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Effect of timing of artificial insemination with conventional or sex-sorted semen on fertility of lactating dairy cows

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ABSTRACT

The effect on pregnancy per artificial insemination (P/AI) of interval from induction of ovulation to timed artificial insemination (TAI) for cows submitted to a fertility program and the interval from the onset of estrous alert to artificial insemination (AI) for cows inseminated to estrous alert was analyzed in 3 experiments. In experiment 1, multiparous lactating Holstein cows (n = 1,924) from 6 herds were submitted to a Double-Ovsynch protocol to receive their first TAI using conventional semen. On the day of the last GnRH treatment, cows were randomly assigned to TAI at the time of the last GnRH treatment (Cosynch-56: 0 h) or TAI 16 h after the last GnRH treatment (Ovsynch-56: 16 h). Ovsynch-56 cows had more P/AI than Cosynch-56 cows (46% vs. 36%) 32 d after TAI. In experiment 2, lactating Holstein cows (n = 13,318) from 2 herds were submitted to a Double-Ovsynch protocol to receive their first TAI (n = 14,089; during more than one year) or to a GGPPG protocol for second and greater TAI (n = 6,806) using either sex-sorted Holstein semen or conventional beef semen. Overall, TAI varied from 13 to 23 h after the last GnRH treatment, and there was no linear or quadratic effect of time from the last GnRH treatment to TAI on P/AI 32 d after TAI for cows receiving AI with sex-sorted Holstein semen or conventional beef semen. In experiment 3, lactating dairy cows (n = 10,927) were fitted with an activity-monitoring tag mounted to a neck collar, and the timing of AI relative to the onset of estrous alert was recorded. Timing of AI varied from 0 to 40 h after the onset of estrous alert, and there were both linear and quadratic effects of interval from the onset of estrous alert to AI in which cows inseminated early (≤3 h) or late (≥24 h) after the onset of estrous alert had fewer P/AI 32 d after AI than cows inseminated 13 to 23 h after the onset of estrous alert. We conclude that lactating dairy cows inseminated too early relative to a synchronized ovulation or too early or too late relative to the onset of estrous alert had fewer P/AI than cows inseminated from 13 to 23 h. Further, optimal timing of AI relative to a synchronized ovulation or the onset of estrous alert in lactating dairy cows did not differ between conventional and sex-sorted semen.

Key words: time of insemination, fertility, estrus, timed artificial insemination, dairy cow

INTRODUCTION

Over the past decades, research has aimed to understand and improve reproductive performance in lactating dairy cows (Fricke et al., 2014; Santos et al., 2017; Carvalho et al., 2018) and nonlactating dairy heifers submitted to timed artificial insemination (**TAI**; Lima et al., 2011; Santos et al., 2016; Moore et al., 2023), or a detected estrus (**ED**, Bombardelli et al., 2016; Macmillan et al., 2020; Tippenhauer et al., 2021), or a combination of both (Santos et al., 2017; Chebel and Cunha, 2020; Lauber et al., 2020). Research on hormonal synchronization protocols that allow for TAI led to the development of fertility programs that increase fertility of high-producing dairy cows as measured by pregnancies per artificial insemination (**P/AI**). The increase in fertility was primarily achieved by manipulating progesterone concentrations during the growth of the ovulatory follicle (Wiltbank et al., 2011, 2014; Carvalho et al., 2018). Several randomized-controlled studies have directly compared P/AI for cows inseminated after ED or after synchronization of ovulation and TAI (reviewed by Fricke and Wiltbank, 2022). Submission of high-producing cows to a fertility program yielded more P/AI than cows inseminated after

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a synchronized estrus (Santos et al., 2017; Sitko et al., 2023; Lauber and Fricke, 2024).

When AI programs (1940s) and TAI programs (1990s) were first being developed and validated, classic studies were conducted in dairy cows to determine the optimal time of AI relative to estrus (Trimberger and Davis, 1943; Trimberger, 1944) or the final GnRH treatment of the Ovsynch protocol (Pursley et al., 1998). Many studies have now evaluated the timing of AI in cows and heifers inseminated after a synchronized ovulation or a detected estrus. Key differences among these studies include the type of animals inseminated (lactating dairy cows vs. nonlactating heifers; Lima et al., 2011; Ribeiro et al., 2012; Moore et al., 2023), semen type (sex-sorted semen vs. conventional semen; Chebel and Cunha, 2020; Drake et al., 2020), type of hormone used to induce ovulation (GnRH vs. EB; Pursley et al., 1998; Sales et al., 2011), timing between induction of luteal regression and induction of ovulation (48 h vs. 56 h vs. 72 h; Sterry et al., 2007; Brusveen et al., 2008; Lauber et al., 2020), and the use of ED and AI during the synchronization protocol (Chebel and Cunha, 2020; Macmillan et al., 2021). Due to these differences and the critical role that timing of AI plays in pregnancy outcomes and organization of labor (ease of AI management) on dairy farms, this study was designed to provide more information on the optimal timing of AI in lactating dairy cows inseminated relative to onset of estrous alert or after a TAI protocol in dairies using conventional or sex-sorted semen.

New studies are needed to evaluate the optimal timing of TAI because previous studies generally used Ovsynch to synchronize ovulation. We are unaware of any published study that evaluated the optimal timing of AI after submission of cows to fertility programs such as the Double-Ovsynch protocol (Fricke and Wiltbank, 2022). In addition, no study directly compared a Cosynch-56 program (GnRH given at 56 h after $PGF_{2\alpha}$ concurrent with TAI) with Ovsynch-56 (GnRH at 56 h after $PGF_{2\alpha}$ and TAI 16 h later). Furthermore, most studies have focused on altering the timing of the last GnRH treatment relative to the time of the $PGF_{2\alpha}$ treatment (Sterry et al., 2007; Brusveen et al., 2008; Hillegass et al., 2008), whereas only a few studies have altered the timing of AI relative to the time of the last GnRH treatment (Pursley et al., 1998; Cornwell et al., 2006; Moore et al., 2023). This may be problematic because the interval between the $PGF_{2\alpha}$ and last GnRH treatments of an Ovsynch protocol affects fertility (Peters and Pursley, 2003). Moreover, under field conditions, it may not be possible to inseminate all cows at an exact time after the last GnRH treatment due to variation in farm routines such as milking and feeding. In this regard, only the study by Chebel and Cunha (2020) reported the exact time of insemination relative to the last GnRH treatment. Thus, further research is needed on the optimal timing of AI, particularly using optimized TAI programs that are used on many dairy farms today.

The primary sign of estrus behavior in cattle is standing to be mounted by a male or a herd mate. Many farms rely on detection of estrus by visual observation of standing behavior, activity-monitoring systems that continuously monitor and record increased physical activity associated with estrus in cows, or both (Fricke et al., 2014). Several studies have evaluated the effect of timing of AI after a standing event or onset of estrous alert on P/AI. The ideal timing of insemination after the first standing event of estrus or onset of estrous alert varies among studies; however, AI around 12 to16 h after onset of estrus seems to result in the most P/AI when inseminating with conventional semen (Maatje et al., 1997; Stevenson et al., 2014; Tippenhauer et al., 2021). Sex-sorted semen has been widely adopted by dairy farms (González-Marín et al., 2018; Vishwanath and Moreno, 2018; Ross et al., 2022) and the use of sexed semen has continued to increase over time (Lauber et al., 2023). Recent advancements in semen sorting technology has yielded improved P/AI (Vishwanath and Moreno, 2018; Reese et al., 2021); however, the impact of these improvements on the effect of timing of insemination on P/AI has not been reported.

