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Fertility programs for lactating dairy cows: A novel presynch + timed artificial insemination program (Double E-Synch) produces similar ovarian dynamics, synchronization, and fertility as Double-Ovsynch

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ABSTRACT

Fertility programs were implemented for the first postpartum timed artificial insemination (TAI) in 800 (primiparous and multiparous) lactating dairy cows, evaluating 2 presynchronization (presynch) strategies and 2 TAI protocols, in a 2 × 2 factorial arrangement. Weekly, cows were enrolled into 1 of 4 groups (Ovs+Ovs [Double-Ovsynch], Ovs+OvsP4/E2, PreP4/E2+Ovs, and PreP4/E2+OvsP4/E2 [Double E-Synch]). On d −17 (34 ± 3 DIM), the Ovsynch [Ovs] presynch was initiated with 10 µg of buserelin acetate (GnRH), and cows received 0.5 mg of cloprostenol sodium $PGF_{2\alpha}$ analog (PGF) on d −10, and 10 µg of GnRH on d −7. The PreP4/E2 presynch was initiated on d −17 with a used 2-g progesterone (P4) insert, which was removed on d −10, together with 0.5 mg of PGF and 1 mg of estradiol (E2) cypionate (EC). For TAI protocols, Ovs group received the following: on d 0, 20 µg of GnRH (double dose); on d 7, PGF; on d 8, PGF; on d 9.5, 10 µg of GnRH; and on d 10, TAI (16 h after GnRH). Cows submitted to OvsP4/E2 received the following: on d 0, 20 µg of GnRH (double dose) and a new 2-g P4 insert; on d 7, PGF; on d 8, P4 insert removal, PGF, and EC; and on d 10, TAI (48 h after P4 insert removal). The GLIMMIX procedure of SAS 9.4 was used for statistical analyses ($P \leq 0.05$). The presence of corpus luteum (CL) on d −17 (average = 68.8% [550/800]) was similar among treatments. The presence of CL on d 0 of TAI protocols was high, and Ovs as a presynch increased percentage of cows with CL (95.5% [382/400] vs. 90.8% [363/400]). However, at the first PGF of the breeding (TAI) protocols (d 7), there was no effect of presynchronization program and 98.5% (788/800) of the cows had at least 1 CL. Ovulation after d 0 was greater in cows submitted to PreP4/E2 than Ovs (72.0% [288/400] vs. 64.3% [257/400]), and those ovulating had greater pregnancies per artificial insemination (P/AI; 51.0% [278/545] vs. 41.6% [106/255]). Overall, multiple ovulations after TAI were low and similar between TAI protocols and presynch strategies (7.2% [54/753]). Expression of estrus in OvsP4/E2 protocols was greater than Ovs (69.4% [274/395] vs. 41.5% [168/405]), and an interaction was detected, in which cows not expressing estrus ovulated more after TAI in Ovs compared with OvsP4/E2 protocol (93.3 [221/237] vs. 77.7% [94/121]). Cows expressing estrus had greater P/AI in both Ovs (58.3 [98/168] vs. 42.2% [100/237]) and OvsP4/E2 (57.3 [157/274] vs. 24.0% [29/121]). There was no interaction between presynch and TAI protocol on P/AI on d 32 of cows that ovulated after TAI (48.4%, 49.7%, 53.3%, and 52.5% for Ovs+Ovs, Ovs+OvsP4/E2, PreP4/E2+Ovs, and PreP4/E2+OvsP4/E2, respectively), and no differences in pregnancy loss between d 32 and 90 (average = 24.0% [92/384]). In conclusion, the study validated 2 presynchronization strategies and 2 TAI protocols, establishing 4 possible fertility programs, all of them producing well-controlled ovarian dynamics, excellent synchronization, and high fertility. Moreover, Double-Ovsynch and Double E-Synch both produced similar results, despite differences in pharmacological bases.

Key words: dairy cow, timed artificial insemination, fertility, synchronization

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

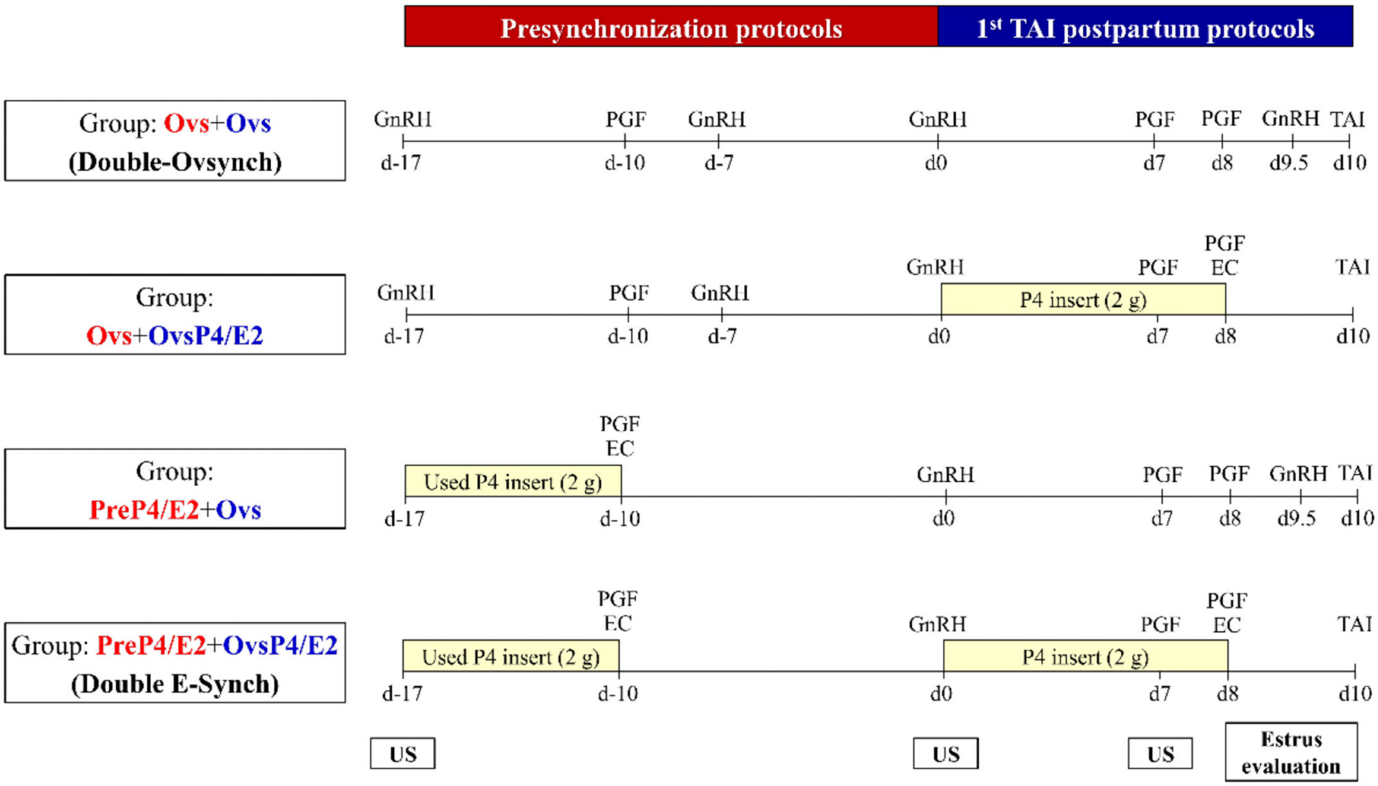

Figure 1. Experimental design with hormonal treatments and procedures performed for the 4 different synchronization programs. In each program, a presynchronization (either Ovs or PreP4/E2) and timed artificial insemination (TAI) protocol (either Ovs or OvsP4/E2) was performed. GnRH = buserelin acetate; PGF = cloprostenol sodium, $PGF_{2\alpha}$; P4 = progesterone; EC = estradiol cypionate; and US = ultrasound.

