Evaluation of delayed timing of artificial insemination with sex-sorted sperm on pregnancy per artificial insemination in seasonal-calving, pasture-based lactating dairy cows

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ABSTRACT

The objective was to use ovulation synchronization with timed artificial insemination (TAI) to evaluate the effect of timing of artificial insemination (AI) with frozen sex-sorted sperm on fertility performance in pasture-based compact calving herds. Ejaculates from 3 Holstein-Friesian bulls were split and processed to provide frozen sex-sorted sperm (SS) at 4×10^6 sperm per straw, and frozen conventional sperm at 15×10^6 sperm per straw (CONV). A modified Progesterone-Ovsynch protocol was used for estrous synchronization, with TAI occurring 16 h after the second GnRH injection for cows assigned to CONV, and either 16 h (SS-16) or 22 h (SS-22) for cows assigned to SS. Pregnancy diagnosis was conducted by transrectal ultrasound scanning of the uterus 35 to 40 d after TAI ($n = 2,175$ records available for analysis). Generalized linear mixed models were used to examine the effects of treatment on pregnancy per artificial insemination (P/AI). Fixed effects included treatment ($n = 3$), bull ($n = 3$), treatment by bull interaction, parity ($n = 4$), days-in-milk category ($n = 3$), and treatment by days-in-milk category, with herd ($n = 24$) included as a random effect. Pregnancy per AI was greater for CONV compared with both SS-16 and SS-22 (61.1%, 49.0%, and 51.3%, respectively), and the SS treatments did not differ from each other (relative P/AI for SS-16 and SS-22 vs. CONV were 80.2% and 84.0%, respectively). There were significant bull and treatment by bull interaction effects. Additional analysis was undertaken using a model that included herd as a fixed effect. This analysis identified marked herd-to-herd variation (within-herd relative P/AI for the combined SS treatments vs. CONV ranged from

48–121%). The tertile of herds with the best performance achieved a mean relative P/AI of 100% (range = 91–121%), indicating that P/AI equivalent to CONV is achievable with SS. Conversely, the tertile of herds with the poorest performance achieved a mean relative P/AI of 67% (range = 48–77%). We found that SS resulted in poorer overall P/AI compared with CONV sperm regardless of timing of AI. Marked variation existed between herds; however, one-third of herds achieved P/AI results equal to CONV. Identification of factors responsible for the large herd-to-herd variation in P/AI with SS, and development of strategies to reduce this variation, warrant further research.

Key words: sex-sorted sperm, synchronization, fixed time artificial insemination, seasonal calving

INTRODUCTION

Seasonal-calving pasture-based systems of milk production focus on maximizing the use of grazed grass in the diet (Dillon et al., 1995; Shalloo et al., 2004). The target is for 90% of the herd to calve within 6 wk after the planned start of calving, with every 1% increase in 6-wk calving rate resulting in a corresponding increase in profitability of €822 for a 100-cow herd (Shalloo et al., 2014). When conventional (CONV) sperm is used, approximately 50% of the calves born will be male and of low economic value, raising potential animal welfare issues (Holden and Butler, 2018).

The potential benefits of sex-sorted sperm (SS) have been well documented and include an estimated 20% reduction in the occurrence of dystocia, improved biosecurity arising from on-farm breeding of replacement heifers, and the facilitation of crossbreeding to yield predominantly female calves (Seidel, 2003; Weigel, 2004). Furthermore, targeted usage of SS to generate replacement heifers at the start of the breeding season and use of beef sires for all other inseminations can increase the value of beef output from the dairy herd

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(Butler et al., 2014). Despite these potential benefits, the uptake of SS remains low in pasture-based systems of milk production that are reliant on excellent fertility and a concentrated period of calving. This is primarily due to concerns regarding reduced fertility of sex-sorted sperm and the associated economic losses.