The objectives of the present analyses were 3-fold: (1) to evaluate the effect of time of AI (0 h vs. 16 h) relative to the last GnRH treatment of the Breeding-Ovsynch portion of a Double-Ovsynch protocol using conventional semen on P/AI, (2) to evaluate the effect of the interval between the time of the last GnRH treatment of a presynchronized Breeding-Ovsynch-56 protocol and the time of AI on P/AI for cows inseminated with either sex-sorted semen or conventional semen, and (3) to evaluate the effect of the interval between the onset of estrous alert measured by an activity-monitoring system and the time of AI on P/AI. Our hypothesis was that insemination approximately 16 h after the onset of estrous alert or after the last GnRH treatment of a fertility program would result in more P/AI compared with insemination too early or too late.

MATERIALS AND METHODS

All animal handling and experimental procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin–Madison.

Experiment 1

Cows, Housing, and Feeding. This study was conducted in collaboration with 6 commercial dairy farms located in Wisconsin from January to October 2016. Multiparous lactating Holstein cows (n = 2,225 with 300

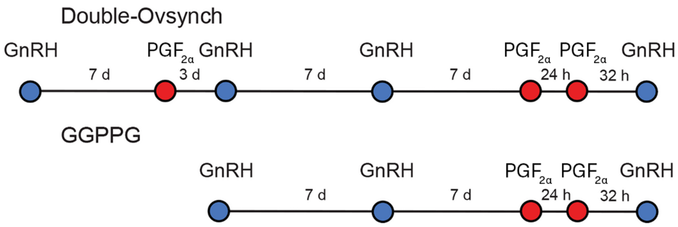

Figure 1. Schematic representation of the synchronization protocols for submission of lactating cows for first insemination (Double-Ovsynch) or second and greater insemination (GGPPG). In both experiments 1 and 2, cows were submitted to a Double-Ovsynch protocol to receive their first timed AI. In experiment 1, on the day of the last GnRH treatment of the Breeding-Ovsynch protocol cows were randomly assigned to receive AI with conventional semen at the same time as the last GnRH treatment (Cosynch-56: AI at 0 h) or to receive AI 16 h later (Ovsynch-56: AI at 16 h). In experiment 2, cows received AI with either sex-sorted or conventional semen at 13 to 23 h after the last GnRH treatment. In experiment 2, cows submitted for second or greater TAI were synchronized with a GGPPG protocol, and cows were inseminated with either sex-sorted or conventional semen at 13 to 23 h after the last GnRH treatment.

to 385 cows per farm used in the study) were milked thrice daily at approximately 8-h intervals and were fed a TMR once daily consisting of corn and alfalfa silage as forage with corn and soybean meal–based concentrate formulated to meet or exceed the minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Cows were housed in freestall barns bedded with sand and had ad libitum access to feed and water.

Synchronization Protocol and AI. Each week, lactating multiparous Holstein cows were submitted to a Double-Ovsynch protocol for first TAI as described by Souza et al. (2008) and later modified by Brusveen et al. (2009). The GnRH (100 µg/dose of gonadorelin hydrochloride, Factrel) and $PGF_{2\alpha}$ (25 mg/dose of dinoprost tromethamine, Lutalyse) were manufactured by Zoetis (Madison, NJ). Briefly, cows received the first GnRH treatment of the Pre-Ovsynch portion of the Double-Ovsynch protocol, followed by a treatment with $PGF_{2\alpha}$ 7 d later and treatment with GnRH 72 h after administration of $PGF_{2\alpha}$. Seven days later, cows began the Breeding-Ovsynch portion of the Double-Ovsynch protocol in which cows received a GnRH treatment, followed by 2 $PGF_{2\alpha}$ treatments administered 7 and 8 d later and the last GnRH treatment administered 56 h after the first $PGF_{2\alpha}$ treatment at 76 ± 3 DIM (Figure 1). On the day of the last GnRH treatment of the Breeding-Ovsynch protocol, cows were randomly assigned to receive AI with conventional dairy semen (20 million sperm cells per straw) at the same time as the last GnRH treatment (Cosynch-56: AI at 0 h) or to receive AI 16 h after the last GnRH treatment (Ovsynch-56: AI at 16 h). For the randomization procedure, a list of multiparous cows was printed on the day of the last GnRH treatment of the synchronization protocol, and cows were randomly assigned to treatment based on the sequence in which they appeared on the list. A total of 2,200 cows (1,100 per group) were initially enrolled in the study. Nonetheless, during the period between insemination and the first pregnancy diagnosis, some cows were removed from the herds, leaving 1,026 cows in the Ovsynch-56 and 989 cows in the Covsynch-56 groups for statistical analysis.

Pregnancy Diagnosis. Pregnancy diagnosis was performed 32 d after TAI for Ovsynch-56 cows and 32.5 d for Cosynch-56 cows using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a corpus luteum on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat.

Statistical Analyses. The experiment was conducted as a complete randomized design with 2 treatments: insemination at 0 h versus 16 h relative to the last GnRH treatment of the Double-Ovsynch protocol. Sample size determination for 2 independent proportions with power = 0.80 and α = 0.05 was performed using the Power and Sample Size application of SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). In a previous study by Pursley et al. (1998), insemination 16 h after the last GnRH treatment resulted in an 8-percentage-point increase in P/AI compared with TAI at the time (0 h) of the last GnRH treatment (45% vs. 37%). Inclusion of a minimum of 593 inseminations per treatment was required to detect an 8-percentage-point difference in P/AI. Analysis of P/AI was performed by logistic regression using the GLIMMIX procedure of SAS. The model included the fixed effect of treatment. Cow within farm was considered the random effect in the model.

Experiment 2

Cows, Housing, Feeding, and Milk Production. Experiment 2 was conducted in collaboration with 2 commercial dairy farms located in Texas from May 2020 to August 2023. Lactating Holstein cows (n = 13,318 cows were enrolled in the study, 6,140 from farm 1 and 7,178 from farm 2) were milked thrice daily at approximately 8-h intervals and were fed a TMR once daily consisting of corn and alfalfa silage as forage with corn and soybean meal–based concentrate formulated to meet or exceed the minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Cows were housed in open dry lot corrals with access to shaded areas and bedded with sand and had ad libitum access to feed and water. Milk production (kg/d) was obtained from the official milk test closest to the AI date. For statistical analysis of P/AI, cows were categorized within parity (primiparous vs. multiparous) as being above or below average milk production.