INTRODUCTION

Timed artificial insemination (**TAI**) programs are divided into 2 main pharmacological bases: Ovsynch-type programs (GnRH and cloprostenol sodium $PGF_{2\alpha}$ analog [**PGF**]-based), and estradiol (**E2**) plus progesterone (**P4**)–based programs. Different combinations of these hormones in various modified versions of these programs have been tested in past research (Wiltbank and Pursley, 2014; Consentini et al., 2021). Currently, TAI programs that result in greater pregnancy per artificial insemination (**P/AI**) than achieved in cows inseminated after estrus are termed fertility programs (Pursley and Martins, 2011; Carvalho et al., 2018; Fricke and Wiltbank, 2022). Although simple Ovsynch is not a fertility program because it produces P/AI that is similar to artificial insemination (**AI**) after estrus (Pursley et al., 1997a,b), other programs are considered fertility programs such as Presynch-Ovsynch (Moreira et al., 2001; Strickland et al., 2024), G7G (Peters and Pursley, 2002; Kim et al., 2020), G6G (Bello et al., 2006), and Double-Ovsynch (**DO**; Souza et al., 2008; Santos et al., 2017; Sitko et al., 2023). Generally, these fertility programs presynchronize cows so that the "breeding" TAI Ovsynch protocol initiates on d 6 or 7 of the estrous cycle. The key is that P4 is not excessively high so that the GnRH-induced LH surge is not inhibited (Giordano et al., 2012) and a dominant follicle is present that can ovulate in response to the LH surge (Vasconcelos et al., 1999; Wiltbank and Pursley, 2014). Ovulation to the first GnRH of the TAI Ovsynch protocol results in the initiation of a new follicular wave and the presence of a second corpus luteum (**CL**) throughout the development of the preovulatory follicular wave, which tends to reduce follicle codominance (Lopez et al., 2005), improve oocyte and embryo quality (Wiltbank et al., 2011, 2014, 2016), and increase P/AI (Carvalho et al., 2018; Cardoso Consentini et al., 2021, 2025).

The fertility programs that have been reported use GnRH and PGF combinations in both the presynchronization protocol and the protocol for the TAI (Fricke and Wiltbank, 2022). In some countries, such as Brazil, E2 products are available for use in lactating dairy cows and these products may allow development of fertility programs that have similar ovarian dynamics but that are either easier to implement or produce greater fertility. Previous studies have shown that, among the many

Table 1. Comparison of 2 different presynchronization programs for the presence of CL on d −17 (start of presynchronization), d 0 (start of TAI protocol), and d 7 (first PGF treatment of TAI protocol) and ovulation after the first GnRH (d 0) of TAI protocol

		Presynchronization strategy		
Item, % (n/n)	Overall	Ovsynch	PreP4/E2	*P*-value
CL on d −17 (start of presynch)	68.8 (550/800)	68.5 (274/400)	69.0 (276/400)	0.85
CL on d 0 (start of TAI program)	93.1 (745/800)	95.5 (382/400)	90.8 (363/400)	0.04
Ovulation after first GnRH (d 0)	68.1 (545/800)	64.3 (257/400)	72.0 (288/400)	0.04
CL on d 7 (first PGF of TAI protocol)	98.5 (788/800)	99.0 (396/400)	98.0 (392/400)	0.54

factors that can affect fertility of lactating dairy cows submitted to TAI programs (Carvalho et al., 2018; Consentini et al., 2021; Sartori et al., 2023), circulating E2 and expression of estrus at the end of TAI protocols are 2 potentially fertility-enhancing factors that may be altered by E2 treatments during the protocols (Bello et al., 2006; Brusveen et al., 2009; Souza et al., 2009; Consentini et al., 2025; Laplacette et al., 2025). Thus, this study was designed to test protocols for presynchronization or for final synchronization that used E2 cypionate (**EC**) to induce ovulation. Our rationale was that producers and veterinarians may want to use EC in fertility programs, if it is allowed and effective, due to the reduction of one handling event compared with Ovsynch-56 (EC is given at the time of the final PGF treatment, removing the handling of the GnRH treatment 16 h before TAI), greater circulating E2 during the proestrous periods, and greater expression of estrus during the protocol.

Our research group has recently developed a program for TAI using EC for induction of ovulation after the presynchronization protocol and at the end of the final TAI protocol (Consentini et al., 2025). This novel protocol has been termed Double E-Synch (**DES**) and it appears to produce similar ovarian dynamics as DO, and the DES (from E2) name was given due to the use of an E2 ester to induce ovulation in both presynchronization and TAI protocols. To validly compare the 2 types of presynchronization protocols (presynchronization Ovsynch vs. presynchronization E-Synch) and the final TAI protocols (TAI Ovsynch vs. TAI E-Synch) a 2 × 2 factorial experimental arrangement was used. The final TAI protocols use hormonal optimizations that have been previously found to produce high fertility including double dose of GnRH at the beginning (Giordano et al., 2013; Silva et al., 2024) and 2 PGF treatments at the end (Wiltbank et al., 2015) of the TAI protocol. Thus, this study was designed to test 2 different presynchronization protocols and 2 different TAI protocols and not to directly test any specific hormonal treatment (e.g., P4 insert or EC vs. GnRH). Four TAI programs were compared in this research, including DO and DES. Three main hypotheses were proposed for the study: (1) Both presynchronization strategies would produce a similar percentage of cows with CL at the beginning and at the time of the first PGF of the TAI protocols, and similar ovulation response after the GnRH treatment at the beginning of the TAI protocol (d 0), (2) the expression of estrus at the end of the TAI protocols would be greater in the TAI protocols including EC and that estrus would be associated with greater fertility in both TAI protocols, and (3) the TAI protocol with P4 insert and EC at the end would produce better fertility, mainly due to a greater percentage of cows expressing estrus indicating greater circulating E2 before AI.

MATERIALS AND METHODS

The Animal Care and Use Committee of Luiz de Queiroz College of Agriculture of the University of São Paulo (ESALQ/USP) approved all procedures involving cows in this study (protocol # 5112290720).

The experiment was conducted on a commercial dairy farm located in the Midwest of Brazil, from January to December of 2021, in which all cows available for first TAI postpartum during that year were enrolled in the study, without, necessarily, a priori sample size calculation. The total number of cows enrolled in the study was 895, but the final number of 800 were used for the analysis. The reasons cows were removed from the experiment were (1) use of sex-sorted sperm (n = 21), and (2) severe health problems or being culled from the herd before TAI or any of the pregnancy diagnosis (n = 74). A power calculation for a 10–percentage point difference (50% vs. 40%) in binomial variables such as P/AI, using α = 0.05 and a one-tailed test, and a sample size per group of 200, yielded a power of 0.6. The study followed a 2 × 2 factorial arrangement, and assuming no interaction between the factors, it results in a total of 400 animals available to evaluate the main effect of presynchronization protocol (2 comparisons) or TAI protocol (2 comparisons) in the binary variables studied. Thus, for these comparisons and a 10–percentage point difference (50% vs. 40%) using α

Table 2. Effect of the presynchronization strategy on the expression of estrus at the end of TAI protocols and ovulation after TAI

Item, % (n/n)	Presynchronization strategy		P-value
	Ovsynch	PreP4/E2	
Estrus by d 9.5	17.3 (69/400)	18.0 (72/400)	0.61
Estrus by TAI (d 10)	54.3 (217/400)	56.3 (225/400)	0.58
Ovulation after TAI	93.3 (373/400)	95.0 (380/400)	0.28
Multiple ovulation after TAI	5.9 (22/373)	8.4 (32/380)	0.15

Table 3. Effect of timed artificial insemination (TAI) protocols on the expression of estrus and ovulation after TAI

Item, % (n/n)	TAI protocol		P-value
	Ovsynch	OvsynchP4/E2	
Estrus by d 9.5	10.9 (44/405)	24.6 (97/395)	<0.001
Estrus by TAI (d 10)	41.5 (168/405)	69.4 (274/395)	<0.001
Ovulation after TAI	96.1 (389/405)	92.2 (364/395)	0.02
Multiple ovulation after TAI	8.0 (31/389)	6.3 (23/364)	0.36

= 0.05, and a one-tailed test, a sample size per group of 400 produced a power calculated at 0.87.

Animals and Herd Management

Cows were housed in freestall barns with sand bedding and had free access to water, mineral salt, and were fed ad libitum with a TMR diet balanced to meet or exceed the nutritional requirements of lactating dairy cows producing 40 kg of milk/d (NRC, 2001). Throughout the experiment, cows were milked thrice daily, and the 305-d average milk production of the herd was 9,500 kg of milk. Daily milk production by individual cows was measured and recorded automatically by the milking system.

A total of 800 lactating Holstein cows (389 multiparous and 411 primiparous) were used for their first postpartum TAI. The presynchronization protocols (d −17; Figure 1) were initiated at 34 ± 3 DIM for cows to be inseminated at 61 ± 3 DIM.

The high proportion of primiparous cows enrolled in the study was due to the structure of the herd and the arrival of a high number of pregnant heifers to the farm before the experiment. The average daily milk yield for primiparous cows on d −17, d 0, and at TAI was 27.9 ± 0.3, 31.8 ± 0.3, and 33.7 ± 0.3 kg/d, respectively, whereas multiparous cows yielded 39.4 ± 0.3, 41.1 ± 0.3, and 41.6 ± 0.3 kg/d. The average BCS (scale from 1 to 5, according to Ferguson et al., 1994) on d −17 was 3.32 ± 0.02 for primiparous and 3.15 ± 0.02 for multiparous cattle.

