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Supplementation with Estradiol-17β Before the Last Gonadotropin-Releasing Hormone Injection of the Ovsynch Protocol in Lactating Dairy Cows

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ABSTRACT

The aim of this study was to determine whether an increase in circulating estrogen concentrations would increase percentage pregnant per artificial insemination (PP/AI) in a timed AI protocol in high-producing lactating dairy cows. We analyzed only cows having a synchronized ovulation to the last GnRH of the Ovsynch protocol (867/1,084). The control group (n = 420) received Ovsynch (GnRH – 7 d – $PGF_{2\alpha}$ – 56 h – GnRH – 16 h – timed AI). The treatment group (n = 447) had the same timed AI protocol with the addition of 1 mg of estradiol-17β (E2) at 8 h before the second GnRH injection. Ovarian ultrasound and blood samples were taken just before E2 treatment of both groups. In a subset of cows (n = 563), pressure-activated estrus detection devices were used to assess expression of estrus at 48 to 72 h after $PGF_{2\alpha}$ treatment. Ovulation was confirmed by ultrasound 7 d after timed AI. Treatment with E2 increased expression of estrus but overall PP/AI did not differ between E2 and control cows. There was an interaction between treatment and expression of estrus such that PP/AI was greater in E2-treated cows that showed estrus than in E2-treated or control cows that did not show estrus and tended to be greater than control cows that showed estrus. There was evidence for a treatment by ovulatory follicle size interaction on PP/AI. Supplementation with E2 improved PP/AI in cows ovulating medium (15 to 19 mm) but not smaller or larger follicles. The E2 treatment also tended to improve PP/AI in primiparous cows with low (≤2.5) body condition score, and in cows at first postpartum service compared with Ovsynch alone. In conclusion, any improvements in PP/AI because of E2 treatment during a timed AI protocol appear to depend on expression of estrus, parity, body condition score, and size of ovulatory follicle.

Key words: Ovsynch, estradiol, dairy cow, conception rate

INTRODUCTION

The increase in milk yield of lactating dairy cows has been associated with a decline in reproductive efficiency (Sartori et al., 2002a; Washburn et al., 2002; Lopez et al., 2004). One of the factors decreasing reproductive efficiency in high-producing herds is the decrease in expression and detection of estrus (Nebel et al., 1997; Dransfield et al., 1998; Lopez et al., 2004). Timed AI (**TAI**) protocols such as Ovsynch have been developed to decrease reliance on detection of estrus in reproductive management programs (Pursley et al., 1995). However, even with increased AI submission rates, the low fertility of lactating dairy cows continues to be a problem after TAI. One of the reasons for low fertility may be the lower circulating estradiol-17β (**E2**) concentrations near AI in high-producing cows (Sartori et al., 2002a, 2004; Lopez et al., 2004; Wolfenson et al., 2004) apparently because of increased metabolism of E2 (Sangsritavong et al., 2002). This problem is exacerbated during Ovsynch because the second GnRH treatment causes ovulation of the dominant follicle before the peak E2 concentrations in most cows. Absent or reduced expression of estrus during Ovsynch is probably caused by this reduction in circulating E2. In addition, other reproductive problems could be caused by reduced E2, including inefficient sperm transport, suboptimal oviductal or uterine environment, impaired oocyte fertilization, and poor embryonic quality (Hawk and Cooper, 1975; Ryan et al., 1993; Sartori et al., 2002b).

This study was designed to test the effects of increasing circulating E2 during Ovsynch. A previous study (Sellars et al., 2006) incorporated 0.25 mg of estradiol cypionate (**ECP**) into an Ovsynch protocol and there were no differences in fertility when ECP was administered at the same time as the second GnRH treatment

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of Ovsynch. In this study, we chose to use E2 rather than a longer-acting estrogen such as ECP. In a recent experiment (Souza et al., 2005), we compared several types of estrogens (estradiol benzoate, estradiol cypionate, and estradiol-17β) and doses (0, 0.5, and 1 mg) in the presence or absence of dominant follicles. We concluded that 1 mg of E2 produced a physiological increase in circulating E2 without disrupting the normal decline in E2 concentrations following the LH surge (basal concentrations by ~10 h after LH peak). We also chose to administer the E2 treatment 8 h before the final GnRH injection of Ovsynch to mimic more closely the final surge in E2 concentrations that normally precedes the onset of estrus and the GnRH/LH surge.

The influence of the size of the ovulatory follicle in TAI programs has been investigated in a number of studies (Vasconcelos et al., 1999, 2001; Lamb et al., 2001; Perry et al., 2005) with somewhat inconsistent results. No previous study has provided adequate information on the effects of ovulatory follicle size on conception rate in high-producing dairy cows. Moreover, previous studies have not provided information on the mechanisms producing any observed changes in fertility because of differences in ovulatory follicle size. For example, lower fertility in cows ovulating smaller follicles may be caused by reduced circulating E2 concentrations. In this study, we evaluated the ovulatory follicle size in a large number of high-producing dairy cows and tested whether supplementing E2 concentrations would increase fertility in cows that ovulate different-sized follicles.

This experiment, therefore, was not designed to test a practical protocol for synchronizing ovulation in dairy cattle but was specifically designed to test whether low circulating E2 concentrations were an important component of the reduced fertility in lactating dairy cows, particularly during the Ovsynch protocol. Another objective was to compare percentage pregnant per AI (**PP/AI**) by ovulatory follicle size in cows that were submitted to Ovsynch or Ovsynch with E2 supplementation. Our first hypothesis was that cows receiving E2 treatment 24 h before TAI would express more estrus near AI and would have increased PP/AI compared with untreated cows receiving Ovsych. A second hypothesis was that cows ovulating medium-sized follicles would have greater PP/AI than cows ovulating smaller or larger follicles and that an E2-induced increase in PP/AI would be greatest in cows ovulating smaller and medium-sized rather than larger follicles.

MATERIALS AND METHODS

Materials

Prostaglandin $F_{2\alpha}$ (dinoprost tromethamine, 25 mg/dose; Lutalyse) was from Pfizer Animal Health (Kalamazoo, MI). The GnRH (gonadorelin diacetate tetrahydrate; 100 μg/dose; Ovacyst) was from Phoenix Scientific Inc. (St. Joseph, MO). Kamar heat mount detectors were from Kamar Inc. (Steamboat Springs, CO). Sesame oil and E2 were from Sigma Chemical Co. (St. Louis, MO). Benzyl alcohol was from EM Science (Cherry Hill, NJ). The E2 solution was prepared as follows: E2 was weighed and benzyl alcohol added to bring the solution to 5 mg/mL. Sesame oil was then added to the preparation to obtain a final solution of 0.5 mg/mL.