Synchronization Protocol and AI. The GnRH (100 μg/dose of gonadorelin hydrochloride, Factrel) and $PGF_{2α}$ (25 mg/dose of dinoprost tromethamine, Lutalyse) were manufactured by Zoetis (Madison, NJ). All cows were submitted to a Double-Ovsynch (n = 14,089) protocol as described in experiment 1 (Figure 1); however, TAI at 76 ± 3 DIM varied from 13 to 23 h after the last GnRH treatment. For second and greater AI, all cows were submitted to a GGPPG (n = 6,806) protocol (Figure 1) as described by Carvalho et al. (2015). No cows were inseminated after estrus detection. Cows on farm 1 started the GGPPG protocol 18 d after a previous AI, and cows on farm 2 started the GGPPG protocol 25 d after a previous AI. On farm 1, cows were treated with GnRH on both 18 and 25 d after a previous AI. After a nonpregnancy diagnosis 32 d after a previous AI, nonpregnant cows received 2 $PGF_{2α}$ treatments 24 h apart with the last GnRH treatment administered 56 h after the first $PGF_{2α}$ treatment followed by TAI 13 to 23 h after the last GnRH treatment 35 d after the previous AI. Cows on farm 2 received a GnRH treatment 25 d after AI, and after a nonpregnant diagnosis 32 d after previous AI, cows received a GnRH treatment 32 d after previous AI followed by 2 $PGF_{2α}$ treatments 24 h apart administered 7 and 8 d later and the last GnRH treatment administered 56 h after the first $PGF_{2α}$ treatment followed by TAI 13 to 23 h after the last GnRH treatment 42 d after the previous AI. Cows were inseminated with either sex-sorted (4 million sperm cells per straw) dairy semen (cows, n = 5,666; AI, n = 6,813; sires, n = 79) or conventional (20 million sperm cells per straw) beef semen (cows, n = 8,253; AI, n = 14,083; sires, n = 38). Cows received AI at a single time. Sires and semen type were chosen based on the specific genetic mating plan of each collaborating herd as defined by their genetic consultants.

Each cow was fitted with an electronic ID tag that was read at the time of the last GnRH treatment and at the time of AI, and the times were recorded with the format year-month-day-hour-minute-second in the herd management software program. The interval from the last GnRH treatment to AI was calculated by subtracting the time of the last GnRH treatment from the time of AI. For statistical analysis, time interval was rounded to hour.

Pregnancy Diagnosis. Pregnancy diagnosis was performed 32 d after TAI for all cows in both treatments using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a corpus luteum on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant was reconfirmed at 70 d after TAI using the same ultrasound machine. Cows diagnosed pregnant and then diagnosed not pregnant at the subsequent pregnancy examination were considered to have undergone pregnancy loss.

Statistical Analyses. The experiment was conducted as a prospective observational design. All statistical analyses were performed using SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). Sample size determination for logistic regression with binary outcome, power = 0.80 and α = 0.05, was performed using the Power and Sample Size application of SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). Inclusion of a minimum of 8,959 total inseminations was required to detect a quadratic effect of timing of insemination on P/AI using the results by Pursley et al. (1998) as a reference. The average and standard deviation for time of insemination was set at 17.15 and 5.75, respectively. In addition, the intercept value of 0.1113 and regression coefficient of −0.01099 were used. Analyses of binary response data (P/AI and pregnancy loss) were performed by logistic regression using the GLIMMIX procedure of SAS fitting a binomial distribution. The model included the fixed effects of time (continuous effect) from the last GnRH treatment and AI (linear and quadratic effect), the categorical effect of parity (primiparous vs. multiparous), the categorical effect of insemination number (first vs. second and greater), the categorical effect of milk production (above or below average), as well as the interaction between time and parity, and the interaction between level of milk production and time. Cow within farm was considered the random effect in the model. Because cows were not randomized to semen type, cows inseminated with conventional semen were analyzed separately from cows inseminated with sex-sorted semen. Continuous variables including DIM at AI, and milk production were analyzed by ANOVA using the MIXED procedure of SAS. The model included the fixed effect of lactation number (except for average lactation number) and semen type. The proportion of cows inseminated with sex-sorted semen or conventional semen was analyzed with FREQ procedure of SAS.

A significant difference between levels of a classification variable was declared when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were declared a statistical tendency. Binary response data are presented as proportion (no./no.) in the text and Figures 2 and 3 and as LSM in Figure 4, and continuous variables are presented as means ± SEM.

Experiment 3

Cows, Housing, Feeding, and Milk Production. Experiment 3 was conducted in collaboration with 2 commercial dairy farms located in Texas and Arizona, from August 2019 to July 2023. Lactating Holstein (n = 927), Jersey (n = 3,238), and crossbred (n = 6,762) cows (5,459

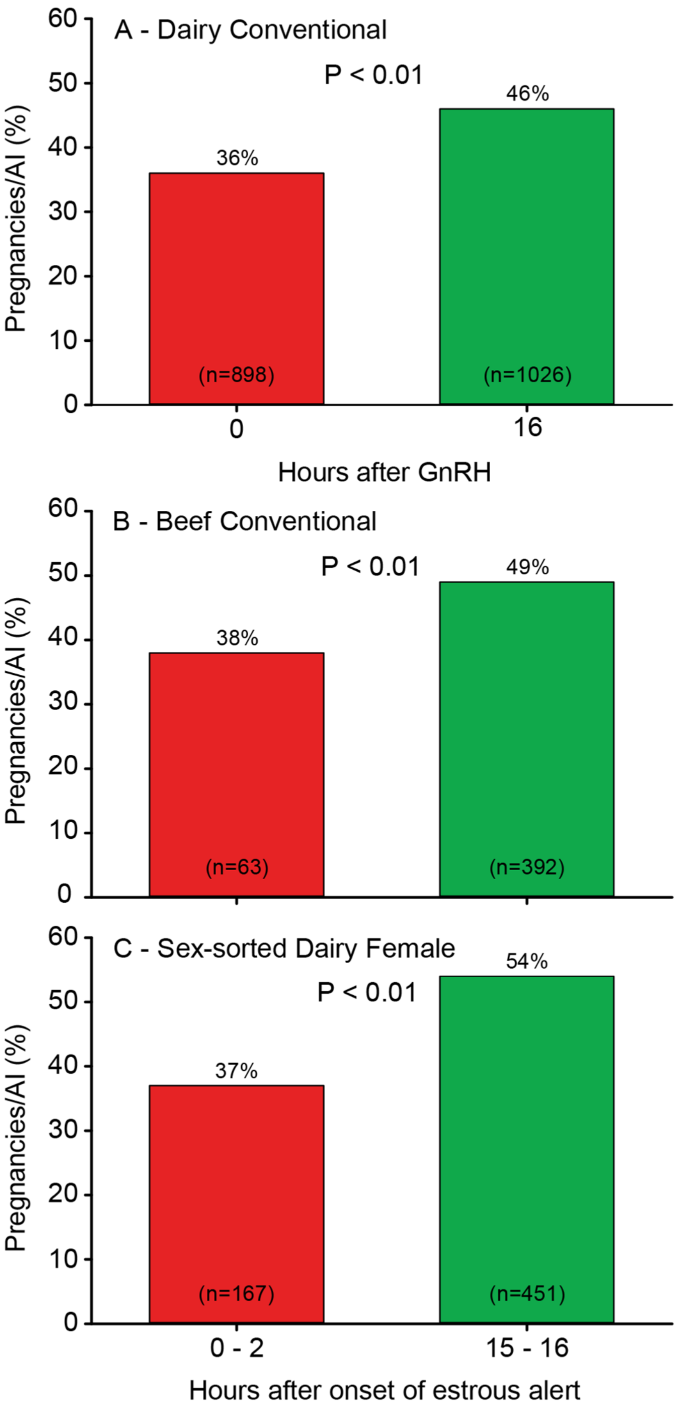

Figure 2. Effect of timed AI at the time of the last GnRH treatment (Cosynch-56) versus AI 16 h after the last GnRH treatment (Ovsynch-56) of a Double-Ovsynch protocol on P/AI using dairy conventional semen (panel A, experiment 1). Effect of AI near (0 to 2 h) the onset of estrous alert versus AI at 15 to 16 h after onset of estrous alert on P/AI for cows inseminated with either conventional beef (panel B, experiment 3) or sex-sorted dairy (panel C, experiment 3) semen.