Treatments and Experimental Design

Following the 2 × 2 factorial arrangement, weekly, cows with 33 ± 3 DIM were randomly distributed into 1 of 4 TAI programs (Ovs+Ovs [DO; n = 202], Ovs+OvsP4/E2 [n = 198], PreP4/E2+Ovs [n = 203], and PreP4/E2+OvsP4/E2 [DES; n = 197]) according to number of lactations and milk production on d −17. Thus, the 2 presynchronization protocols were Ovs and PreP4/E2, and TAI protocols were Ovs and OvsP4/E2 (Figure 1). Cows in the Ovs presynchronization received 10 µg of buserelin acetate (GnRH, Maxrelin, GlobalGen, Jaboticabal, Brazil) on d −17, 0.5 mg of PGF (Induscio, GlobalGen) on d −10, and 10 µg of GnRH on d −7. The PreP4/E2 presynchronization was initiated on d −17 with a previously used once (for 8 d) or twice (16 d) 2-g P4 insert (Reprosync, GlobalGen), which was removed on d −10, with simultaneous treatments of 0.5 mg of PGF and 1 mg of EC (Cipion, GlobalGen). Regarding TAI protocols, the Ovs program was initiated with 20 µg of GnRH (double dose) on d 0, the first PGF was given on d 7 followed by a second dose on d 8, and 10 µg of GnRH was given on d 9.5 (16 h before TAI on d 10). Cows submitted to OvsP4/E2 protocol received 20 µg of GnRH (double dose) and a new intravaginal 2-g P4 insert on d 0, the first PGF on d 7, and a second dose on d 8, concomitant with P4 insert removal and 1 mg of EC, and the TAI was performed on d 10. All injectables were given i.m. During the entire year of the study, randomization and hormonal treatments were performed by 2 graduate students, and AI procedures were performed by the same technician and only conventional frozen–thawed Holstein semen was used. All cows received TAI regardless of expression of estrus before the scheduled time of AI.

BCS, Ovarian Structures, Expression of Estrus, and Pregnancy Diagnosis

Information related to BCS, ovarian dynamics, and expression of estrus were recorded for all cows during the study. Transrectal ovarian ultrasound (**US**) to evaluate the presence (independent of its size) and number of CL was performed on d −17 (initiation of presynch programs), d 0 (beginning of TAI protocols), d 7 (time of first PGF treatment), and d 17 (7 d after TAI). Ovulation (after d 0 or after TAI) was considered based on the appearance of a new CL on d 7 or d 17 and if the cow had a CL with a cavity in the same ovary that had a compact CL in the previous US. All cows received a tail-head patch (BOViFLAG, Bovitime Animal Products LTD, Stellenbosch, South Africa) for detection of estrus at the time of first PGF, and were evaluated on d 8, d 9.5, and at TAI. Cows were considered to have expressed estrus if the device was activated (>50% of the silver scratch-off layer was rubbed off, changing the patch color) or based on other signs of estrus, such as standing to be mounted. The first

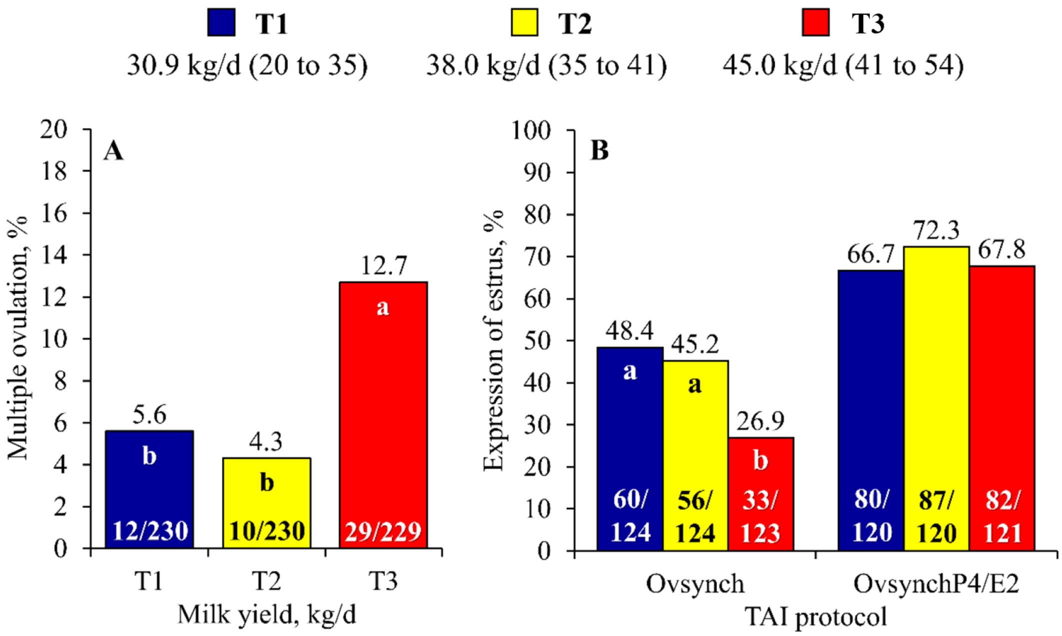

Figure 2. Effect of milk yield (divided into terciles) on multiple ovulation after TAI (panel A) and on expression of estrus at the end of TAI protocols (panel B). The terciles of milk yield are indicated as T1, T2, and T3. The lowercase letters (a,b) indicate an effect ($P < 0.01$) of milk yield on multiple ovulation (panel A) and an effect of milk tercile on expression of estrus in cows bred to Ovsynch (a,b) but not in cows bred to OvsynchP4/E2 protocol (panel B). There was an interaction between milk yield and TAI protocol ($P < 0.05$), in which expression of estrus was affected by milk yield in the Ovsynch protocol ($P = 0.001$), but not in OvsynchP4/E2 protocol ($P = 0.60$).

pregnancy diagnosis was performed 32 d after TAI by transrectal US of the reproductive tract by confirming an embryonic heartbeat. Subsequent confirmation of pregnancy was performed by US 60 and 90 d after TAI.

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). The analyses of binary variables were performed using the GLIMMIX procedure, fitting a binomial distribution with Link Logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

For the presence of CL on d −17, d 0, and d 7, the model included the effects of presynchronization, parity, and BCS class (≤2.75 vs. >2.75). The model for ovulation after d 0 included effects of presynchronization, parity, BCS and presence of CL on d 0. For expression of estrus, the variables studied were presynchronization, TAI protocol, parity, and ovulation after d 0. Regarding ovulation rate and multiple ovulations after TAI, the model included effects of presynchronization, protocol, and parity, and a separate analysis was performed to study the effect of expression of estrus and its interaction with TAI protocol.

To study the relationship between milk production (average of the 7 d before TAI) and multiple ovulation rate after TAI and expression of estrus at the end of the TAI protocol, milk production was divided into terciles. All cows were included in the analysis of the effect of milk yield on multiple ovulation rate, due to no effect of either presynchronization or TAI protocol. Regarding expression of estrus, due to the interaction between milk yield and TAI protocol, the effect of milk yield was evaluated within TAI protocol.

The models for P/AI and pregnancy loss included effects of presynchronization, TAI protocol, parity, ovulation after d 0, and expression of estrus. Moreover, for pregnancy loss, effect of multiple ovulation was studied. A separate analysis was performed to study the effect of milk yield (below or above the median, regardless of parity) and its interactions. Analyses were performed considering all cows and only cows ovulating after TAI. The interaction between presynchronization strategies and TAI protocols was maintained in the models, and other potentially important interactions between treatments and the variables were evaluated, and they are presented and discussed throughout the manuscript.

When an interaction was detected, the SLICE command within the LSMEANS was used to interpret the results. Tukey honest significant difference post hoc test was performed to determine differences. Values are

Table 4. Effect of the timed artificial insemination (TAI) protocol and expression of estrus on ovulation after TAI

	TAI protocol		*P*-value[1]		
Item, % (n/n)	Ovsynch	OvsynchP4/E2	P	E	I
Ovulation after TAI					
No estrus	93.3 (221/237)	77.7 (94/121)	<0.001	<0.001	<0.001
Estrus	100 (168/168)	98.5 (270/274)	0.97		
P-value[2]	0.0005	<0.001			
Multiple ovulation after TAI					
No estrus	9.5 (21/221)	8.5 (8/94)	0.88	0.18	0.83
Estrus	6.0 (10/168)	5.6 (15/270)	0.65		
P-value[2]	0.22	0.35			

[1]P = effect of TAI protocol within class of cows; E = main effect of expression of estrus; I = interaction between TAI protocol and expression of estrus.

[2]The *P*-values are related to the effect of expression of estrus within TAI protocols.

presented as percentage and significant differences were declared for $P \leq 0.05$, whereas tendencies were considered for $0.10 > P > 0.05$.