Animals, Management, and Experimental Design

Seven hundred seventeen lactating Holstein cows (466 multiparous and 251 primiparous), during 1,084 Ovsynch treatments, were housed in free-stall barns on a commercial dairy farm in Juneau, Wisconsin. Only synchronized breedings (criteria described subsequently) were analyzed in this study (867/1,084). The experimental period began in April 2004 and ended in October 2004. Cows were 105.2 ± 1.4 DIM and produced 39.4 ± 0.3 kg of milk/d. Cows in the study started to receive bST (500 mg/dose; Posilac, Monsanto Co., St. Louis, MO) at about 60 d postpartum. Cows were milked thrice daily and fed a TMR twice daily that consisted of corn silage and alfalfa silage as forage with a corn-soybean meal-based concentrate. The TMR was balanced to meet or exceed minimum nutritional requirements for lactating dairy cows (NRC, 2001).

Before first postpartum insemination, cows were presynchronized with 2 $PGF_{2\alpha}$ treatments given 14 d apart, with the first given on d 37 to 43 postpartum and the second on d 51 to 57 postpartum. This was followed by the first GnRH of the Ovsynch protocol 11 d later (d 62 to 68 postpartum). Cows detected in estrus by tail chalking (twice-daily examination of mounting activity followed by rechalking) between the second $PGF_{2\alpha}$ of the presynch and the first GnRH of the Ovsynch were inseminated. Animals were assigned to 2 groups in a completely randomized experimental design. The control group (n = 420) received Ovsynch (GnRH – 7 d – $PGF_{2\alpha}$ – 56 h – GnRH – 16 h – TAI). The treatment group (n = 447) had the same TAI protocol with the addition of 1 mg of E2 (i.m.; Ovsynch + E2) at 8 h before the second GnRH injection. Randomization of cows to treatment occurred at 48 h after the $PGF_{2\alpha}$ treatment (time of E2 treatment). Pregnancy diagnosis was performed by palpation of uterine contents per rectum at 35 to 41 d after AI and again at 58 to 64 d after AI. Pregnancy losses were calculated based on these 2 pregnancy exams. Cows diagnosed not pregnant were re-randomized to receive Ovsynch or Ovsynch + E2 without presynchronization treatments. To evaluate expression of estrus near AI (48 to 72 h after $PGF_{2\alpha}$), a subset (n = 563) of cows throughout the entire trial received a pressure-activated heat mount detector (Kamar). The device was checked for signs of mounting activity at the time of AI.

Cows were examined by a veterinarian before Ovsynch. Animals with detectable health or uterine disorders were not used in the experiment. Inseminations were performed with commercial semen from multiple sires; all inseminations were performed by 2 technicians. All animal procedures were approved by the Animal Care Committee of the College of Agriculture and Life Sciences at the University of Wisconsin–Madison.

Data Collection

Ovarian ultrasound evaluations were performed at the time of presynchronization injections (d –35 and d –21; to detect anovular cows), at the time of E2 treatment (d 0; to measure ovulatory follicle size), and 8 d later (d 8; to determine ovulation to Ovsynch) with an Aloka 500V equipped with a 7.5-MHz linear array transducer (Corometrics Medical Systems Inc., Wallingford, CT). Ovulation response was determined with ultrasound images from d 0 and d 8. Body condition scores were recorded at the time of ultrasound evaluation on d 0 (scale of 1 to 5; Edmonson et al., 1989). Blood samples were also collected on d 0 for evaluation of circulating E2 and progesterone concentrations in serum. Environmental maximum temperatures on the day of TAI (d 1) were collected by using records from the Wisconsin Weather Station. Daily milk yield from the day of the beginning of Ovsynch (d –10) until timed AI (d 1) were collected on Dairy Comp 305 (Valley Agricultural Software, Tulare, CA). Milk production from cows with more than 30% of values unrecorded or with a sudden decrease (>25%) in daily milk production were not used (n = 213) for analysis of milk production (n = 654).

Hormone Assays

Serum progesterone concentration was evaluated by double extraction with petroleum ether followed by ELISA as previously reported (Rasmussen et al., 1996). Estradiol samples were extracted twice with diethyl ether and analyzed with RIA as previously reported (Kulick et al., 1999). The intra- and interassay coefficients of variation (**CV**) for progesterone were 8.2 and 10.5%. The E2 assay had intra- and interassay CV of 11.7 and 13.0%. The sensitivity (calculated as the average from all of the assays using 2 standard deviations above the total binding) was 0.08 ng/mL for the progesterone assay and 0.6 pg/mL for the estradiol assay.

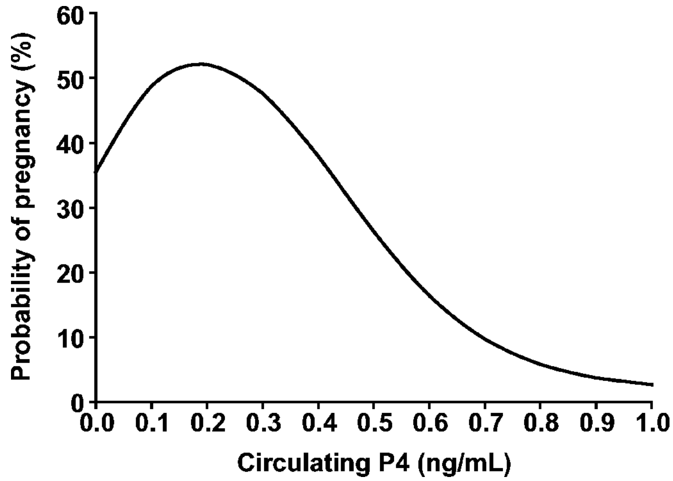

Figure 1. Effect of circulating progesterone (P4) concentration at 48h after $PGF_{2\alpha}$ treatment during the Ovsynch protocol on the estimated probability of pregnancy at 58 to 64 d after AI. Cows with ≥0.5 ng/mL (n = 52) were not used in the final analysis because of the 50% or more decrease in percentage pregnant/AI (PP/AI) compared with maximum PP/AI.

Analyzed Breedings

To rigorously test our experimental hypothesis that increasing E2 would increase PP/AI in cows with synchronized ovulation, we used objective criteria to eliminate any nonsynchronized cows. Thirty-two cows had no dominant follicle (follicle ≥10 mm diameter) 48 h after $PGF_{2\alpha}$ and 64 cows showed estrus before randomization to E2 treatment; these cows were eliminated from the study. In addition, retrospective analysis indicated that 52 cows (n = 28 in E2 group; n = 24 in control group) had elevated circulating progesterone (>0.5 ng/mL) at 48 h after $PGF_{2\alpha}$, indicating incomplete luteal regression. The value of 0.5 ng/mL was chosen as the separation point for use in this experiment based on logistic regression showing that this was the point at which PP/AI dropped to less than half the normal PP/AI (Figure 1). A further 44 cows (n = 19 in E2 group; n = 25 in control group) did not ovulate after the last GnRH of Ovsynch, and 25 animals (n = 9 in E2 group; n = 16 in control group) were sold or had died before the first pregnancy diagnosis. The breedings associated with all of these conditions (n = 217) were not studied further.