from farm 1 and 5,468 from farm 2) were milked thrice daily at approximately 8-h intervals and were fed a TMR once daily consisting of corn and alfalfa silage as forage with corn and soybean meal–based concentrate formulated to meet or exceed the minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Cows were housed in open dry lot corrals bedded with sand and had ad libitum access to feed and water. Milk production (kg/d) was obtained from the official milk test closest to the AI date. For statistical analysis of P/AI, cows were categorized within parity (primiparous vs. multiparous) as being above or below average milk production.

Estrus Detection and AI. All cows were fitted with activity-monitoring tags (Heatime; SCR Engineers Ltd., Netanya, Israel) mounted to a neck collar. Data collected by the activity-monitoring system was read by receiver units placed over the water trough in the pens and in the archway at the milking parlor entrance, and scans from individual cows were recorded by the activity-monitoring system software (Data Flow; SCR Engineers Ltd.) installed on an on-farm computer. All settings of the herd management software were based on those being used by the farms at initiation of the experiment. The software settings for activity change threshold for heat index calculation were set at 25 and were not changed during the experiment. The time to AI interval was calculated from the moment this threshold was exceeded and an estrous alert was generated. Because of the genetic programs of the farms, we could not randomize cows to be inseminated with sex-sorted semen or conventional semen. Therefore, we included a much larger sample size with the goal of obtaining sufficient observations within each semen type. Inseminations (n = 20,461) were performed when cows with active automated estrous alert were detected by the activity-monitoring system or when observed standing to be mounted by herd mates when cows were between 50 and 300 DIM. Only data from inseminations conducted in cows with automated estrous alert were included in analyses. Once daily in the morning, a list of cows with activity levels that exceeded the system threshold to consider a cow in estrus was generated by the activity-monitoring system software. The onset of estrous alert was recorded by the activity-monitoring system software and exported every 8 h using the format year-month-day-hour-minute-second. In addition, each cow was fitted with an electronic ID tag that was read at the time of AI, and the time was recorded using the format year-month-day-hour-minute-second. The interval from the onset of estrous alert to AI was calculated by subtracting the time of onset of estrus from the time of AI. For statistical analysis, time interval was rounded to hour. Cows were inseminated with either sex-sorted (4 million sperm cells per straw) dairy semen (cows, n = 5,436; AI, n = 7,565; sires, n = 77) or conventional

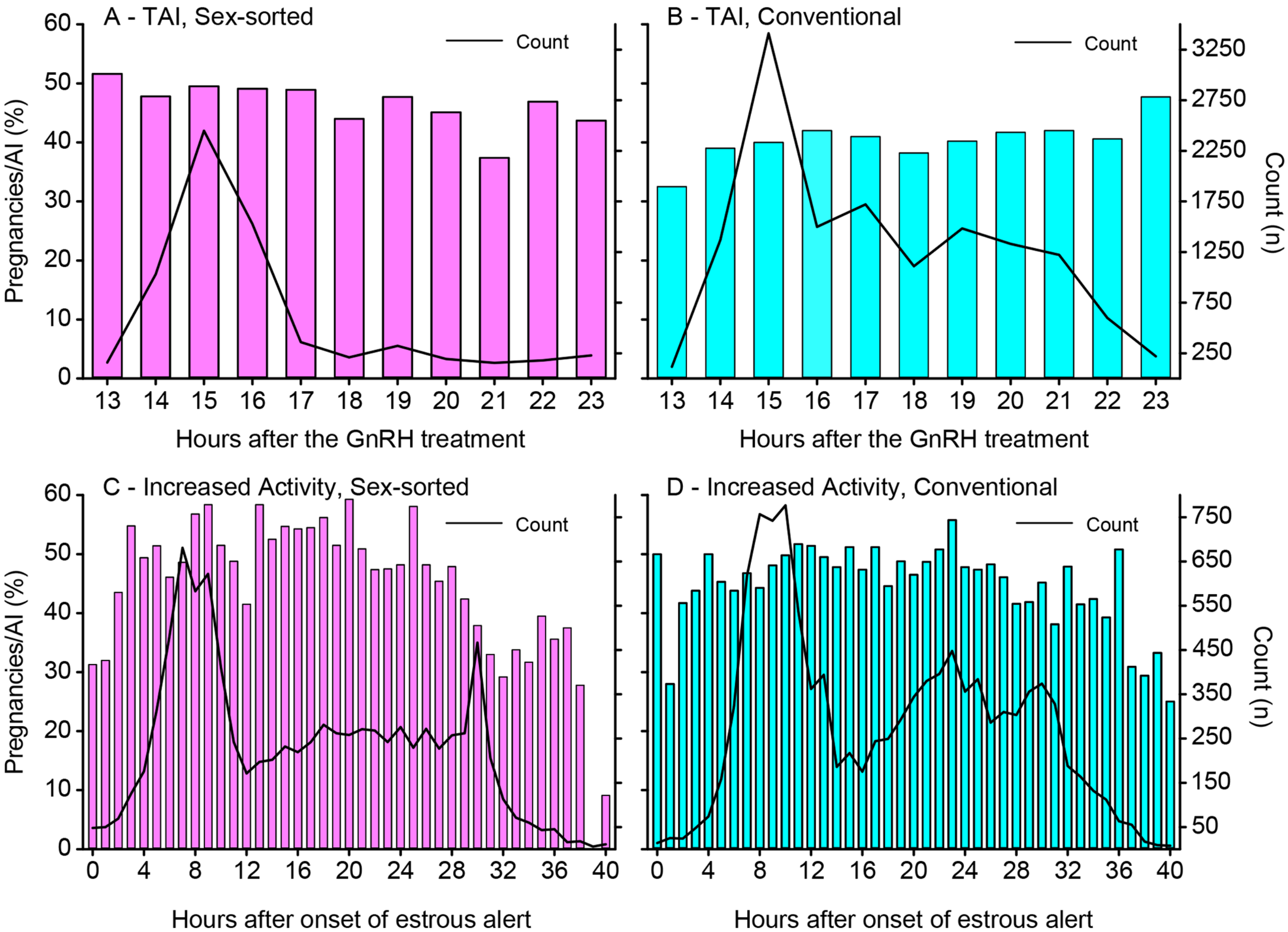

Figure 3. Effect of timing of AI from 13 to 23 h after the last GnRH treatment of a fertility protocol on P/AI (bars) and number of AI (lines) for cows inseminated with sex-sorted (panel A, experiment 2) or conventional semen (panel B, experiment 2). Effect of timing of AI from 0 to 40 h after estrous alert on P/AI (bars) and number of AI (lines) for cows inseminated with sex-sorted (panel C, experiment 3) or conventional semen (panel D, experiment 3).