RESULTS AND DISCUSSION

The 2 presynchronization strategies and both TAI protocols evaluated in the study, although differing in their pharmacological bases, produced similar reproductive outcomes in terms of ovarian dynamics and synchronization, and relatively high fertility.

Ovarian Dynamics During the TAI Programs

The overall presence of CL at the beginning of presynchronization programs (34 ± 3 DIM) was 68.8% (550/800, Table 1), which is similar to studies reporting ~30% of cows in anovulatory condition at the beginning of lactation, up to 60 DIM (Gümen et al., 2003; Manríquez et al., 2021; Monteiro et al., 2021). Parity and BCS influenced the presence of CL on d −17. More multiparous cows had CL on d −17 compared with primiparous cows (71.7 [279/389] vs. 65.9% [271/411]; $P = 0.03$) and fewer thin cows (≤2.75) had CL than cows with BCS > 2.75 (62.2 [84/135] vs. 70.1% [466/665]; $P = 0.03$).

All cows were submitted to presynchronization protocols including strategies to induce ovulation, and 93.1% (745/800) of the cows initiated the TAI protocols with CL. Our first hypothesis, related to the ovarian dynamics, was not confirmed. The Ovs presynchronization program increased the percentage of cows with CL on d 0 compared with the PreP4/E2 protocol (Table 1) perhaps due to the use of GnRH rather than EC to induce ovulation at the end of the presynchronization program. More multiparous cows had CL on d 0 compared with primiparous cows (95.4% [371/389] vs. 91.0% [374/411]; $P = 0.005$), and on d 0, there was no effect of BCS (≤or > 2.75) on CL incidence (91.9% [124/135] vs. 93.4% [621/665]; $P = 0.34$). The lack of effect of BCS on presence of CL on d 0 indicates that both presynchronization strategies, with either EC or GnRH as ovulation inducers, were efficient in inducing high ovulatory response, including in thinner cows that were more likely to be in an anovulatory condition in early lactation (fewer thin cows had CL on d −17). Thus, both presynchronization strategies were very effective in inducing ovulation in cows and therefore the TAI protocols were initiated in cows with CL, similar to previous studies reporting ≥90% of cows with CL at first GnRH of TAI protocols in programs such as DO (Ayres et al., 2013; Luchterhand et al., 2019).

Correspondingly, ovulatory response after GnRH treatment on d 0 was greater in cows submitted to PreP4/E2 than Ovs (Table 1), partially explained by the lower percentage of cows with CL on d 0 and potentially lower circulating P4 in some cows, although this was not measured in our study. Indeed, regardless of experimental group, cows without CL on d 0 had greater ovulation than cows with CL (85.5% [47/55] vs. 66.9% [498/745]; $P = 0.002$). The high proportion of CL at the time of first GnRH (93%) may have reduced ovulation in some cows, because high P4 (CL) is known to reduce the magnitude of the LH surge and ovulatory responses (Giordano et al., 2012; Carvalho et al., 2015).

A key aspect positively associated with fertility in lactating dairy cows is the presence of CL and high circulating P4 at the time of PGF treatment during the TAI protocols (Bisinotto et al., 2015a; Consentini et al., 2021). The 4 complete TAI programs implemented in the study were effective in having 98.5% of cows with at least one CL at the time of first PGF treatment of the TAI protocols (Table 1). Therefore, both presynchronization strategies were efficient in controlling ovarian dynamics, assuring adequate P4 (presence of CL), and producing high synchronization during the TAI protocols.

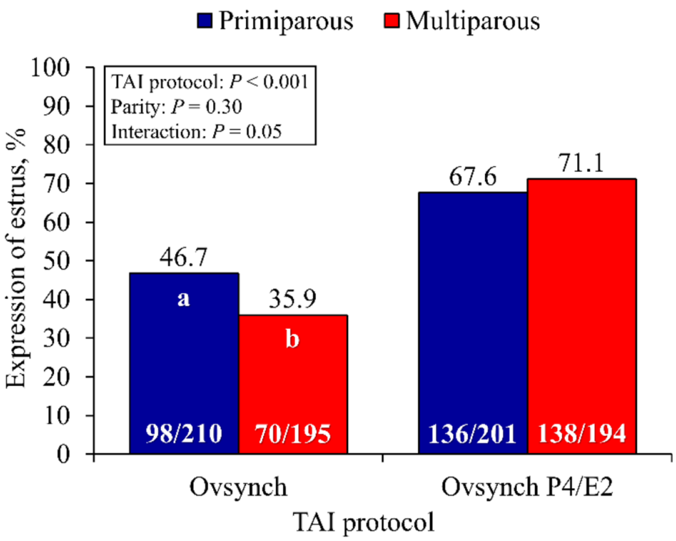

Figure 3. Effect of parity on expression of estrus at the end of different TAI protocols: Ovsynch or OvsynchP4/E2. [a,b]There was an interaction between parity and TAI protocol ($P = 0.05$). Primiparous cows had greater expression of estrus than multiparous cows in Ovsynch protocol ($P = 0.03$) but there was no effect ($P = 0.54$) of parity in OvsynchP4/E2 protocol.

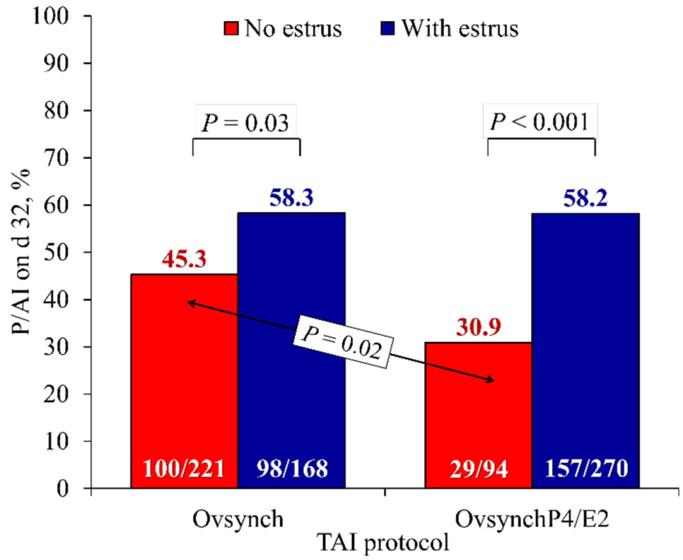

Figure 4. Effect of the timed artificial insemination (TAI) protocol and expression of estrus on pregnancy per AI (P/AI) at d 32 in lactating dairy cows ovulating after TAI. There was a main effect of expression of estrus ($P < 0.001$), but no main effect of TAI protocol ($P = 0.96$). Moreover, there was an interaction between estrus and TAI protocol ($P = 0.04$). The $P = 0.02$ indicates the difference in fertility between TAI protocols in cows not showing estrus.

Expression of Estrus at the End of the TAI Protocols and Ovulation After TAI

There was no difference between presynchronization strategies in expression of estrus or ovulation at the end of the TAI protocols (Table 2). Moreover, there was a low incidence of multiple ovulations after TAI (Table 2) compared with TAI programs without presynchronization or programs that do not provide adequate circulating P4 concentrations during development of the preovulatory follicle (Wiltbank et al., 2014; Gomez-León et al., 2022). This result indicates that the programs in the study likely produced elevated circulating P4 during development of the preovulatory follicle, which is essential for minimizing multiple ovulations (Wiltbank et al., 2012; Gomez-León et al., 2022). It is important to prevent multiple ovulations, as twinning is undesirable in dairy operations due to an increase in pregnancy loss (Martins et al., 2018), higher proportion of postpartum health problems, and impaired calf development and mortality (Cabrera and Fricke, 2021).

As expected, for the final TAI protocols, there was a greater expression of estrus near TAI in cows receiving EC compared with GnRH (Table 3), confirming our second hypothesis. A greater proportion of cows submitted to the Ovs protocol ovulated after TAI compared with OvsP4/E2 (96.1% vs 92.2%; $P = 0.02$); however, incidence of multiple ovulations did not differ between TAI protocols (Table 3). The difference in final ovulation is likely resulting from more cows having appropriate LH surges to cause ovulation when they received GnRH compared with EC. Using OvsP4/E2 protocols, some cows with small ovulatory follicles do not have a GnRH/LH surge and do not ovulate after receiving EC (Souza et al., 2005; Silva et al., 2018). Induction of a "pharmacologic estrus" is a possibility in protocols that use E2 to induce ovulation. For example, treatment with a higher dose of EC (2 vs. 1 mg) increased expression of estrus but did not increase percentage ovulation (Pereira et al., 2022). The high ovulation percentage after EC treatment (92.2% [364/395]; Table 3) and the observation that practically all cows that showed estrus also ovulated (98.5 [270/274]; Table 3) indicates that this was not a problem in our study with this dose of EC.