Statistical Analyses

Normality was assumed for the continuous response variables, such as DIM at breeding, E2 concentration, and milk yield, and PROC MIXED of SAS (Littell et al., 1996; SAS Institute, Cary, NC) was used for the analyses. The binomial distribution was assumed for

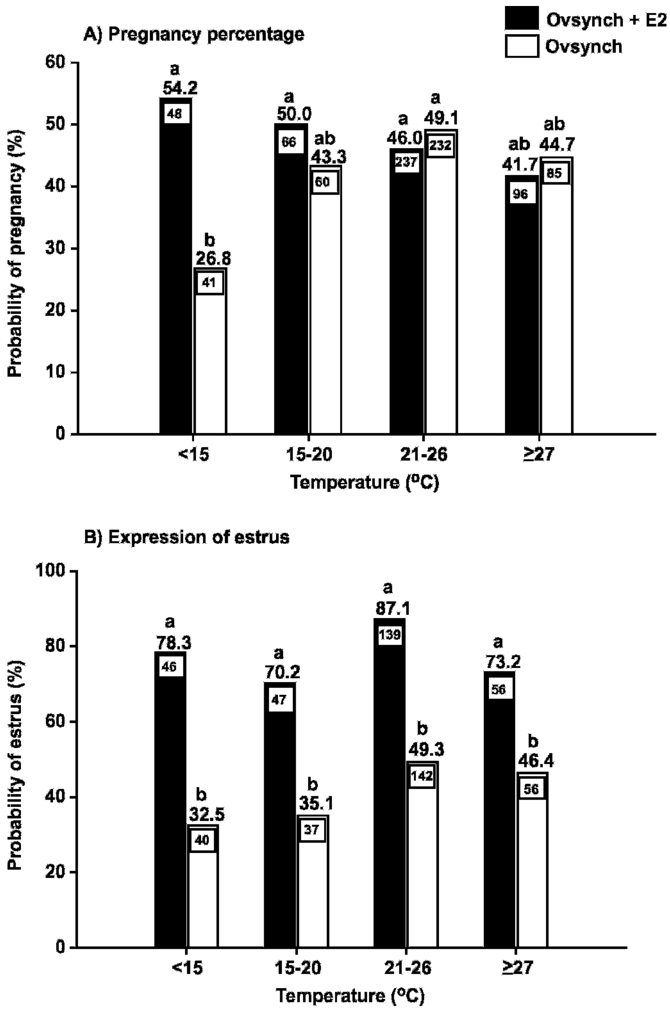

Figure 2. Effect of the maximum ambient temperature on the day of AI on the percentage pregnant per AI (A) and percentage that displayed estrus (B) during Ovsynch + estradiol-17β (E2) (black bars) and Ovsynch (white bars) treatments.

the binary response variables such as expression of estrus and conception, and these variables were analyzed with the GLIMMIX macro of SAS with cow as a random effect. This allowed us to use the terminology cow or breeding interchangeably to define our experimental unit (breeding). The procedure GENMOD of SAS was used for some of the binary response variables, when cow was not considered as a random effect, such as pregnancy loss. The logit link was used for the analyses of these binary variables and the resulting values were converted back to probabilities by the formula $P = 1/(1 + e^{-(b_0+b_1X_1+b_2X_2+\ldots+b_iX_i)})$. In all analyses, maximal temperature, lactation number, DIM, and BCS were initially used in the analyses. If significant ($P > 0.10$), then they were kept in the final analysis.

Table 1. Overall estrous response, percentage pregnant per AI (PP/AI) at 35 to 41 d and 58 to 64 d after AI, and pregnancy losses for cows receiving Ovsynch + estradiol-17β (E2) or Ovsynch alone

	Treatment	
End point	Ovsynch + E2	Ovsynch
	% (n/n)	
Estrus[1]	80.2[a] (231/288)	44.4[b] (122/275)
PP/AI, d 35 to 41	46.5 (208/447)	45.0 (189/420)
PP/AI, d 58 to 64[2]	42.4 (188/443)	39.4 (161/409)
Pregnancy loss	7.8 (16/204)	9.6 (17/178)

[a,b]Means with different superscripts within a row are different; $P < 0.05$.

[1]Determined by pressure-activated heat mount detector (Kamar) in a subset of cows.

[2]The total number of animals at this later pregnancy diagnosis was reduced due to culling, selling, or death of a cow between the first and second pregnancy exams.

RESULTS AND DISCUSSION

General Results

Milk production for E2-treated (39.6 ± 0.5 kg/d) and control (39.2 ± 0.5 kg/d) cows did not differ ($P = 0.27$). In addition, there was no relationship between milk production and PP/AI.

There was no effect of ambient temperature on PP/AI. It appears that temperatures were not sufficiently high during this experiment to produce a reduction in fertility. Nevertheless, there was an interaction of temperature with treatment ($P = 0.057$). As shown in Figure 2A, this interaction was because of cows treated with Ovsynch + E2 having a decrease in PP/AI as temperature increased, whereas cows treated with only Ovsynch appeared to demonstrate the opposite trend. At low temperatures, therefore, there was a difference in PP/AI between cows treated with Ovsynch + E2 vs. Ovsynch. At higher temperatures, there was no difference between groups. In addition, there was an effect of temperature on expression of estrus ($P = 0.01$) but no temperature by treatment interaction. At all temperatures (Figure 2B), more cows treated with Ovsynch + E2 expressed estrus than cows treated with Ovsynch.

Expression of Estrus

Supplementation with E2 increased ($P < 0.01$) expression of estrus in cows receiving Ovsynch (Table 1). Although treatment with E2 nearly doubled the percentage of cows expressing estrus during the day before timed AI, overall PP/AI and pregnancy losses did not differ ($P > 0.10$) between E2-treated and control cows (Table 1). Nevertheless, there was a treatment by expression of estrus interaction ($P < 0.01$) on PP/AI (Table 2). The cows expressing estrus in the E2-treated group

Table 2. Percentage pregnant per AI (PP/AI) at 35 to 41 or 58 to 64 d after AI, and pregnancy losses by estrous response (analyzed only in the subset of cows receiving a Kamar device; n = 563) for cows receiving Ovsynch + estradiol-17β (E2) or Ovsynch alone

	Ovsynch + E2		Ovsynch	
	% (n/n)			
Estrous response[1]	Estrus	No estrus	Estrus	No estrus
PP/AI, d 35 to 41	53.7[a] (124/231)	22.8[c] (13/57)	45.1[ab] (55/122)	40.5[b] (62/153)
PP/AI, d 58 to 64	49.3[A,a] (113/229)	19.3[c] (11/57)	40.3[B,ab] (48/119)	35.1[b] (53/151)
Pregnancy loss	7.4 (9/122)	15.4 (2/13)	7.7 (4/52)	11.7 (7/60)

[a–c]Means with different superscripts within a row are different; $P < 0.05$.

[A,B]Means with different superscripts within a row are different; $P < 0.10$.