(20 million sperm cells per straw) beef semen (cows, n = 7,266; AI, n = 12,896; sires, n = 62). Cows received AI in a single time. Sires and semen type were chosen based on the specific genetic mating criteria of each collaborating herd as defined by their genetic consultants.

Pregnancy Diagnosis. Pregnancy diagnosis was performed 32 ± 3 d after TAI for all cows in both treatments using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5-MHz linear-array transducer. A positive pregnancy diagnosis was based on visualization of a corpus luteum on the ovary ipsilateral to the uterine horn containing an embryo with a heartbeat. Pregnancy status for cows diagnosed pregnant was reconfirmed at 90 ± 3 d after TAI using the same ultrasound machine. Cows diagnosed pregnant and then diagnosed not pregnant at the subsequent pregnancy examination were considered to have undergone pregnancy loss.

Statistical Analyses. The experiment was conducted as a prospective observational design. All statistical analyses were performed using SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). Sample size determination for logistic regression with binary outcome, power = 0.80 and α = 0.05, was performed using the Power and Sample Size application of SAS computational software version 9.4 (SAS Institute Inc., Cary, NC). Inclusion of a minimum of 8,959 total inseminations was required to detect a quadratic effect of timing of insemination on P/AI using the results by Pursley et al. (1998) as a reference. The average and standard deviation for time of insemination was set at 17.15 and 5.75, respectively. In addition, the intercept value of 0.1113 and regression coefficient of −0.01099 were used. Analyses of binary response data (P/AI and pregnancy loss) were performed by logistic regression using the GLIMMIX procedure of

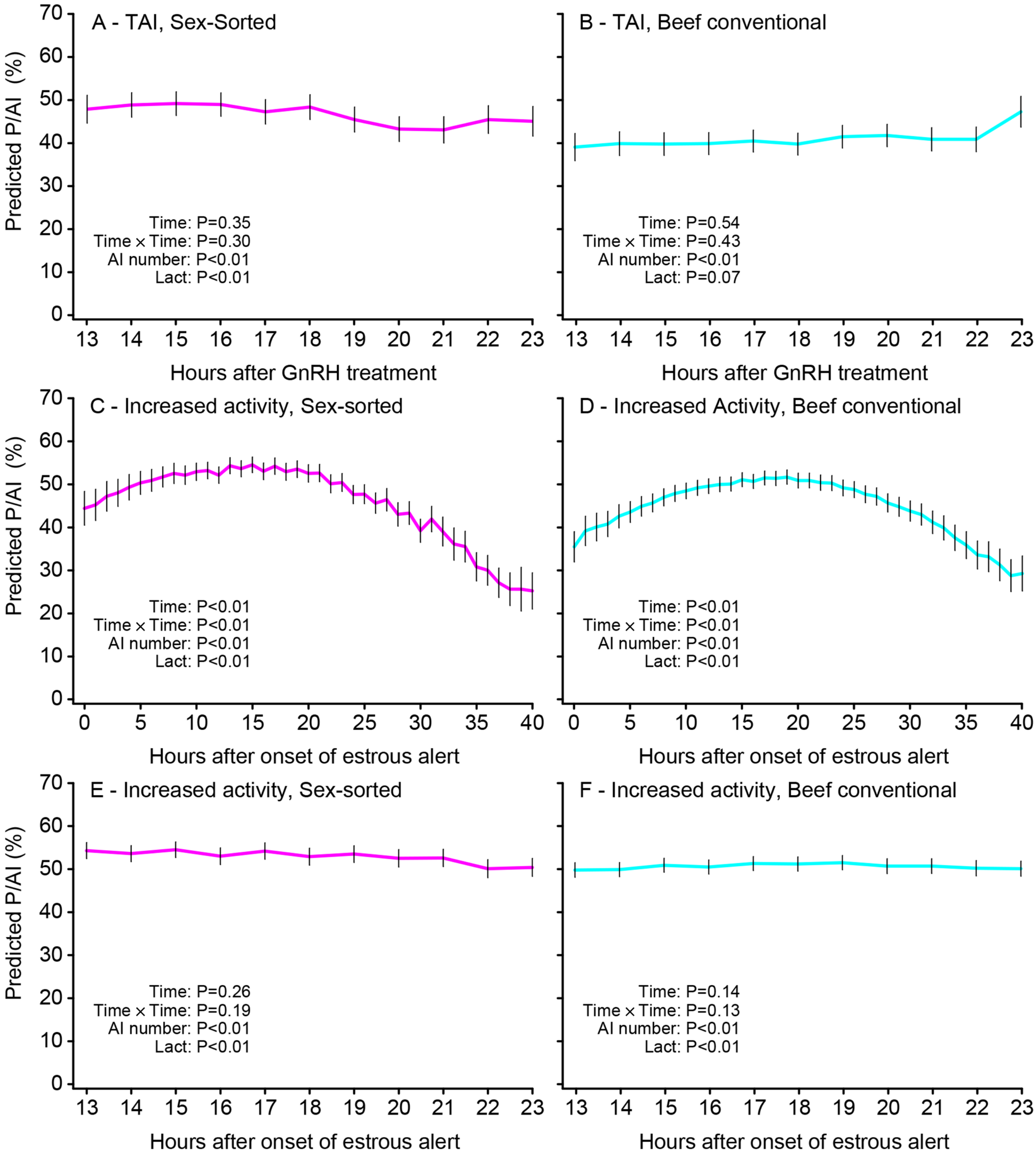

Figure 4. Effect of timing of AI from 13 to 23 h after the last GnRH treatment of a fertility protocol on P/AI for cows inseminated with sex-sorted (panel A, experiment 2) or conventional semen (panel B, experiment 2). Effect of time of AI from 0 to 40 h after estrous alert on P/AI for cows inseminated with sex-sorted (panel C, experiment 3) or conventional semen (panel D, experiment 3). Effect of timing of AI from 13 to 23 h after estrous alert on P/AI for cows inseminated with sex-sorted (panel E, experiment 3) or conventional (panel F, experiment 3) semen. Values are LSMeans ± SEM.

SAS fitting a binomial distribution. The model included the fixed effects of time (continuous effect) after onset of estrous alert (linear and quadratic), the categorical effect of parity (primiparous vs. multiparous), the categorical effect of number of inseminations (first vs. second and greater), the categorical effect of breed, the categorical effect level of milk production (above or below average), as well as the interaction between time and lactation group, the interaction between breed and time, and the interaction between level of milk production and time. Cow within farm was considered the random effect in the model. A second analysis of P/AI was performed using only inseminations that occurred between 13 and 23 h, a time range similar to that observed in experiment 2. Also, cows inseminated at 0 to 2 h were also compared with cows inseminated at 15 to 16 h, a time range similar to that observed in experiment 1. Cows inseminated with conventional semen were analyzed separately from cows inseminated with sex-sorted semen. Continuous variables such as DIM at AI, and milk production near AI were analyzed by ANOVA using the MIXED procedure of SAS. The model included the fixed effect of lactation number (except for average lactation number) and semen type. The proportion of cows inseminated with sex-sorted semen or conventional semen was analyzed with FREQ procedure of SAS.