There was an interaction between TAI protocol and expression of estrus and ovulation after TAI, with cows expressing estrus having high ovulation percentage in either protocol, whereas cows not expressing estrus had greater ovulation to Ovs than to OvsP4/E2 (Table 4). Previous studies (Souza et al., 2009; Ferreira et al., 2017) did not report differences in ovulation after TAI between EC and GnRH, although fewer cows were used in those studies, and the TAI protocols did not include optimizations that increase fertility such as GnRH on d 0 (Consentini et al., 2021), 2 PGF treatments (Wiltbank et al., 2015), and presynchronization (Fricke and Wiltbank, 2022). Multiple ovulation incidence was not influenced by expression of estrus or TAI protocol (Table

Table 5. Pregnancy per AI (P/AI) and pregnancy loss of lactating Holstein cows submitted to 4 fertility programs (Presynch + TAI protocol) for the first postpartum service

Item, % (n/n)	Reproductive program (Presynch + TAI protocol)				*P*-value[1]		
	Ovs+Ovs	Ovs+OvsP4/E2	PreP4/E2+Ovs	PreP4/E2+OvsP4/E2	Pres	Prot	Int
All cows							
P/AI on d 32	46.0 (93/202)	45.5 (90/198)	51.7 (105/203)	48.7 (96/197)	0.22	0.60	0.66
P/AI on d 60	39.1 (79/202)	36.9 (73/198)	41.4 (84/203)	40.6 (80/197)	0.41	0.64	0.91
P/AI on d 90	36.1 (73/202)	34.3 (68/198)	37.0 (75/203)	38.6 (76/197)	0.48	0.95	0.70
Pregnancy loss d 32 to 90	21.5 (20/93)	24.4 (22/90)	28.6 (30/105)	20.8 (20/96)	0.70	0.61	0.26
Ovulated cows[2]							
P/AI on d 32	48.4 (93/192)	49.7 (90/181)	53.3 (105/197)	52.5 (96/183)	0.33	0.96	0.71
P/AI on d 60	41.2 (79/192)	40.3 (73/181)	42.6 (84/197)	43.7 (80/183)	0.54	0.99	0.86
P/AI on d 90	38.0 (73/192)	37.6 (68/181)	38.1 (75/197)	41.5 (76/183)	0.63	0.71	0.66
Pregnancy loss d 32 to 90	21.5 (20/93)	24.4 (22/90)	28.6 (30/105)	20.8 (20/96)	0.70	0.61	0.26

[1]Pres = main effect of presynchronization protocol; Prot = main effect of TAI protocol; Int = interaction between presynchronization and TAI protocol.
[2]Only cows with CL 7 d after TAI were considered to have ovulated after TAI.

4). Nevertheless, multiparous cows had greater ovulation than primiparous cows (96.7% [376/389] vs. 91.7% [377/411]; $P = 0.001$) and incidence of multiple ovulation was almost 3 times greater in multiparous cows than primiparous (11.4% [43/376] vs. 2.9% [11/377]; $P < 0.0001$). It is likely that the increased incidence of multiple ovulation is related to greater milk production in multiparous than primiparous cows (41.6 ± 0.31 vs. 33.7 ± 0.25 kg/d) consistent with previous reports of a strong association of milk yield with multiple ovulation (Lopez et al., 2005; Martins et al., 2018). The present study demonstrated a low (7.2%) overall multiple ovulation rate in cows submitted to fertility programs, although milk yield still had some effect, with cows in the highest tercile of milk yield having greater multiple ovulation after TAI (Figure 2). Nevertheless, the 12.7% (29/229) multiple ovulation incidence in cows producing more than 41.0 kg/d (highest tercile) in our study can be considered to be low compared with the 25% to 52% multiple ovulations after a natural estrus in cows producing >40 kg/d (Lopez et al., 2005). These results reinforce the idea that these fertility programs could partially overcome the high multiple ovulation rate, even in high-producing cows.

Interestingly, there was an interaction between parity and TAI protocol in expression of estrus up to the time of AI. In the Ovs protocol, more primiparous cows expressed estrus than multiparous cow; however, there was no effect of parity in the OvsP4/E2 protocol (Figure 3). Previous research in cows without any synchronization protocol found that cows with greater milk production had reduced expression of estrus (Lopez et al., 2004). Thus, inclusion of EC in the protocol increases the number of cows expressing estrus and eliminates the effect of parity. Similarly, after an analysis to study the effect of milk production, the expression of estrus was affected by milk yield only in the Ovs protocol but not in cows treated with OvsP4/E2 as the TAI protocol (Figure 2).

Effect of Reproductive Programs and Other Variables on Fertility

Interestingly, despite differences in pharmacology, the outcomes for fertility and pregnancy loss were similar among the TAI programs (Table 5). When only cows that ovulated to the TAI protocol were considered for the fertility analyses, there also were no differences detected (Table 5). Thus, similar fertility was observed for the established DO and the new DES program.

The presence of CL at d −17 (~34 DIM) had no effect on fertility (49.5% [272/550] vs. 44.8% [112/250]; $P = 0.20$). The effects of anovulatory condition, BCS loss, and health problems on fertility are not necessarily eliminated by using optimized TAI programs (Carvalho et al., 2014, 2019; Barletta et al., 2017). Cows ovulating at the beginning of the TAI protocols had greater fertility than cows not ovulating (51.0% [278/545] vs. 41.6% [106/255]; $P = 0.01$) likely related to differences in circulating P4 and follicular wave synchrony during the preovulatory follicular wave (Giordano et al., 2013; Borchardt et al., 2020). One factor that could not be tested in our experimental design was whether the P4 insert during the TAI program was positive for fertility, as it has been evaluated in conventional Ovsynch protocols without presynchronizations (Bisinotto et al., 2015a,b). The high synchrony in the fertility programs tested in this research (98.5% with CL at first PGF) may reduce the need for a P4 insert during the TAI program.

There was no difference between cows ovulating or not ovulating at the beginning of the TAI protocols on pregnancy loss (22.7% [63/278] vs. 27.4% [29/106]; $P = 0.34$). The number of cows with multiple ovulations

Table 6. Effect of expression of estrus at the end of the TAI protocols on pregnancy loss of lactating dairy cows submitted to fertility programs (Presynch + TAI protocol)

Pregnancy loss	Expression of estrus, % (n/n) No estrus	Estrus	*P*-value
Between d 32 and 60	20.2 (26/129)	16.5 (42/255)	0.37
Between d 60 and 90	11.7 (12/103)	5.6 (12/213)	0.04
Between d 32 and 90	29.5 (38/129)	21.2 (54/255)	0.05

was reduced (n = 54), but considering only cows that conceived after AI, multiple ovulation was associated with higher pregnancy loss (39.3% [11/28] vs. 22.8% [81/356]; $P = 0.04$), as expected based on previous research (Martins et al., 2018).

To evaluate the effect of expression of estrus on fertility, only cows that ovulated to the protocol were included in an analysis of the effect of expression of estrus on fertility. Expression of estrus increased fertility for both TAI protocols, although the effect was more marked for OvsP4/E2 than the Ovs program (Figure 4). For example, lack of expression of estrus decreased fertility 23% for Ovs but 47% for OvsP4/E2. When all cows were considered (ovulated and nonovulated), the difference in fertility between TAI protocols in cows without estrus was even greater (42.2% [100/237] vs. 24.0% [29/121] for Ovs and OvsP4/E2, respectively; $P < 0.001$), which was expected due to the differences in ovulation. As expected, the cows that did not ovulate after TAI did not become pregnant in either group. Thus, the differences in ovulation (78% vs. 93%) can explain some of the differences between TAI protocols in fertility in cows that did not show estrus. However, when only cows that ovulated were considered, the P/AI was still greater for the Ovs protocol, perhaps due to a suboptimal timing of ovulation and TAI for OvsP4/E2 cows compared with the precise timing when using GnRH to induce ovulation in an Ovsynch protocol (Pursley et al., 1998; Saacke, 2008; Souza et al., 2009).

Pregnancy loss did not differ among the reproductive programs as presented in Table 5. It is important to mention that the overall pregnancy loss of the farm was high, at least compared with other studies with large number of animals that reported ~12% pregnancy loss in this time period (Wiltbank et al., 2016, 2018; Fernandez-Novo et al., 2020; Sigdel et al., 2021). Although the protocol with EC resulted in higher expression of estrus, our hypothesis of lower pregnancy loss in cows submitted to the OvsP4/E2 protocol was not supported, as there was no significant difference in pregnancy loss between OvsP4/E2 and Ovs (22.6% [42/186] vs. 25.3% [50/198]; $P = 0.61$). In a recent study from our research group that compared strategies to induce final ovulation, cows receiving EC at the end of TAI protocols had lower pregnancy loss compared with cows receiving only GnRH (11.2% vs. 19.8%; Consentini et al., 2025). In contrast, expression of estrus did not interact with TAI protocol or parity, and it was associated with decreased pregnancy loss (Table 6). Cows not expressing estrus up to the time of TAI had 39.2% ([29.5 − 21.2]/21.2 = 39.2%) greater pregnancy loss between d 32 and 90 after TAI compared with cows expressing estrus (Table 6).

