous regression of the CL before the injection of PGF_{2α}, resulted in increased premature ovulation at the end of the synchronization program and reduced fertility (Vasconcelos et al., 1999). In the present study, 11.6% of the cows did not bear a CL at the injection of PGF_{2α}, which reduced P/AI. A greater than anticipated proportion of cows did not have a visible CL by ultrasonography on the day of the first GnRH of the resynchronization (27.8%). This is similar to results reported by Bartolome et al. (2009), but also indicates that a large proportion of the resynchronized cows was not on the expected days of the new estrous cycle as one might expect.

Although ovulation to the first GnRH has been shown to decline in cows that receive a CIDR (Galvão et al., 2004; Stevenson et al., 2006), this response has not been consistent (Lima et al., 2009). A possible reason for lack

of effect of CIDR on ovulation to GnRH in the current study was the day when the protocol initiated. Overall, the ovulatory response was low (only 35.6%), which indicates that most cows did not have a follicle responsive to a GnRH/LH surge, thereby limiting a potential effect of progesterone blocking ovulation. Progesterone released by the CIDR insert reduces LH pulsatility and blocks the preovulatory LH surge (Rathbone et al., 2001); therefore, it potentially reduces the occurrence of premature ovulations during a timed AI protocol. In fact, the odds of ovulating before the final GnRH injection tended to be lower for cows supplemented with progesterone during the resynchronization protocol in experiment 2. It is possible that a portion of cows that did not ovulate prematurely as assessed here had an endogenous surge of LH before the final injection of GnRH, which would result in ovulation either before or early after AI and reduce P/AI (Saacke, 2008). These cows are also expected to benefit from the supplementation with progesterone, which could account for some of the increased fertility observed in RCIDR cows in the present study.

High-producing dairy cows have reduced plasma concentrations of progesterone during follicular development (Sartori et al., 2004), which may increase LH pulsatility and reduce oocyte competence and embryo quality (Inskeep, 2004). In fact, greater concentrations of progesterone during development of the ovulatory follicle have been associated with improved fertility in dairy cows (Bisinotto et al., 2010). Use of a single CIDR insert increased the concentration of progesterone in plasma by approximately 0.9 ng/mL (Lima et al., 2009); therefore, it is inadequate to increase progesterone in cows not bearing a CL to concentrations comparable to those of cows in diestrus (Lima et al., 2009). Nonetheless, it is possible that the additional progesterone from the CIDR insert complemented luteal synthesis of progesterone and allowed for adequate follicular maturation, which resulted in increased P/AI. Similar to our findings, supplementation with progesterone tended to improve P/AI only in cows that had an active CL at the initiation of the Ovsynch protocol (Bartolome et al., 2009).

CONCLUSIONS

Fertility of lactating dairy cows subjected to a 5-d timed AI protocol was not affected by the time of administration of the final GnRH relative to AI. As opposed to standard timed AI programs with an interval of 7 d from the first GnRH to induction of luteolysis, in the 5-d program, induction of ovulation with GnRH concurrently with AI resulted in similar fertility compared with inducing ovulation 16 h before AI. During

resynchronization starting on d 34 after the previous AI, the incorporation of a CIDR insert to the 5-d timed AI program improved P/AI, primarily in cows with a CL at the initiation of the resynchronization, and the benefits were observed despite major changes in synchronization of the estrous cycle after AI.

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REFERENCES

- Bartolome, J. A., J. J. Van Leeuwen, M. Thieme, O. G. Sá Filho, P. Melendez, L. F. Archbald, and W. W. Thatcher. 2009. Synchronization and resynchronization of inseminations in lactating dairy cows with the CIDR insert and the Ovsynch protocol. *Theriogenology* 72:869–878.
- Bisinotto, R. S., R. C. Chebel, and J. E. P. Santos. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. *J. Dairy Sci.* 93:3578–3587.
- Bridges, G. A., L. A. Helsen, D. E. Grum, M. L. Mussard, C. L. Gasser, and M. L. Day. 2008. Decreasing the interval between GnRH and PGF_{2α} from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. *Theriogenology* 69:843–851.
- Brusveen, D. J., A. P. Cunha, C. D. Silva, P. M. Cunha, R. A. Sterry, E. P. B. Silva, J. N. Guenther, and M. C. Wiltbank. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. *J. Dairy Sci.* 91:1044–1052.
- Cerri, R. L., 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.
- Chebel, R. C., M. J. Al-Hassan, P. M. Fricke, J. E. P. Santos, J. R. Lima, C. A. Martel, J. S. Stevenson, R. Garcia, and R. L. Ax. 2010. Supplementation of progesterone via controlled internal drug release inserts during ovulation synchronization protocols in lactating dairy cows. *J. Dairy Sci.* 93:922–931.
- Chebel, R. C., J. E. P. Santos, R. L. A. Cerri, H. M. Rutigliano, and R. G. S. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. *J. Dairy Sci.* 89:4205–4219.
- Dalton, J. C., S. Nadir, J. H. Bame, M. Noftsinger, R. L. Nebel, and R. G. Saacke. 2001. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. *J. Dairy Sci.* 84:2413–2418.
- Dransfield, M. B. G., R. L. Nebel, R. E. Pearson, and L. D. Warnick. 1998. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. *J. Dairy Sci.* 81:1874–1882.
- Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. *J. Dairy Sci.* 77:2695–2703.
- Fricke, P. M., D. Z. Caraviello, K. A. Weigel, and M. L. Welle. 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. *J. Dairy Sci.* 86:3941–3950.
- Galvão, K. N., J. E. P. Santos, S. O. Juchem, R. L. A. Cerri, A. C. Coscioni, and M. Villaseñor. 2004. Effect of addition of a progesterone intravaginal insert to a timed insemination protocol using estradiol cypionate on ovulation rate, pregnancy rate, and late embryonic loss in lactating dairy cows. *J. Anim. Sci.* 82:3508–3517.