[1]Determined by pressure-activated heat mount detector (Kamar).

had greater ($P < 0.02$) PP/AI at first (35 to 41 d after AI) and second (58 to 64 d) pregnancy diagnosis than E2-treated or control cows that did not show estrus. Interestingly, cows that received E2 and showed estrus also tended ($P = 0.06$) to have better PP/AI in the later pregnancy diagnosis than control cows that expressed estrus (Table 2).

Expression of estrus in 44.4% of Ovsynch-treated cows may be slightly greater than other reports (~20%; Jobst et al., 2000; Pancarci et al., 2002; Perry et al., 2005), possibly because of treatment with the second GnRH at 56 h, rather than 48 h, after $PGF_{2\alpha}$. The greater expression of estrus in cows receiving estrogen supplementation near AI is consistent with previous studies that used ECP to substitute for the last GnRH treatment in the Ovsynch protocol (Pancarci et al., 2002; Cerri et al., 2004; Galvao et al., 2004; Kasimanickam et al., 2005). A recent study (Souza et al., 2005) reported that treatment with 1 mg of E2 will not cause standing estrus in cows that had all follicles >5 mm removed by aspiration in spite of a dramatic increase in circulating E2 concentrations. Endogenous E2 may be essential for the expression of estrus induced by this dose of E2.

About 20% of cows did not show estrus in response to E2 treatment in our study. This group had much lower PP/AI than all other groups. Previous studies comparing the Heatsynch protocol (ECP treatment) with Ovsynch also reported greater PP/AI in cows that expressed estrus during Heatsynch compared with cows that did not express estrus (Lopes et al., 2000; Pancarci et al., 2002; Cerri et al., 2004; Galvao et al., 2004; Kasimanickam et al., 2005). This reduction in PP/AI is not because of lack of ovulation to the protocol because only ovulating cows were included in our analyses. It appears that lack of expression of estrus after E2 treatment designates a group of cows with extremely low fertility. We were unable to find one variable that clearly defined the cows in this group, although E2-treated cows that did not show estrus had somewhat lower ($P < 0.05$) BCS (2.68 ± 0.05) compared with cows that showed estrus (2.85 ± 0.03).

Expression of estrus in cows treated with Ovsynch alone was not associated with an increase in PP/AI. This result contrasts with some previous studies (Jobst et al., 2000; Perry et al., 2005). For example, Jobst et al. (2000) found about a 2-fold increase in PP/AI in cows expressing estrus compared with cows that did not show estrus after Ovsynch. Nevertheless, our results are similar to some other studies that did not find an effect of expression of estrus during Ovsynch on fertility (Pancarci et al., 2002; Kasimanickam et al., 2005). In the Ovsynch group, there were no differences ($P > 0.10$) in BCS between cows that did (BCS = 2.83 ± 0.04) or did not (BCS = 2.79 ± 0.03) show estrus.

The factors regulating expression of estrus appeared to be different in Ovsynch compared with Ovsynch + E2 cows. Overall, cows with lower BCS were less likely to express estrus ($P < 0.03$). However, there was an interaction ($P = 0.05$) between treatment and BCS in expression of estrus (Figure 3). Regardless of BCS at time of AI, about 40% of cows in the control group showed estrus. Thus, BCS did not affect expression of estrus in the control group. In contrast, cows treated with E2 had increasing ($P < 0.01$) expression of estrus with increasing BCS (Figure 3, Table 3).

Previous studies (Pancarci et al., 2002; Gümen et al., 2003; Galvao et al., 2004) have shown that cows with lower BCS are more likely to be anovular. For instance, Gümen et al. (2003) used ultrasound examinations and serum progesterone concentrations at 47 to 53 and 54 to 60 d postpartum to determine cyclicity and reported that approximately 40% of cows with BCS ≤2.5 were anovular, compared with approximately 20% of cows with BCS ≥2.75. Galvao et al. (2004) reported that anovular cows had impaired estrous responses after the Heatsynch protocol, possibly because of a lack of progesterone priming of the hypothalamus (Britt et al., 1986; Vailes et al., 1992; Caraty and Skinner, 1999). Lower expression of estrus in cows with lower BCS during

Table 3. Estrous response, percentage pregnant per AI (PP/AI) at 35 to 41 d and 58 to 64 d after AI, and pregnancy losses in cows with low (≤2.5) or high (>2.5) BCS for cows receiving Ovsynch + estradiol-17β (E2) or Ovsynch alone

End point	Treatment			
	Ovsynch + E2		Ovsynch	
	% (n/n)			
BCS[1] (mean ± SEM)	2.84 ± 0.02		2.83 ± 0.02	
BCS	Low	High	Low	High
Estrus[2]	68.5[b] (61/89)	85.2[a] (167/196)	42.5[c] (34/80)	45.5[c] (85/187)
PP/AI, d 35 to 41	42.9[ab] (54/126)	48.3[a] (153/317)	34.7[b] (41/118)	49.1[a] (144/293)
PP/AI, d 58 to 64	40.0[A,a] (50/125)	43.9[a] (138/314)	28.1[B,b] (32/114)	43.7[a] (125/286)
Pregnancy loss	5.7 (3/53)	8.0 (12/150)	13.5 (5/37)	8.8 (12/137)

[a–c]Means with different superscripts within a row are different; $P < 0.05$.

[A,B]Means with different superscripts within a row are different; $P < 0.10$.

[1]On a scale from 1 to 5.

[2]Determined by pressure-activated heat mount detector (Kamar) in a subset of cows.

Ovsynch + E2, therefore, could be because of the cyclicity status before TAI.

Estrous response in the control group (Ovsynch alone) was strongly affected ($P < 0.01$) by size of the ovulatory follicle (Figure 4). The increase in expression of estrus was most apparent as follicles increased from 15 (~30% estrus) to 20 mm (>60% estrus) in diameter.

The relationship between expression of estrus and follicle size was less evident ($P = 0.09$) in E2-treated cows probably because E2 treatment increased ($P < 0.01$) probability of estrus in cows ovulating smaller follicles (Figure 4). Expression of estrus is highly dependent on the circulating E2 concentrations during proestrus (Kyle et al., 1992; Lyimo et al., 2000). Cows that ovulated follicles <13 mm in diameter had lower ($P < 0.05$) circulating E2 (1.4 ± 0.3), and cows ovulating follicles ≤15 mm tended ($P = 0.07$) to have lower circulating E2 (1.8 ± 0.3) compared with cows ovulating follicles >15 mm (2.2 ± 0.1). A reduction in circulating E2 is likely to be the hormonal basis for the reduced expression of estrus in cows ovulating smaller follicles during Ovsynch.