Expression of estrus near TAI has previously been associated with pregnancy loss. In protocols using EC to induce ovulation, cows expressing estrus had 28.5% lower pregnancy loss (14.4% [255/1,785] vs. 20.1% [43/222]; Pereira et al., 2016). Similarly, cows submitted to Ovsynch-type protocols and expressing estrus at the end of the protocol also had reduced pregnancy loss compared with cows without estrus (6.5% [68/1,041] vs. 10.9% [161/1,482]; Carlos E. C. Consentini, Roberto Sartori, and Milo C. Wiltbank, unpublished data). Expression of estrus is associated with several aspects favorable to the establishment and maintenance of pregnancy. For instance, cows expressing estrus had greater ovulatory follicle size and subsequent circulating P4 after TAI (Rodrigues et al., 2018; Cooke et al., 2019; Laplacette et al., 2025), and greater circulating pregnancy associated glycoproteins on d 28 (Pohler et al., 2016), all associated with increased fertility. Moreover, expression of estrus is associated with changes in the reproductive tract and expression of genes favorable to embryo implantation, placentation, and pregnancy (Davoodi et al., 2016; Cooke et al., 2019).

Primiparous cows had greater fertility than multiparous cows (51.6% [212/411] vs. 44.2% [172/389]; $P = 0.03$). Moreover, there was no interaction between parity and presynchronization ($P = 0.48$) or TAI protocols ($P = 0.42$), and no effect of parity on pregnancy loss between d 32 and 60 ($P = 0.88$) or d 32 and 90 ($P = 0.16$). The effect of milk yield on fertility in other TAI studies has been variable (Santos et al., 2009; Pereira et al., 2020). Our study provided results regarding the interaction of milk yield and fertility programs, particularly the interaction with presynchronization strategy. Regardless of TAI protocol, when cows were divided by the median milk yield (< or ≥37.9 kg/d during the protocol), there was no overall effect of milk yield on fertility (Table 7); however, cows with higher milk production had greater P/AI at d 32 when submitted to PreP4/E2 compared with Ovs (Table 7). Thus, one advantage of the DES program is that the PreP4/E2 increased P/AI in the high milk production cows in response to the final TAI program (regardless of TAI program) by 10 percentage points (40.4% vs 50.8%) or over 25% relative P/AI. We were not able to identify the explanations underlying the greater fertility of PreP4/E2 group, because it did not produce substantial effects

Table 7. Effect of milk yield and its interaction with presynchronization strategy and TAI protocol on P/AI on d 32 of lactating dairy cows submitted to fertility programs (Presynch + TAI protocol)

Item[2]	Milk yield		*P*-value[1]		
	<37.9 kg/d	≥37.9 kg/d	M	V	I
Average milk yield, kg/d ± SEM	32.3 ± 0.20	43.1 ± 0.20	<0.001	—	—
Overall P/AI	50.0 (182/364)	45.7 (168/368)	0.25	—	—
Presynchronization					
Ovs	50.3 (92/183)	40.4 (74/183)	0.05	0.18	0.10
PreP4/E2	49.7 (90/181)	50.8 (94/185)	0.83		
P-value[3]	0.92	0.04			
TAI protocol					
Ovs	50.3 (89/177)	46.9 (91/194)	0.52	0.68	0.78
OvsP4/E2	49.7 (93/187)	44.3 (77/174)	0.30		
P-value[3]	0.92	0.61			
Fertility program					
Double-Ovsynch	49.5 (45/91)	42.1 (40/95)	0.31	0.87	0.66
Ovs+OvsP4/E2	51.1 (47/92)	38.6 (34/88)	0.09		
PreP4/E2+Ovs	51.2 (44/86)	51.5 (51/99)	0.96		
Double E-Synch	48.4 (46/95)	50.0 (43/86)	0.83		
P-value[3]	0.98	0.24			

[1]M = milk yield effect within classes of cows; V = main effect of presynchronization; TAI protocol or fertility program; I = interaction between milk yield and the variable.

[2]Values reported as % (n/n) unless stated otherwise.

[3]Effect of presynchronization, TAI protocol, and fertility program within classes of milk yield.

on ovarian dynamics, synchronization, and expression of estrus in those cows with greater milk yield. The potential effects of utilizing E2 and inducing higher expression of estrus during the presynchronization protocols on uterine recovery, health, and embryo development post-TAI, which was one of our speculations that could not be tested, still require further investigation. There was no interaction between TAI protocol and milk yield on fertility (Table 7), even with OvsP4/E2 increasing expression of estrus, which could have had positive effects for cows with greater milk production. Previous studies already showed the benefits of using fertility programs for first service, in both service rates and fertility, compared with management based on estrus detection (Santos et al., 2017; Fricke and Wiltbank, 2022; Sitko et al., 2023). The results from the present study indicate that a variety of TAI programs can be used with various hormonal treatments, some that may be easier to implement on some dairies, can result in high fertility. One major advantage is that there is little effect of milk production, indicating that these programs mitigate or potentially eliminate the effect of milk yield on fertility. Nevertheless, the experimental design that we chose allowed us only to test different complete programs for presynchronization and final TAI and did not differentiate the effect of specific hormones such as EC versus GnRH for final ovulation.

CONCLUSIONS

This study validated 2 presynchronization strategies and 2 TAI protocols, establishing 4 possible fertility programs, all of them producing well-controlled ovarian dynamics, excellent synchronization, and high fertility. Moreover, one of our goals was to compare Double-Ovsynch with DES, and despite differences in pharmacological bases, both produced similar results. Finally, the data generated from this study provide multiple effective synchronization strategies for TAI in high-producing dairy cows that can be selected according to management preferences.

NOTES

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Nonstandard abbreviations used: AI = artificial insemination; CL = corpus luteum; DES = Double E-Synch; DO = Double-Ovsynch; E2 = estradiol; EC = estradiol cypionate; Ovs = Ovsynch; P4 = progesterone; P/AI = pregnancies per AI; PL = pregnancy loss; TAI = timed AI; PGF = cloprostenol sodium $PGF_{2\alpha}$ analog; US = ultrasound.

REFERENCES

Ayres, H., R. M. Ferreira, A. P. Cunha, R. R. Araújo, and M. C. Wiltbank. 2013. Double-Ovsynch in high-producing dairy cows: Effects on progesterone concentrations and ovulation to GnRH treatments. Theriogenology 79:159–164. https://doi.org/10.1016/j.theriogenology.2012.10.001.

Barletta, R. V., M. Maturana Filho, P. D. Carvalho, T. A. Del Valle, A. S. Netto, F. P. Rennó, R. D. Mingoti, J. R. Gandra, G. B. Mourão, P. M. Fricke, R. Sartori, E. H. Madureira, and M. C. Wiltbank. 2017. Association of changes among body condition score during the transition period with NEFA and BHBA concentrations, milk production, fertility, and health of Holstein cows. Theriogenology 104:30–36. https://doi.org/10.1016/j.theriogenology.2017.07.030.

Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. J. Dairy Sci. 89:3413–3424. https://doi.org/10.3168/jds.S0022-0302(06)72378-5.

Bisinotto, R. S., L. O. Castro, M. B. Pansani, C. D. Narciso, N. Martinez, L. D. Sinedino, T. L. Pinto, N. S. Van de Burgwal, H. M. Bosman, R. S. Surjus, W. W. Thatcher, and J. E. Santos. 2015a. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. J. Dairy Sci. 98:2515–2528. https://doi.org/10.3168/jds.2014-9058.

Bisinotto, R. S., I. J. Lean, W. W. Thatcher, and J. E. Santos. 2015b. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. J. Dairy Sci. 98:2472–2487. https://doi.org/10.3168/jds.2014-8954.

Borchardt, S., A. Pohl, and W. Heuwieser. 2020. Luteal presence and ovarian response at the beginning of a timed artificial insemination protocol for lactating dairy cows affect fertility: A meta-analysis. Animals (Basel) 10:1551. https://doi.org/10.3390/ani10091551.

Brusveen, D. J., A. H. Souza, and M. C. Wiltbank. 2009. Effects of additional prostaglandin F-2 alpha and estradiol-17β during Ovsynch in lactating dairy cows. J. Dairy Sci. 92:1412–1422. https://doi.org/10.3168/jds.2008-1289.

Cabrera, V. E., and P. M. Fricke. 2021. Economics of twin pregnancies in dairy cattle. Animals (Basel) 11:552. https://doi.org/10.3390/ani11020552.