A significant difference between levels of a classification variable was declared when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were declared a statistical tendency. Binary response data are presented as proportion (no./no.) in the text and Figures 2 and 3 and as LSM in Figure 4, and continuous variables are presented as means ± SEM.

RESULTS

Experiment 1

Cows inseminated with conventional semen 16 h after the last GnRH treatment of the Double-Ovsynch protocol had more ($P < 0.01$) P/AI 32 d after TAI than cows inseminated at the same time as the last GnRH treatment of the Double-Ovsynch protocol (46% [472/1,026] vs. 36% [323/898], Figure 2A).

Experiment 2

A total of 20,895 insemination records were available for this analysis in which the time of the last GnRH treatment of the synchronization protocol and the time of AI were both recorded. Of those inseminations, 14,089 were from first TAI, whereas 6,806 were from second or greater TAI. The average insemination number was 1.6 and ranged from 1 to 8. Primiparous cows had fewer ($P < 0.01$) inseminations than multiparous cows (1.5 ± 0.01 vs. 1.7 ± 0.01, respectively). In addition, cows inseminated with sex-sorted semen had fewer ($P < 0.01$) inseminations than cows inseminated with conventional semen (1.2 ± 0.01 vs. 1.8 ± 0.01, respectively). Average DIM on the day of insemination was 102 and ranged from 77 to 598 DIM. Primiparous cows had fewer ($P < 0.01$) DIM at AI than multiparous cows (100 ± 0.4 vs. 104 ± 0.4, respectively). In addition, cows inseminated with sex-sorted semen had fewer ($P < 0.01$) DIM at AI than cows inseminated with conventional semen (85 ± 0.3 vs. 111 ± 0.4, respectively). For primiparous cows receiving their first AI service, 78.7% were inseminated with sex-sorted semen and 21.3% were inseminated with conventional beef semen. For primiparous cows receiving their second or greater AI service 38.2% were inseminated with sex-sorted semen and 61.8% were inseminated with conventional beef semen. For multiparous cows receiving their first AI service, 18.8% were inseminated with sex-sorted semen and 81.2% were inseminated with conventional beef semen. For multiparous cows receiving their second or greater AI service 2.2% were inseminated with sex-sorted semen and 97.8% were inseminated with conventional beef semen. Based on the milk test closest to AI, cows averaged 42.0 kg of milk/d, which differed ($P < 0.01$) between parities (36.2 ± 0.1 vs. 44.3 ± 0.1 kg/d for primiparous and multiparous cows, respectively). Cows inseminated with sex-sorted semen produced less ($P < 0.01$) milk than cows inseminated with conventional semen (38.1 ± 0.1 vs. 43.1 ± 0.1 kg/d, respectively).

Overall, P/AI was 43.0% (8,995/20,895). For cows inseminated with sex-sorted semen, there was no linear ($P = 0.54$) or quadratic ($P = 0.43$) effect of time of AI after the last GnRH treatment of the synchronization protocol on P/AI (Figures 3A and 4A). Cows receiving first AI had more ($P < 0.01$) P/AI than cows receiving second or greater AI (49.1% [2,921/5,951] vs. 42.5% [366/862], respectively). Primiparous cows tended ($P = 0.07$) to have more P/AI than multiparous cows (48.7% [2,478/5,089] vs. 46.9% (809/1,724), respectively). There was no effect ($P = 0.53$) of level of milk production and there was no interaction ($P = 0.46$) between the level of milk production and time of AI after the last GnRH treatment of the synchronization protocol on P/AI.

For cows inseminated with conventional semen, there was no linear ($P = 0.35$) or quadratic ($P = 0.30$) effect of time of AI after the last GnRH treatment of the synchronization protocol on P/AI (Figures 3B and 4B). Cows receiving first AI had more ($P < 0.01$) P/AI than cows receiving second or greater AI (42.1% [3,427/8,138] vs. 38.4% [2,281/5,944], respectively). Primiparous cows had more ($P = 0.03$) P/AI than multiparous cows (42.3% [1,011/2,389] vs. 40.2% [4,697/11,693], respectively). There was no effect ($P = 0.34$) of level of milk production and there was no interaction ($P = 0.28$) between the level of milk production and time of AI after the last GnRH treatment of the synchronization protocol on P/AI.

Overall, pregnancy loss from 32 to 70 d after TAI averaged 5.2% (461/8,855). For cows inseminated with sex-sorted semen there was no linear ($P = 0.14$) or quadratic ($P = 0.24$) effect of time of AI after the last GnRH treatment of the synchronization protocol on pregnancy loss. Pregnancy loss did not differ ($P = 0.34$) for cows receiving their first TAI and cows receiving their second and greater TAI (5.7% [166/2,893] vs. 4.3% [16/366], respectively). In addition, pregnancy loss did not differ ($P = 0.44$) between primiparous and multiparous cows (5.3% [328/6,246] vs. 5.1% [133/2,609], respectively).

For cows inseminated with conventional semen, there was no linear ($P = 0.70$) or quadratic ($P = 0.94$) effect of time of AI after the last GnRH treatment of the synchronization protocol on pregnancy loss. Pregnancy loss did not differ ($P = 0.93$) for cows receiving their first TAI compared with cows receiving their second and greater TAI (4.8% [162/3,353] vs. 5.2% [117/2,243], respectively). Pregnancy loss tended to be less ($P = 0.08$) for primiparous than for multiparous cows (3.9% [39/997] vs. 5.2% [241/4,599], respectively).

Experiment 3

A total of 20,461 AI records were available for analysis in which the onset of estrus and the time of AI were both recorded. Of those inseminations, 10,686 were from first AI, whereas 9,775 were from second or greater AI. The average insemination number was 1.9 and ranged from 1 to 8. Primiparous cows had more ($P < 0.01$) inseminations than multiparous cows (2.0 ± 0.2 vs. 1.8 ± 0.01, respectively). For primiparous cows receiving their first AI service, 74.7% were inseminated with sex-sorted semen and 25.3% were inseminated with conventional beef semen. For primiparous cows receiving their second or greater AI service 44.3% were inseminated with sex-sorted semen and 55.7% were inseminated with conventional beef semen. For multiparous cows receiving their first AI service, 51.4% were inseminated with sex-sorted semen and 48.6% were inseminated with conventional beef semen. For multiparous cows receiving their second or greater AI service, 25.5% were inseminated with sex-sorted semen and 74.5% were inseminated with conventional beef semen. The average DIM on the day of insemination was 99 and ranged from 51 to 296 DIM. Primiparous cows had more ($P < 0.01$) DIM at AI than multiparous cows (107 ± 0.64 vs. 97 ± 0.3, respectively). In addition, cows inseminated with sex-sorted semen had fewer ($P < 0.01$) DIM at AI than cows inseminated with conventional semen (88 ± 0.3 vs. 107 ± 0.4, respectively). Overall, lactation number averaged 2.4 and ranged from 1 to 9. Cows inseminated with sex-sorted semen had fewer ($P < 0.01$) lactations than cows inseminated with conventional semen (2.1 ± 0.01 vs. 2.6 ± 0.01, respectively). Based on the milk test closest to AI, cows averaged 32.1 kg of milk/d, which differed between parities (28.9 ± 0.1 vs. 33.1 ± 0.1 kg/d for primiparous and multiparous cows, respectively). Cows inseminated with sex-sorted semen produced less ($P < 0.01$) milk than cows inseminated with conventional semen (31.3 ± 0.1 vs. 33.1 ± 0.1 kg/d, respectively).