Our results are consistent with prior studies (Cerri et al., 2004) that reported increased expression of estrus near TAI in cows with larger follicles. Cerri et al. (2004) indicated, however, that this association was also true in cows receiving 1 mg of ECP. Conversely, in our study, there was no interaction between E2 treatment and follicle size on expression of estrus. These contrasting results between our study and Cerri et al. (2004) might be related to markedly lower circulating E2 concentra-

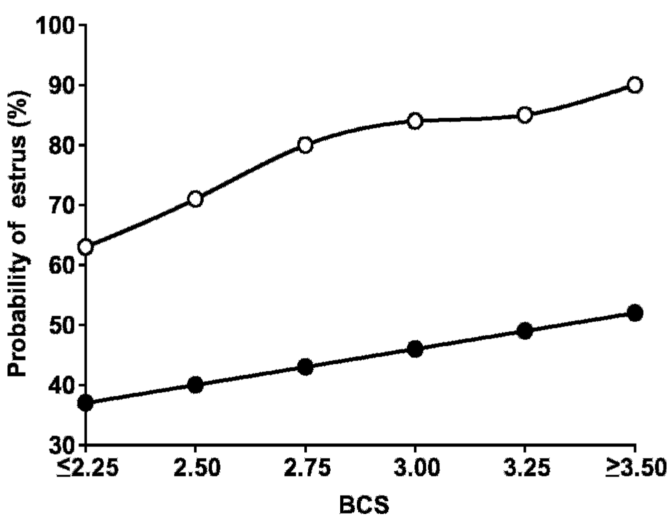

Figure 3. Effect of BCS at the time of AI on the estimated probability of expression of estrus during Ovsynch + estradiol-17β (E2) (○) and Ovsynch (●). Distribution of BCS: ≤2.25 = 11.2%; 2.5 = 17.3%; 2.75 = 27.2%; 3.0 = 25.2%; 3.25 = 10.4%; ≥3.5 = 8.7%.

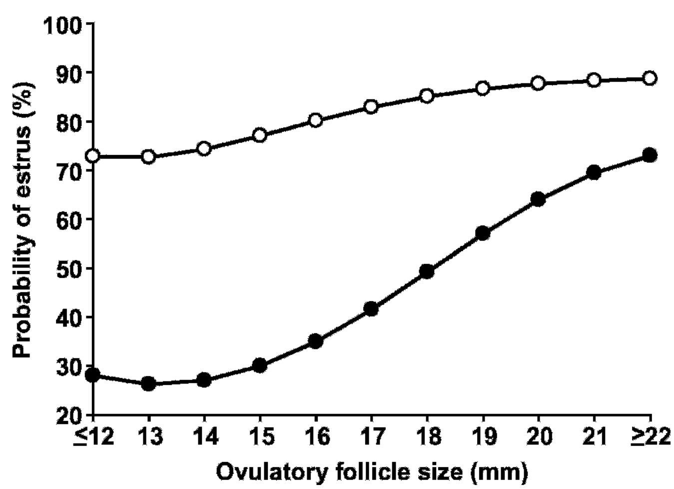

Figure 4. Effect of ovulatory follicle size (mm) at 48 h after $PGF_{2\alpha}$ treatment of the Ovsynch protocol on the estimated probability of expression of estrus during Ovsynch + estradiol-17β (E2) (○) and Ovsynch (●). Only cows with single ovulation were analyzed.

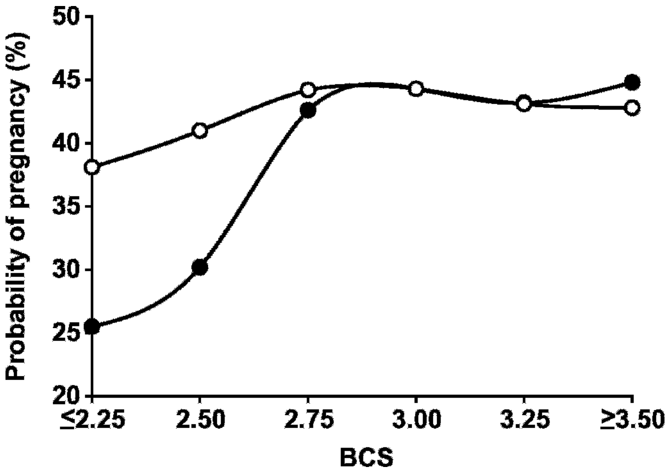

Figure 5. Effect of BCS at the time of AI on the estimated probability of pregnancy at 58 to 64 d after AI in cows treated with Ovsynch + estradiol-17β (E2) (○) or Ovsynch (●).

tions achieved in cows treated with ECP compared with cows treated with native E2 (Souza et al., 2005). The E2 treatment increased expression of estrus during Ovsynch and seemed to change the primary factor regulating the probability of expression of estrus. In E2-treated cows, BCS appeared to have the greatest influence on expression of estrus, whereas the size of the ovulatory follicles was the greatest predictor of expression of estrus in cows treated with Ovsynch alone.

Effect of BCS on Fertility

In contrast to expression of estrus, lower BCS was most clearly associated with reduced PP/AI in cows treated with Ovsynch alone (Figure 5, Table 3). A reduced (≤2.5) BCS was associated with lower PP/AI (P = 0.02) when all cows were used in the analysis. This association, however, was primarily due to the effect (P = 0.01) of low BCS (≤2.5) on PP/AI in cows treated with Ovsynch alone. Cows with lower BCS treated with E2 had comparable ($P > 0.10$) PP/AI to cows with greater BCS. At the later pregnancy exam, E2-treated cows with low BCS tended (P = 0.07) to have greater PP/AI than cows in the control group with lower BCS (Table 3). In cows with BCS ≥2.75 there was no effect of E2 treatment or BCS on PP/AI. Supplementing E2, therefore, had a beneficial effect in cows with low BCS but not in cows with normal BCS.

Our finding of reduced PP/AI in cows with reduced BCS treated with Ovsynch alone is consistent with previous findings (Moreira et al., 2000; Gümen et al., 2003). In contrast to our results, Pancarci et al. (2002) found that cows with low BCS that were treated with ECP had lower PP/AI than comparable cows with low BCS receiving the typical Ovsynch protocol. These authors argued that cows with lower BCS are more likely to be anovular and less likely to respond with a GnRH-induced LH surge after estrogen treatment possibly because of hypothalamic unresponsiveness (Wiltbank et al., 2002). The opposite effects of E2 treatment on cows with low BCS in our study compared with Pancarci et al. (2002) could be because of the differences in estrogen used (ECP vs. E2) or could be because of the use of a final GnRH in our study. The mechanisms mediating the E2-induced increase in PP/AI in Ovsynch-treated cows with low BCS could be related to optimization of sperm transport (Hawk and Cooper, 1975; Gaddum-Rosse, 1981; Orihuela et al., 1999), reduction in the incidence of short cycles (Mann and Lamming, 2000; Mann and Haresign, 2001), or a beneficial effect on the uterine capacity for maintaining pregnancy (Miller et al., 1977).