Carvalho, M. R., F. Peñagaricano, J. Santos, T. J. DeVries, B. W. McBride, and E. S. Ribeiro. 2019. Long-term effects of postpartum clinical disease on milk production, reproduction, and culling of dairy cows. J. Dairy Sci. 102:11701–11717. https://doi.org/10.3168/jds.2019-17025.

Carvalho, P. D., V. G. Santos, J. O. Giordano, M. C. Wiltbank, and P. M. Fricke. 2018. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. Theriogenology 114:165–172. https://doi.org/10.1016/j.theriogenology.2018.03.037.

Carvalho, P. D., A. H. Souza, M. C. Amundson, K. S. Hackbart, M. J. Fuenzalida, M. M. Herlihy, H. Ayres, A. R. Dresch, L. M. Viera, J. N. Guenther, R. R. Grummer, P. M. Fricke, R. D. Shaver, and M. C. Wiltbank. 2014. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. J. Dairy Sci. 97:3666–3683. https://doi.org/10.3168/jds.2013-7809.

Carvalho, P. D., M. C. Wiltbank, and P. M. Fricke. 2015. Manipulation of progesterone to increase ovulatory response to the first GnRH treatment of an Ovsynch protocol in lactating dairy cows receiving first timed artificial insemination. J. Dairy Sci. 98:8800–8813. https://doi.org/10.3168/jds.2015-9968.

Consentini, C. E. C., L. F. Melo, J. C. L. Motta, L. O. E. Silva, N. P. Folchini, R. L. O. R. Alves, G. Madureira, T. O. Carneiro, T. Abadia, L. Moroz, R. V. S. Fortes, S. Soriano, B. Gonzales, M. C. Wiltbank, and R. Sartori. 2025. Reproductive outcomes of lactating dairy cows submitted to first timed artificial insemination protocols with different strategies to induce final ovulation. J. Dairy Sci. 108:1138–1149. https://doi.org/10.3168/jds.2024-25188.

Consentini, C. E. C., M. C. Wiltbank, and R. Sartori. 2021. Factors that optimize reproductive efficiency in dairy herds with an emphasis on timed artificial insemination programs. Animals (Basel) 11:301–331. https://doi.org/10.3390/ani11020301.

Cooke, R. F., K. G. Pohler, J. L. M. Vasconcelos, and R. L. A. Cerri. 2019. Estrous expression during a fixed-time artificial insemination protocol enhances development and interferon-tau messenger RNA expression in conceptuses from *Bos indicus* beef cows. Animal 13:2569–2575. https://doi.org/10.1017/S1751731119000636.

Davoodi, S., R. F. Cooke, A. C. C. Fernandes, B. I. Cappellozza, J. L. M. Vasconcelos, and R. L. A. Cerri. 2016. Expression of estrus modifies the gene expression profile in reproductive tissues on day 19 of gestation in beef cows. Theriogenology 85:645–655. https://doi.org/10.1016/j.theriogenology.2015.10.002.

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. https://doi.org/10.3168/jds.S0022-0302(94)77212-X.

Fernandez-Novo, A., O. Fargas, J. M. Loste, F. Sebastian, N. Perez-Villalobos, J. L. Pesantez-Pacheco, R. Patron-Collantes, and S. Astiz. 2020. Pregnancy loss (28–110 days of pregnancy) in Holstein cows: A retrospective study. Animals (Basel) 10:925. https://doi.org/10.3390/ani10060925.

Ferreira, R. M., H. Ayres, L. U. Gimenes, F. P. Torres, F. A. Lima, M. B. Veras, T. G. Guida, R. V. Sala, and P. S. Baruselli. 2017. Inducing ovulation with estradiol cypionate allows flexibility in the timing of insemination and removes the need for gonadotrophin-releasing hormone in timed AI protocols for dairy cows. Reprod. Fertil. Dev. 29:468–475. https://doi.org/10.1071/RD15270.

Fricke, P. M., and M. C. Wiltbank. 2022. Symposium review: The implications of spontaneous versus synchronized ovulations on the reproductive performance of lactating dairy cows. J. Dairy Sci. 105:4679–4689. https://doi.org/10.3168/jds.2021-21431.

Giordano, J. O., P. M. Fricke, J. N. Guenther, G. Lopes, M. M. Herlihy, A. B. Nascimento, and M. C. Wiltbank. 2012. Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. J. Dairy Sci. 95:3781–3793. https://doi.org/10.3168/jds.2011-5155.

Giordano, J. O., M. C. Wiltbank, P. M. Fricke, S. Bas, R. Pawlisch, J. N. Guenther, and A. B. Nascimento. 2013. Effect of increasing GnRH and PGF 2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology 80:773–783. https://doi.org/10.1016/j.theriogenology.2013.07.003.

Gomez-León, V. E., A. D. Beard, O. J. Ginther, and M. C. Wiltbank. 2022. Effect of elevating LH action using low doses of hCG on double ovulation, follicle dynamics, and circulating FSH in lactating dairy cows. J. Dairy Sci. 105:7023–7035. https://doi.org/10.3168/jds.2021-21767.

Gümen, A., J. N. Guenther, and M. C. Wiltbank. 2003. Follicular size and response to Ovsynch versus detection of estrus in anovular and ovular lactating dairy cows. J. Dairy Sci. 86:3184–3194. https://doi.org/10.3168/jds.S0022-0302(03)73921-6.

Kim, I. H., J. K. Jeong, and H. G. Kang. 2020. Reproductive performance following a modified Presynch-Ovsynch, Double-Ovsynch, or conventional reproductive management program in Korean dairy herds. Theriogenology 156:27–35.

Laplacette, A. L., C. Rial, E. Sitko, M. M. Perez, S. Tompkins, M. L. Stangaferro, M. J. Thomas, and J. O. Giordano. 2025. Delaying induction of ovulation and timed artificial insemination in a Double-Ovsynch protocol increased expression of estrus and altered first-service reproductive outcomes of lactating dairy cows. J. Dairy Sci. 108:1103–1124. https://doi.org/10.3168/jds.2024-24994.

Lopez, H., D. Z. Caraviello, L. D. Satter, P. M. Fricke, and M. C. Wiltbank. 2005. Relationship between level of milk production and mul-