Overall, P/AI was 48.1% (9,851/20,461). For cows inseminated with sex-sorted semen, there was a linear ($P < 0.01$) and a quadratic ($P < 0.01$) effect of time of AI after the onset of estrous alert on P/AI (Figures 3C and 4C). Cows submitted to first AI had more ($P < 0.01$) P/AI than cows submitted to second or greater AI (50.4% [3,110/6,176] vs. 47.2% [1,441/3,052], respectively). Primiparous cows had more ($P = 0.01$) P/AI than multiparous cows (50.9% [1,784/3,505] vs. 48.3% [2,767/5,723], respectively). There was no effect ($P = 0.70$) of level of milk production and there was no interaction ($P = 0.69$) between the level of milk production and time of AI after the onset of estrous alert on P/AI.

For cows inseminated with conventional semen, there was a linear ($P < 0.01$) and quadratic ($P < 0.01$) effect of time of AI after the onset of estrous alert on P/AI (Figures 3D and 4D). Cows submitted for first AI had more ($P < 0.01$) P/AI than cows submitted for second or greater AI (52.8% [2,383/4,510] vs. 43.4% [2,917/6,723], respectively). Primiparous cows had fewer ($P < 0.01$) P/AI than multiparous cows (34.3% [821/2,395] vs. 50.7% [4,479/8,838], respectively). In addition, there was no effect ($P = 0.48$) of level of milk production and there was no interaction ($P = 0.25$) between the level of milk production and time of AI after the onset of estrous alert on P/AI.

When cows were grouped based on timing of AI that occurred early (between 0 and 2 h after onset of estrus) versus AI that occurred at the recommended time (between 15 and 16 h after the onset of estrus based on the results of Pursley et al., 1998), cows inseminated early had fewer ($P < 0.01$) P/AI than cows inseminated at the recommended time (conventional: 38% vs. 49%, Figure 2B; sex-sorted: 37% vs. 54%, Figure 2C, respectively). In addition, when the time of AI was limited to inseminations occurring only from 13 to 23 h after the onset of estrous alert, there was no linear or quadratic effect of time of AI after the onset of estrus on P/AI for cows inseminated with sex-sorted semen ($P = 0.26$ and $P = 0.19$, respectively, Figure 4E) or conventional semen ($P = 0.14$ and $P = 0.13$, respectively, Figure 4F). Furthermore, cows inseminated after 23 h after the onset of estrous alert had fewer ($P < 0.01$) P/AI than cows inseminated between 15 and 16 h after the onset of estrous alert (conventional:

44% vs. 49%, respectively, and sex-sorted: 42% vs. 54%, respectively). There was no effect ($P = 0.20$) of breed and there was no interaction ($P = 0.60$) between breed and time of AI after the onset of estrous alert on P/AI.

Overall, pregnancy losses from 32 to 90 d after AI averaged 2.0% (196/9,851). For cows inseminated with sex-sorted semen there was no linear ($P = 0.34$) or quadratic ($P = 0.43$) effect of time of AI after the onset of estrus on pregnancy loss. Pregnancy loss did not differ ($P = 0.84$) between cows receiving their first AI and cows receiving their second and greater AI (0.8% [25/3,110] vs. 0.9% [13/1,441], respectively). In addition, pregnancy loss did not differ ($P = 0.16$) between primiparous and multiparous cows (1.1% [19/1,784] vs. 0.7% [19/2,767], respectively).

For cows inseminated with conventional semen, there was no linear ($P = 0.18$) or quadratic ($P = 0.28$) effect of time of AI after the onset of estrus on pregnancy loss. Cows submitted for first AI had fewer ($P = 0.05$) pregnancy losses than cows submitted for second and greater AI (2.4% [58/2,383] vs. 3.4% [100/2,917], respectively). Pregnancy loss was greater ($P < 0.01$) for primiparous than for multiparous cows (5.8% [48/821] vs. 2.5% [110/4,479], respectively).

DISCUSSION

The present study was designed to assess the effect on pregnancy outcomes of the timing of insemination relative to the time of GnRH treatment at the end of the synchronization protocol and relative to the onset of estrous alert. We used electronic ID technology to accurately record the timing of GnRH treatment and AI and electronic activity monitors to determine precise timing of onset of estrous alert. The precise assessment of timing of predicted ovulation and AI for a large number of inseminations (>40,000) allowed for accurate assessment of the AI to ovulation interval on pregnancy outcomes with sex-sorted and conventional semen. A novel result from these analyses is that P/AI did not differ for cows inseminated 13 to 23 h after the last GnRH treatment of a synchronization protocol or from 13 to 23 h after onset of estrous alert. A second novel result is that optimal timing of AI relative to the last GnRH treatment of a synchronization protocol or onset of estrus did not differ between conventional and sex-sorted semen. It is important to note that a direct comparison of the fertility of conventional versus sex-sorted semen in experiments 2 and 3 cannot be made based on these analyses because cows were not randomized to semen type. Thus, relative differences in P/AI between semen types is likely due to the parity of cows inseminated (primiparous vs. multiparous), insemination number, and the genetic merit of cows mated to conventional versus sex-sorted semen. Furthermore, only an association, not a causal relationship, between timing of AI and P/AI can be inferred in experiments 2 and 3, as cows were not randomly assigned to specific insemination times.

Optimal Timing of Insemination

Studies that have evaluated the timing of AI with sex-sorted semen after ED have reported equivocal results. Some studies reported an increase in fertility when the interval from the onset of estrus to AI is increased and AI is performed closer to the presumptive time of ovulation (Sá Filho et al., 2010; Bombardelli et al., 2016; Nebel, 2018), whereas others have found no difference (Macmillan et al., 2020; Tippenhauer et al., 2023). In some studies, the decrease in P/AI associated with inseminations that occur close to the onset of estrus was greater for sex-sorted semen (Sá Filho et al., 2010; Bombardelli et al., 2016; Nebel, 2018) than for conventional semen (Dransfield et al., 1998; LeRoy et al., 2018; Burnett et al., 2022). This has led to the recommendation that AI should occur later relative to the onset of estrus when inseminating with sex-sorted semen. In the present study, a large number of inseminations were performed with both sex-sorted and conventional semen, and our results do not support the recommendation that the optimal timing of AI differs between sex-sorted and conventional semen.