Double vs. Single Ovulation

The number of cows having double ovulations [2 or more corpora lutea (**CL**) detected 7 d after AI] did not differ ($P > 0.10$) between E2-treated (18.1%) and control

Table 4. Circulating estradiol-17β (E2) concentrations, percentage pregnant per AI (PP/AI), and pregnancy losses for 3 classes of ovulatory follicles in cows ovulating single follicles during Ovsynch + E2 or Ovsynch alone

	Ovulatory follicle size					
	≤14 mm		15 to 19 mm		≥20 mm	
Item	Ovsynch + E2	Ovsynch	Ovsynch + E2	Ovsynch	Ovsynch + E2	Ovsynch
Circulating E2 (pg/mL)	1.8 ± 0.3^{b}		1.9 ± 0.2^{b}		2.6 ± 0.2^{a}	
	% (n/n)					
PP/AI, d 35 to 41	41.1^{b} (37/90)	42.7^{b} (38/89)	57.9^{a} (88/152)	47.0^{ab} (71/151)	38.9^{b} (28/72)	47.1^{ab} (32/68)
PP/AI, d 58 to 64	38.2^{bc} (34/89)	34.1^{c} (29/85)	$52.6^{A,a}$ (80/152)	$42.0^{B,ab}$ (63/150)	34.3^{bc} (24/70)	42.4^{abc} (28/66)
Pregnancy loss	5.6 (2/36)	14.7 (5/34)	9.1 (8/88)	10.0 (7/70)	7.7 (2/26)	6.7 (2/30)

[a–c]Means with different superscripts within a row are different; $P < 0.05$.

[A,B]Means with different superscripts within a row are different; $P < 0.10$.

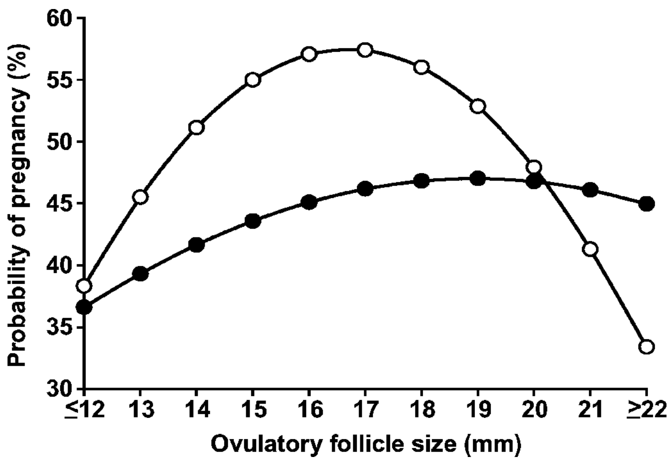

Figure 6. Effect of ovulatory follicle size (mm) at 48 h after $PGF_{2\alpha}$ treatment of the Ovsynch protocol on the estimated probability of pregnancy at 58 to 64 d after AI in cows treated with Ovsynch + estradiol-17β (E2) (○) or Ovsynch (●). Only single ovulating cows were analyzed.

(13.8%) cows. Cows ovulating multiple or single follicles had similar ($P > 0.10$) PP/AI within and between treatments. For example at the second pregnancy exam, cows receiving only Ovsynch had a PP/AI of 39.9% in single ovulators (n = 301) and 42.9% in multiple ovulators (n = 56). Cows receiving Ovsynch + E2 had a PP/AI of 44.4% in single ovulators (n = 304) and 38.3% in multiple ovulators (n = 81).

Effect of Follicle Size on Fertility

The large number of single-ovulating cows in this study allowed a valid test of our second main hypothesis that during Ovsynch, ovulation of medium-sized follicles would result in greater PP/AI than ovulation of smaller or larger follicles and that E2 supplementation would increase PP/AI for cows ovulating smaller, but not larger, follicles. As summarized in Table 4 and shown graphically in Figure 6, there was increasing PP/AI with increasing size of the ovulatory follicle (up to ≥18 mm diameter) in cows treated with Ovsynch alone. Supplementation with E2 tended to increase PP/AI in cows ovulating medium-sized follicles (15 to 19 mm) but not in cows ovulating larger or smaller follicles. Logistic regression indicated that PP/AI was associated ($P < 0.05$) with the size of ovulatory follicle, having a quadratic relationship in E2-treated cows but a linear relationship in control cows (Figure 6). The differences between trend lines are a reflection of a significant ($P < 0.02$) follicle size by treatment interaction on PP/AI. In the Ovsynch group, cows ovulating smaller follicles (≤13 mm) had lesser ($P < 0.05$) PP/AI (28.1%; n = 64) and greater ($P < 0.05$) embryonic losses (21.7%) than cows ovulating larger follicles (>13 mm; 43.0 and 8.1%, respectively) in the same experimental group. The E2-treated cows ovulating follicles ≤13 mm had an acceptable PP/AI (40.0%, n = 60) that was less ($P < 0.05$) than cows ovulating medium-sized follicles (15 to 19 mm; 52.6%; n = 152) but similar to cows ovulating larger follicles (≥20 mm; 34.3%; n = 70).

These results are consistent with some previous studies (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005). These previous studies generally indicated that cows ovulating smaller follicles had lower PP/AI with maximal PP/AI at 16 to 18 mm in Lamb et al. (2001) or at ~14 mm in Perry et al. (2005). In relation to the second hypothesis for this study, ovulation of medium-sized follicles (15 to 19 mm) did produce the greatest fertility if E2 was included in the Ovsynch protocol. The PP/AI of >50% (Table 4) indicated that medium-sized follicles could produce a fertile oocyte and a CL with sufficient progesterone production to support high pregnancy rates but that circulating E2 may be a limiting factor for high-producing dairy cows that ovulate medium-sized follicles. In cows ovulating

Table 5. Average DIM, estrous response, percentage pregnant per AI (PP/AI), and pregnancy losses in cows submitted for first, second, or more services during Ovsynch + estradiol-17β (E2) or Ovsynch alone

	Ovsynch + E2			Ovsynch		
End point, % (n/n)	First	Second	≥Third	First	Second	≥Third
DIM[1]	70.7[c]	108.2[b]	157.5[a]	71.2[c]	110.8[b]	163.8[a]
			% (n/n)			
Estrus[2]	78.8[a] (108/137)	79.3[a] (65/82)	84.1[a] (58/69)	42.6[b] (52/122)	44.7[b] (34/76)	46.8[b] (36/77)
PP/AI, d 35 to 41	49.8 (103/207)	40.9 (54/132)	47.2 (51/108)	43.8 (81/185)	42.1 (51/121)	50.0 (57/114)
PP/AI, d 58 to 64	45.1[a] (93/206)	37.2 (48/129)	43.5 (47/108)	35.4[B,b] (63/178)	39.5 (47/119)	45.5[A] (51/112)
Pregnancy loss	8.8 (9/102)	5.9 (3/51)	7.8 (4/51)	14.9 (11/74)	4.1 (2/49)	7.3 (4/55)

[a–c]Means with different superscripts within a row are different; $P < 0.05$.