tiple ovulations in lactating dairy cows. J. Dairy Sci. 88:2783–2793. https://doi.org/10.3168/jds.S0022-0302(05)72958-1.
Lopez, H., L. D. Satter, and M. C. Wiltbank. 2004. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim. Reprod. Sci. 81:209–223. https://doi.org/10.1016/j.anireprosci.2003.10.009.
Luchterhand, M., C. A. Gamarra, R. S. Gennari, P. D. Carvalho, R. V. Barletta, and A. H. Souza. 2019. Ovulation and fertility response to commercially available GnRH products in lactating cows synchronized with the Double-Ovsynch protocol. Anim. Reprod. Sci. 202:42–48. https://doi.org/10.1016/j.anireprosci.2019.01.006.
Manríquez, D., W. W. Thatcher, J. Santos, R. C. Chebel, K. N. Galvão, G. M. Schuenemann, R. C. Bicalho, R. O. Gilbert, S. Rodriguez-Zas, C. M. Seabury, G. Rosa, and P. Pinedo. 2021. Effect of body condition change and health status during early lactation on performance and survival of Holstein cows. J. Dairy Sci. 104:12785–12799. https://doi.org/10.3168/jds.2020-20091.
Martins, J. P. N., D. Wang, N. Mu, G. F. Rossi, A. P. Martini, V. R. Martins, and J. R. Pursley. 2018. Level of circulating concentrations of progesterone during ovulatory follicle development affects timing of pregnancy loss in lactating dairy cows. J. Dairy Sci. 101:10505–10525. https://doi.org/10.3168/jds.2018-14410.
Monteiro, P. L. J., B. Gonzales, J. N. Drum, J. E. P. Santos, M. C. Wiltbank, and R. Sartori. 2021. Prevalence and risk factors related to anovular phenotypes in dairy cows. J. Dairy Sci. 104:2369–2383. https://doi.org/10.3168/jds.2020-18828.
Moreira, F., C. Orlandi, C. A. Risco, R. Mattos, F. Lopes, and W. W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J. Dairy Sci. 84:1646–1659. https://doi.org/10.3168/jds.S0022-0302(01)74600-0.
NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
Pereira, M. H. C., M. C. Wiltbank, T. G. Guida, F. R. Lopes, B. I. Cappellozza, and J. L. M. Vasconcelos. 2020. Evaluation of presynchronization and addition of GnRH at the beginning of an estradiol/progesterone protocol on circulating progesterone and fertility of lactating dairy cows. Theriogenology 147:124–134. https://doi.org/10.1016/j.theriogenology.2019.11.025.
Pereira, M. H. C., B. I. Cappellozza, W. M. Costa, L. Barbosa, R. L. A. Cerri, and J. L. M. Vasconcelos. 2022. Effects of estradiol cypionate dose as an ovulatory stimulus on reproductive performance of lactating dairy cows during the summer season. Theriogenology 182:110–118. https://doi.org/10.1016/j.theriogenology.2022.02.001.
Pereira, M. H. C., M. C. Wiltbank, and J. L. M. Vasconcelos. 2016. Expression of estrus improves fertility and decreases pregnancy losses in lactating dairy cows that receive artificial insemination or embryo transfer. J. Dairy Sci. 99:2237–2247. https://doi.org/10.3168/jds.2015-9903.
Peters, M. W., and J. R. Pursley. 2002. Fertility of lactating dairy cows treated with Ovsynch after presynchronization injections of PGF2 alpha and GnRH. J. Dairy Sci. 85:2403–2406. https://doi.org/10.3168/jds.S0022-0302(02)74322-1.
Pohler, K. G., R. Peres, J. A. Green, H. Graff, T. Martins, J. L. M. Vasconcelos, and M. F. Smith. 2016. Use of bovine pregnancy-associated glycoproteins to predict late embryonic mortality in postpartum Nelore beef cows. Theriogenology 85:1652–1659. https://doi.org/10.1016/j.theriogenology.2016.01.026.
Pursley, J. R., M. R. Kosorok, and M. C. Wiltbank. 1997a. Reproductive management of lactating dairy cows using synchronization of ovulation. J. Dairy Sci. 80:301–306. https://doi.org/10.3168/jds.S0022-0302(97)75938-1.
Pursley, J. R., and J. P. Martins. 2011. Impact of circulating concentrations of progesterone and antral age of the ovulatory follicle on fertility of high-producing lactating dairy cows. Reprod. Fertil. Dev. 24:267–271. https://doi.org/10.1071/RD11917.
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. https://doi.org/10.3168/jds.s0022-0302(98)75790-x.
Pursley, J. R., M. C. Wiltbank, J. S. Stevenson, J. S. Ottobre, H. A. Garverick, and L. L. Anderson. 1997b. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. J. Dairy Sci. 80:295–300. https://doi.org/10.3168/jds.S0022-0302(97)75937-X.
Rodrigues, A. D., R. F. Cooke, R. S. Cipriano, L. Silva, R. L. A. Cerri, L. H. Cruppe, M. Meneghetti, K. G. Pohler, and J. L. M. Vasconcelos. 2018. Impacts of estrus expression and intensity during a timed-AI protocol on variables associated with fertility and pregnancy success in *Bos indicus*-influenced beef cows. J. Anim. Sci. 96:236–249. https://doi.org/10.1093/jas/skx043.
Saacke, R. G. 2008. Insemination factors related to timed AI in cattle. Theriogenology 70:479–484. https://doi.org/10.1016/j.theriogenology.2008.04.015.
Santos, J. E., H. M. Rutigliano, and M. F. Sá Filho. 2009. Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. Anim. Reprod. Sci. 110:207–221. https://doi.org/10.1016/j.anireprosci.2008.01.014.
Santos, V. G., P. D. Carvalho, C. Maia, B. Carneiro, A. Valenza, and P. M. Fricke. 2017. Fertility of lactating Holstein cows submitted to a Double-Ovsynch protocol and timed artificial insemination versus artificial insemination after synchronization of estrus at a similar day in milk range. J. Dairy Sci. 100:8507–8517. https://doi.org/10.3168/jds.2017-13210.
Sartori, R., C. E. C. Consentini, R. L. O. R. Alves, L. O. Silva, and M. C. Wiltbank. 2023. Review: Manipulation of follicle development to improve fertility of cattle in timed-artificial insemination programs. Animal 17(Suppl. 1):100769. https://doi.org/10.1016/j.animal.2023.100769.
Sigdel, A., R. S. Bisinotto, and F. Peñagaricano. 2021. Genes and pathways associated with pregnancy loss in dairy cattle. Sci. Rep. 11:13329. https://doi.org/10.1038/s41598-021-92525-0.
Silva, E. P., M. C. Wiltbank, A. B. Machado, L. S. Gambin, M. M. Dias, M. F. C. Chaiben, M. L. Bernardi, and J. B. S. Borges. 2018. Optimizing timed AI protocols for Angus beef heifers: Comparison of induction of synchronized ovulation with estradiol cypionate or GnRH. Theriogenology 121:7–12. https://doi.org/10.1016/j.theriogenology.2018.07.019.
Silva, L. O. E., J. C. L. Motta, A. L. Oliva, G. Madureira, R. L. O. R. Alves, N. P. Folchini, M. A. da Silva, T. J. B. da Silva, C. E. C. Consentini, M. C. Wiltbank, and R. Sartori. 2024. Influence of GnRH analog and dose on LH release and ovulatory response in heifers and cows on day seven of the estrous cycle. Theriogenology 214:215–223. https://doi.org/10.1016/j.theriogenology.2023.10.015.
Sitko, E. M., M. M. Perez, G. E. Granados, M. Masello, S. Hernandez, E. M. Cabrera, E. M. Schilkowsky, F. A. D. Croce, A. K. Mcneel, D. J. Weigel, and J. O. Giordano. 2023. Effect of reproductive management programs that prioritized artificial insemination at detected estrus or timed artificial insemination on the reproductive performance of primiparous Holstein cows of different genetic merit for fertility. J. Dairy Sci. 106:6476–6494. https://doi.org/10.3168/jds.2022-22673.
Souza, A. H., H. Ayres, R. M. Ferreira, and M. C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208–215. https://doi.org/10.1016/j.theriogenology.2008.03.014.
Souza, A. H., A. P. Cunha, D. Z. Caraviello, and M. C. Wiltbank. 2005. Profiles of circulating estradiol-17 β after different estrogen treatments in lactating dairy cows. Anim. Reprod. 2:224–232.
Souza, A. H., S. Viechnieski, F. A. Lima, F. F. Silva, R. Araújo, G. A. Bó, M. C. Wiltbank, and P. S. Baruselli. 2009. Effects of equine chorionic gonadotropin and type of ovulatory stimulus in a timed-AI protocol on reproductive responses in dairy cows. Theriogenology 72:10–21. https://doi.org/10.1016/j.theriogenology.2008.12.025.
Strickland, J. M., J. P. N. Martins, L. Neuder, and J. R. Pursley. 2024. When implementing the Presynch-11/Ovsynch reproductive management program, the fertility of lactating dairy cows improved when they received timed artificial insemination compared with the inclusion of estrus detection. Animals (Basel) 14:2235–2245. https://doi.org/10.3390/ani14152235.

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. https://doi.org/10.1016/S0093-691X(99)00195-8.

Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin F-2 alpha during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. J. Dairy Sci. 98:8644–8654. https://doi.org/10.3168/jds.2015-9353.

Wiltbank, M. C., G. M. Baez, A. Garcia-Guerra, M. Z. Toledo, P. L. Monteiro, L. F. Melo, J. C. Ochoa, J. E. P. Santos, and R. Sartori. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 86:239–253. https://doi.org/10.1016/j.theriogenology.2016.04.037.

Wiltbank, M. C., P. D. Carvalho, A. Kaskin, K. S. Hackbart, M. A. Meschiatti, M. R. Bastos, J. N. Guenther, A. B. Nascimento, M. M. Herlihy, M. C. Amundson, and A. H. Souza. 2011. Effect of progesterone concentration during follicle development on subsequent ovulation, fertilization, and early embryo development in lactating dairy cows. Biol. Reprod. 85:685. https://doi.org/10.1093/biolreprod/85.s1.685.

Wiltbank, M. C., M. A. Mezera, M. Z. Toledo, J. N. Drum, G. M. Baez, A. Garcia-Guerra, and R. Sartori. 2018. Physiological mechanisms involved in maintaining the corpus luteum during the first two months of pregnancy. Anim. Reprod. 15(Suppl. 1):805–821. https://doi.org/10.21451/1984-3143-AR2018-0045.

Wiltbank, M. C., and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170–185. https://doi.org/10.1016/j.theriogenology.2013.09.017.

Wiltbank, M. C., A. H. Souza, P. D. Carvalho, A. P. Cunha, J. O. Giordano, P. M. Fricke, G. M. Baez, and M. G. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8:70–81. https://doi.org/10.1017/S1751731114000585.

Wiltbank, M. C., A. H. Souza, J. O. Giordano, A. B. Nascimento, J. M. Vasconcelos, M. H. C. Pereira, P. M. Fricke, R. S. Surjus, F. C. S. Zinsly, P. D. Carvalho, R. W. Bender, and R. Sartori. 2012. Positive and negative effects of progesterone during timed AI protocols in lactating dairy cattle. Anim. Reprod. 9:231–241.

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