The successful development of an embryo and establishment of a pregnancy is dependent on a well-orchestrated series of events in a race against "aging" of both spermatozoa and the oocyte. The LH surge, which induces ovulation, occurs within 1 to 2 h after the onset of estrus (Walton et al., 1987) or treatment with exogenous GnRH (Haughian et al., 2004; Giordano et al., 2012; Pulley et al., 2015). The interval from the LH surge to ovulation, based on hourly blood sampling and hourly detection of ovulation, averages 25.7 h (23 to 29 h, Ginther et al., 2013). In addition, ovulation occurs approximately 26.4 h after the first standing event (20 to 40 h, Stevenson et al., 2014), 28.7 h after onset of estrous alert (6 to 42 h, Valenza et al., 2012), and 28 h after exogenous GnRH treatment (28 to 30 h, Giordano et al., 2012, 24 to 32 h, Pursley et al., 1995). Even though these events are interconnected, there is a wide degree of variation in timing of ovulation among cows, particularly relative to onset of estrous alert, making it difficult to recommend a single time for AI. The results of the present analyses are supported by studies that reported that insemination at 13 to 23 h after the onset of estrus or after exogenous GnRH treatment optimized P/AI (Pursley et al., 1998; Valenza et al., 2012; Stevenson et al., 2014). Additionally, we found that the optimal time of insemination with sex-sorted semen did not differ from that of conventional semen. Insemination either early (<12 h) or late (>24 h) after the onset of estrus or the last GnRH treatment of a synchronization protocol resulted in decreased fertility.

Consequences of Inseminating Early

Semen requires approximately 4 h to undergo capacitation (Parrish et al., 1988), and 8 to 12 h for capacitated spermatozoa to be transported in the female reproductive tract to the site of fertilization in the isthmic-ampullary junction (Hunter and Wilmut, 1983, 1984). When insemination occurs too early relative to ovulation, spermatozoa remain in the caudal portion of the isthmus until ovulation occurs (Hunter and Wilmut, 1984; Hawk, 1987). In addition, when semen is deposited either in the uterine body or uterine horns too early relative to ovulation, most sperm are lost by either retrograde movement within the female reproductive tract or through phagocytosis (Hawk, 1983; Gallagher and Senger, 1989). Less than 1% of the sperm deposited in the female reproductive tract remain 24 h after AI (Hawk, 1987). In addition, because the lifespan of sperm in the female reproductive tract is estimated to be 30 to 36 h (Beschlebnov, 1938; Roelofs et al., 2006), inseminations that occur too early relative to ovulation may result in complete loss of viable sperm reservoirs in the female reproductive tract.

Results from the present study in which inseminations performed too early relative to ovulation or onset of estrous alert resulted in decreased P/AI are supported by the following: (1) decreased fertilization rate when AI is performed at the onset of estrus (Dalton et al., 2001), (2) increased percentage of degenerated embryos when AI is performed from 36 to 32 h before ovulation (Roelofs et al., 2006), (3) increased P/AI in dairy heifers when timing of AI is delayed by 8 h after the last GnRH treatment of a TAI protocol (Moore et al., 2023), (4) increased P/AI in dairy cows inseminated at 16 h after the last GnRH treatment compared with cows inseminated at the time of the last GnRH treatment (Pursley et al., 1998; Brusveen et al., 2008), (5) decreased P/AI in dairy heifers inseminated 24 before the last GnRH treatment compared with AI at the time of the last GnRH treatment (Figueiredo et al., 2020), and (6) increased P/AI, in most (Dransfield et al., 1998; Sá Filho et al., 2010; Nebel, 2018) but not all studies (LeRoy et al., 2018; Macmillan et al., 2020; Burnett et al., 2022) when AI is delayed by at least 4 h after the onset of estrus. Thus, inseminations occurring too early relative to the time of ovulation may cause sperm to lose the ability to fertilize the oocyte or cause sperm to be removed from the female reproductive tract thereby resulting in low fertilization rate, poor embryo development, and decreased P/AI.

Consequences of Inseminating Late

Before the final stages of oocyte maturation, oocytes are arrested at the prophase-I stage of meiosis, the germinal vesicle stage. After the LH surge, the oocyte contained within the dominant follicle will resume meiosis to the metaphase-II stage, where it will be arrested until fertilization. Thus, delayed timing of AI relative to estrus or ovulation results in fertilization of a postovulatory "aged" oocyte. Although, fertilization rate increased when AI occurred closer to the time of ovulation (Dalton et al., 2001), the ability of the zygote to develop to the blastocyst stage is compromised (Dalton et al., 2001; Roelofs et al., 2006), thereby decreasing fertility. In addition, when fertilization is delayed or does not occur, oocytes undergo degradation and apoptosis. Postovulatory aged oocytes are characterized by increased reactive oxygen species; decreased glutathione; decreased expression of BCL2 (an antiapoptotic protein); impaired calcium regulation in the endoplasmic reticulum (lower amplitude and higher frequency); mitochondrial dysfunction; disrupted thick microfilament domain beneath the oolemma; disruption and loss of the meiotic spindle assembly; disruption of the centrosome structure at the meiotic poles, leading to premature chromosomal separation and aneuploidy (Lord and Aitken, 2013; Takahashi et al., 2013; Martin et al., 2022); and abnormal early embryo development in many mammalian species including cows (Dalton et al., 2001; Roelofs et al., 2006), pigs (Hunter, 1967), hamsters (Chang and Fernandez-Cano, 1958; Yanagimachi and Chang, 1961), rabbits (Hammond, 1934), and mice (Marston and Chang, 1964). Therefore, to optimize embryo development and P/AI, semen must be available to fertilize the oocyte at the time of ovulation or soon thereafter.

Although the lifespan of bovine oocytes has been estimated to be 10 h (Hunter, 1988), the lifespan of oocytes in modern high-producing dairy cows may be shorter than previously estimated based on several observations. First, ovulation occurs at approximately 28 h after onset of estrus (Walker et al., 1996; Valenza et al., 2012) or after exogenous GnRH treatment, and P/AI began to decrease when AI was performed at 24 h or later after the onset of estrus, which is less than 4 h before the expected time of ovulation. Additionally, considering that it takes approximately 8 h for the capacitated spermatozoa to reach the site of fertilization (Hunter and Wilmut, 1983, 1984), the ability of the oocyte to establish a viable pregnancy decreases as early as 4 h after ovulation.

CONCLUSIONS

Cows inseminated at the time of the final GnRH treatment of a Double-Ovsynch protocol had fewer P/AI than cows inseminated 16 h after the last GnRH treatment (experiment1). Cows inseminated from 13 to 23 h after the final GnRH treatment of a hormonal synchronization had similar P/AI (experiment 2). Cows inseminated within the first 2 h or later than 24 h after the onset of estrous alert had fewer P/AI than cows inseminated 15 to 16 h after the onset of estrous alert (experiment 3). Finally, optimal timing of AI did not differ between sex-sorted or conventional semen and was optimal when it occurred from 13 to 23 h after the last GnRH treatment of the synchronization protocol or after the onset of estrous alert.

NOTES

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Nonstandard abbreviations used: AI = artificial insemination; ED = detected estrus; P/AI = pregnancy per artificial insemination; TAI = timed artificial insemination.

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