[A,B]Means with different superscripts within a row are different; $P < 0.10$.

[1]Average DIM at breeding.

[2]Determined by pressure-activated heat mount detector (Kamar) in a subset of cows.

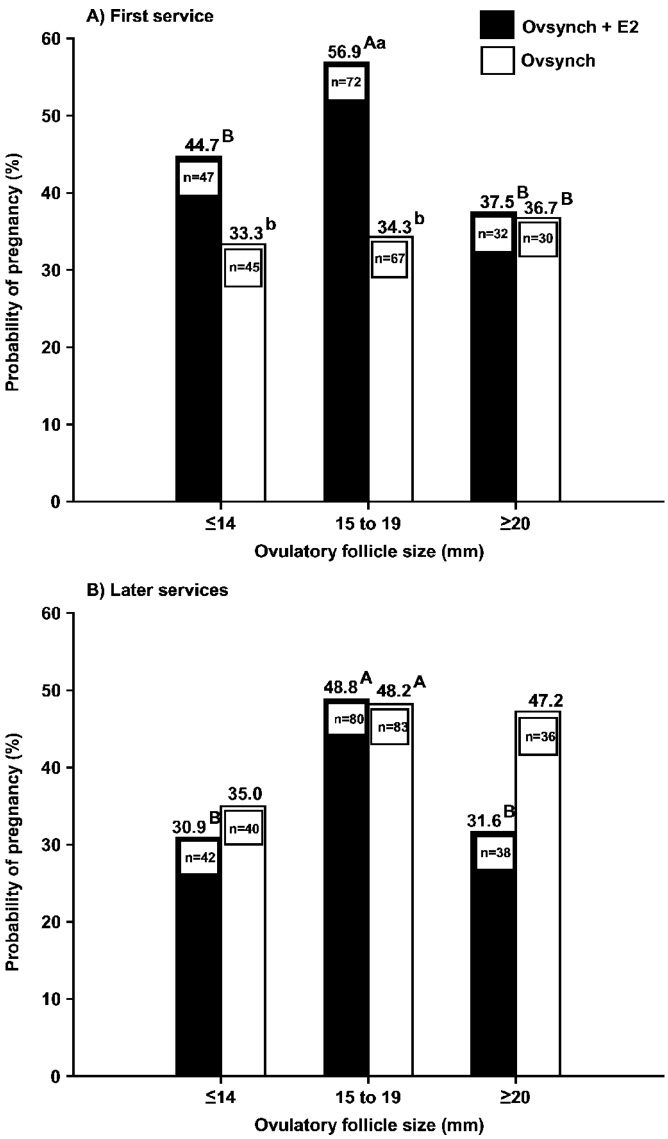

Figure 7. Effect of the ovulatory follicle size (mm) at 48 h after $PGF_{2\alpha}$ treatment during the Ovsynch protocol at first (A) or later (B) postpartum services on the estimated probability of pregnancy at 58 to 64 d after AI following Ovsynch + estradiol-17β (E2) (black bars) or Ovsynch (white bars). Only single ovulating cows were analyzed. [a,b]Means with different superscripts are different ($P < 0.05$); [A,B]Indicates a tendency for differences between means ($P < 0.10$). Comparisons between first vs. later services were not performed.

smaller follicles, supplementation with E2 was not sufficient to increase PP/AI to the high values achieved in cows ovulating 15- to 19-mm follicles, indicating that other factors are still limiting for fertility in these cows.

Vasconcelos et al. (2001) used follicular aspirations to induce ovulation of smaller (~11.5 mm) follicles during the Ovsynch protocol compared with nonaspirated Ovsynch-treated cows (~14.5 mm). They found that cows ovulating smaller follicles had reduced peak E2 concentrations, reduced CL tissue volume, reduced circulating progesterone, and reduced PP/AI. In agreement with these results, an earlier study using ewes (Murdoch and Van Kirk, 1998) and, more recently, a study done in dairy cows (Peters and Pursley, 2003) have described impaired CL function and PP/AI when animals were prematurely induced to ovulate. Cows ovulating small follicles may have reduced circulating progesterone that could impair embryonic development or cause premature uterine $PGF_{2\alpha}$ secretion and luteolysis as previously described (Mann and Lamming, 2001).

There was a numerical decrease in PP/AI in E2-treated cows ovulating larger follicles (≥20 mm). Lower fertility in cows ovulating larger follicles could be related to ovulation of a persistent follicle that may be caused by excessive LH stimulation and ovulation of oocytes with reduced fertility (Ahmad et al., 1995; Kinder et al., 1996; Revah and Butler, 1996). In addition, earlier studies have described detrimental effects of high circulating E2 concentrations before ovulation in ewes (Langford et al., 1980) and rats (Butcher and Pope, 1979).

One explanation of why a positive effect of E2 supplementation on PP/AI was not observed in this study may be that only 48.7% (303/622) of cows ovulated a medium-sized follicle (15 to 19 mm), with many cows ovulating follicles that were too small (28.7%; 179/622; 10 to 14 mm) or too large (22.5%; 140/622; ≥20 mm). It seems clear that Ovsynch does not provide a compact synchronization of ovulatory follicle size. Optimizing fertility in future timed AI programs will require not only optimization of circulating E2 but also improved synchrony of follicle size at time of ovulation.

Effect of Number of Services

Table 5 shows the effect of E2 treatment on PP/AI and pregnancy losses by number of services. At the first pregnancy diagnosis (35 to 41 d), no differences were detected between or within treatments ($P > 0.10$). At the later pregnancy exam, for cows at first service, however, E2 increased ($P < 0.02$) PP/AI compared with untreated cows. Within the control group, cows with 3 or more services tended ($P = 0.09$) to have greater PP/AI than cows at first service.

Chebel et al. (2004) found a decrease in PP/AI as the number of services increased. The E2-induced improvements in PP/AI only during the first service may be because of a greater proportion of anovular cows at first service than at later services. The use of presynch and detection of estrus after the second $PGF_{2\alpha}$ injection would tend to increase the number of anovular cows treated with Ovsynch at the first service, although this

Table 6. Estrous response, percentage pregnant per AI (PP/AI), and pregnancy losses for primiparous and multiparous cows[1] during Ovsynch + estradiol-17β (E2) or Ovsynch alone

	Ovsynch + E2		Ovsynch	
Item	Primiparous	Multiparous	Primiparous	Multiparous
	% (n/n)			
Estrus[2]	80.6[a] (83/103)	80.0[a] (148/185)	44.2[b] (46/104)	44.4[b] (76/171)
PP/AI, d 35 to 41	55.2[A,a] (80/145)	42.4[b] (128/302)	45.3[B] (68/150)	44.8[B] (121/270)
PP/AI, d 58 to 64	52.8[a] (76/144)	37.5[b] (112/299)	39.0[b] (57/146)	39.5[b] (104/263)
Pregnancy loss	3.8 (3/79)	10.4 (13/125)	10.9 (7/64)	8.8 (10/114)

[a–c]Means with different superscripts within a row are different; $P < 0.05$.