- Hillegass, J., F. S. Lima, M. F. Sá Filho, and J. E. P. Santos. 2008. Effect of time of artificial insemination and supplemental estradiol on reproduction of lactating dairy cows. *J. Dairy Sci.* 91:4226–4237.
- Inskeep, E. K. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. *J. Anim. Sci.* 82 (E-Suppl.):E24–E39.
- Lima, J. R., F. A. Rivera, C. D. Narciso, R. Oliveira, R. C. Chebel, and J. E. P. Santos. 2009. Effect of increasing amounts of supplemental progesterone in a timed artificial insemination protocol on fertility of lactating dairy cows. *J. Dairy Sci.* 92:5436–5446.
- Lopes, A. S., S. T. Butler, R. O. Gilbert, and W. R. Butler. 2007. Relationship of pre-ovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. *Anim. Reprod. Sci.* 99:34–43.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Peters, M. W., and J. R. Pursley. 2003. Timing of final GnRH of the Ovsynch protocol affects ovulatory follicle size, subsequent luteal function, and fertility in dairy cows. *Theriogenology* 60:1197–1204.
- Pursley, J. R., M. O. Mee, and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF_{2α} and GnRH. *Theriogenology* 44:915–923.
- Pursley, J. R., R. W. Silcox, and M. C. Wiltbank. 1998. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. *J. Dairy Sci.* 81:2139–2144.
- Rathbone, M. J., J. E. Kinder, K. Fike, F. Kojima, D. Clopton, C. R. Ogle, and C. R. Bunt. 2001. Recent advances in bovine reproductive endocrinology and physiology and their impact on drug delivery system design for the control of the estrous cycle in cattle. *Adv. Drug Deliv. Rev.* 50:277–320.
- Saacke, R. G. 2008. Insemination factors related to timed AI in cattle. *Theriogenology* 70:479–484.
- Sangsrivong, 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-17β 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 AI protocol on reproduction of dairy cows. *J. Dairy Sci.* 93:2976–2988.
- 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.
- Silva, E., R. A. Sterry, D. Kolb, N. Mathialagan, M. F. McGrath, J. M. Ballam, and P. M. Fricke. 2009. Effect of interval to resynchronization of ovulation on fertility of lactating Holstein cows when using transrectal ultrasonography or a pregnancy-associated glycoprotein enzyme-linked immunosorbent assay to diagnose pregnancy status. *J. Dairy Sci.* 92:3643–3650.
- Sterry, R. A., M. L. Welle, and P. M. Fricke. 2006. Effect of interval from timed artificial insemination to initiation of resynchronization of ovulation on fertility of lactating dairy cows. *J. Dairy Sci.* 89:2099–2109.
- Stevenson, J. S., J. R. Pursley, H. A. Garverick, P. M. Fricke, D. J. Kesler, J. S. Ottobre, and M. C. Wiltbank. 2006. Treatment of cycling and noncycling lactating dairy cows with progesterone during ovsynch. *J. Dairy Sci.* 89:2567–2578.
- Vasconcelos, J. L., R. Sartori, H. N. Oliveira, J. G. Guenther, and M. C. Wiltbank. 2001. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. *Theriogenology* 56:307–314.
- 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.
- Walker, W. L., R. L. Nebel, and M. L. McGilliard. 1996. Time of ovulation relative to mounting activity in dairy cattle. *J. Dairy Sci.* 79:1555–1561.