[A,B]Means with different superscripts within a row are different; $P < 0.10$.

[1]Primiparous = first lactation; multiparous = ≥second lactation.

[2]Determined by pressure-activated heat mount detector (Kamar) in a subset of cows.

was not quantified in the present experimental design. Treatment with E2 may be more effective in anovular cows (Gümen et al., 2005).

Figure 7 shows the interactions of E2 treatment within service number and ovulatory follicle size. At the first postpartum service, there was no effect of follicle size in cows treated with Ovsynch alone. The E2 treatment, however, increased PP/AI in cows ovulating medium-sized follicles and numerically increased PP/AI in cows ovulating smaller but not larger follicles. At later services, E2 treatment did not increase PP/AI in any of the PP/AI groups although the higher PP/AI for cows ovulating medium-sized follicles continued to be present. No significant interactions were found between treatment and service number in the number of cows losing pregnancies.

Effect of Parity

There was no interaction ($P > 0.10$) between lactation number and treatment in expression of estrus (Table 6). There was a treatment by parity interaction on PP/AI at the first ($P = 0.08$) and second ($P = 0.03$) pregnancy exams. Primiparous cows treated with E2 tended to have greater PP/AI than primiparous ($P = 0.08$) and multiparous ($P = 0.06$) cows in the control group at the first pregnancy exam (Table 6). In addition, at the first pregnancy exam, primiparous cows that received E2 had greater ($P < 0.03$) PP/AI than multiparous that received E2. At the second pregnancy exam, an interaction between treatment and parity in PP/AI was evident ($P = 0.03$). Primiparous cows treated with E2 had greater PP/AI than multiparous cows ($P < 0.01$) treated with E2 and greater PP/AI than primiparous ($P = 0.01$) and multiparous cows ($P < 0.02$) in the control group. No interactions between treatment and lactation number were detected for pregnancy losses (Table 6).

Figure 8 shows the interactions of E2 treatment within parity and follicle size. In primiparous cows, there was no effect of follicle size in cows treated with Ovsynch alone. Primiparous cows receiving E2 treatment had high PP/AI at all sizes of follicles and E2 treatment increased PP/AI in primiparous cows ovulating medium-sized follicles. Multiparous cows treated only with Ovsynch had greater PP/AI in cows ovulating medium-sized compared with smaller follicles. Treatment with E2 did not improve PP/AI in multiparous cows at any follicle size and tended to decrease ($P = 0.06$) PP/AI in multiparous cows ovulating large follicles. The positive effects of E2 treatment on fertility in primiparous, but not multiparous, cows was unexpected and will require further investigation. A number of previous studies have found parity effects during Ovsynch or Heatsynch protocols (Pursley et al., 1997; Cerri et al., 2004) but this is the first report, that we are aware of, indicating a parity by follicle size and a parity by E2 treatment interaction on PP/AI.

General Discussion

Fertility during Ovsynch protocols may be limited by circulating E2 concentrations in some situations. For example, cows with low BCS had a substantial improvement in PP/AI when Ovsynch was supplemented with E2, such that low-BCS cows had similar fertility to cycling cows. This highlights the key role of deficient E2 in noncycling dairy cows. In addition, this study highlighted the importance of improving the synchrony of ovulatory follicle size in the Ovsynch protocol and demonstrated that E2 is a key limiting factor for fertility in cows ovulating follicles in an ideal range (15 to 19 mm) but does not improve fertility in cows ovulating smaller or larger follicles during Ovsynch. In addition, E2 supplementation was found to improve Ovsynch results in primiparous cows and cows at the first service postpartum perhaps because of greater potential fertility or greater synchrony of ovulatory follicle size in these cows. Cows that did not express estrus during an

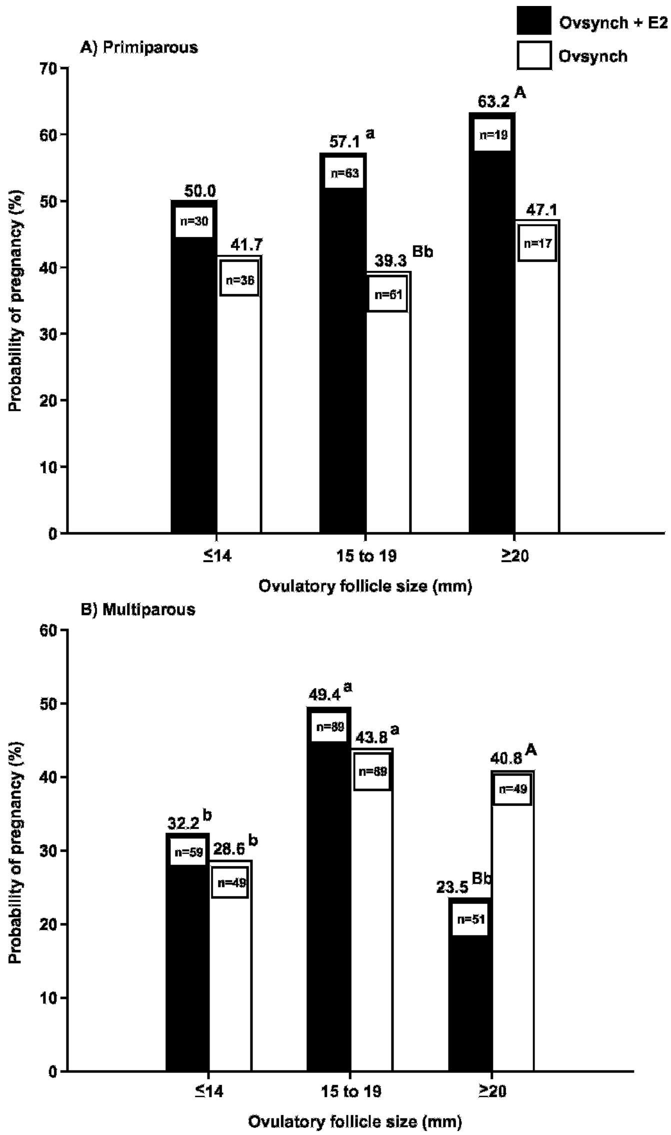

Figure 8. Effect of the ovulatory follicle size (mm) at 48h after $PGF_{2\alpha}$ treatment of the Ovsynch protocol for primiparous (A) or multiparous (B) cows on the estimated probability of pregnancy at 58 to 64 d after AI during Ovsynch + estradiol-17β (E2) (black bars) or Ovsynch (white bars). Only single ovulating cows were analyzed. [a,b]Means with different superscripts are different ($P < 0.05$); [A,B]Indicates a tendency for differences between means ($P < 0.10$). Comparisons between parity were not performed.

Ovsynch + E2 protocol were found to be less fertile. Determination of the underlying cause of this reduction in fertility may allow identification of these cows and development of enhanced reproductive management procedures for these animals.

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