Fluorescence-activated cell sorting via flow cytometry is the most widely used process for sex-sorting sperm based on differences in DNA content (Garner et al., 2013). In cattle, X-chromosome-bearing sperm contain approximately 3.8% more DNA than Y-chromosome-bearing sperm. This method typically results in a ~90% sex-bias semen straw (Johnson, 1995). Sex-sorted sperm has been reported to have a shorter fertile lifespan after the sorting process (Vishwanath and Moreno, 2018). Reasons for this reduced longevity are multifactorial, including nuclear staining, pressure, exposure to lasers, physical stress, extended processing time, and dilution (Maxwell and Johnson, 1997; Maxwell et al., 1998; Maxwell et al., 2004; Suh et al., 2005; Schenk et al., 2009).

Bioeconomic modeling studies have indicated that SS usage in both heifers and cows in expanding herds can increase farm profitability despite reduced fertility (Hutchinson et al., 2013a,b). A recent field trial evaluated the fertility of frozen SS (4×10^6 per straw) compared with frozen CONV (15×10^6 per straw) under seasonal pasture-based conditions. Overall, a relative pregnancy per artificial insemination (P/AI) of 76% (SS relative to CONV) was reported, which was apparently better for bulls that were resident at the sorting laboratory (84%) compared with those that had ejaculates transported for 6 to 7 h to the sorting laboratory (70%; Maicas et al., 2020). That study also identified marked herd-to-herd variation, with 33% of herds achieving a relative P/AI of $\geq 90\%$. Based on this variation, it was hypothesized that herd management (including timing of AI) may have played a role in variable P/AI results between herds (Maicas et al., 2020). Previous studies that used timed AI (TAI) protocols (without a final injection of GnRH to induce ovulation; Sales et al., 2011; Thomas et al., 2014), or that evaluated time of AI relative to an increase in activity (Bombardelli et al., 2016), have suggested that delaying the timing of AI to be closer to the time of ovulation has a favorable effect on P/AI achieved with SS. This presumably reflects the presence of a larger population of viable sperm in the female reproductive tract at the time of ovulation. Ovulation synchronization and TAI have been extensively evaluated in seasonal-calving systems, and have been shown to shorten the interval from the start of the breeding period to pregnancy establishment compared with nonsynchronized animals (Herlihy et al., 2011; Randi et al., 2018). The objective of this

study was to evaluate the effect of timing of AI on P/AI for SS in a pasture-based, compact calving production system. We tested the hypothesis that delaying timing of AI with SS would increase P/AI.

MATERIALS AND METHODS

Semen Collection

This study was carried out in spring of 2019. Three Holstein-Friesian bulls were selected by the collaborating Irish AI companies to produce SS sperm straws for commercial sale, and these bulls were transported to a semen collection center in close proximity to a sperm sorting laboratory (Cogent Breeding Ltd., Chester, United Kingdom). Ejaculates were collected by experienced operators using an artificial vagina and immediately delivered to the sex-sorting laboratory. Semen was processed in the laboratory and subjected to quality control and testing as described in detail by Maicas et al. (2019). To produce straws for the field trial, each ejaculate was split into 2 aliquots and either processed for CONV straws (not SS) with a concentration of 15×10^6 sperm per straw, or SS (90% X-chromosome biased), using the SexedULTRA Genesis III system as described by Vishwanath and Moreno (2018), with a concentration of 4×10^6 total sperm per straw.

Participating Herds

Herds were recruited with the cooperation of the Irish Cattle Breeding Federation (ICBF; Bandon, Co. Cork, Ireland), which maintains the national database for cattle data collection in Ireland, in conjunction with participating Irish AI companies (Dovea Genetics, Thurles, Co. Tipperary; Munster Bovine, Mallow, Co. Cork; and Progressive Genetics, Enfield, Co. Meath). Herds were required to have a mating start date in mid to late April, with 100 cows meeting eligibility criteria based on parity (1–4 inclusive) and DIM (≥ 50 DIM on the day of AI). Herds meeting these criteria ($n = 24$) were enrolled in the study. Enrolled cows were classified as Holstein-Friesian (82.0%), Jersey (10.7%), British-Friesian (5.3%), Norwegian Red (1.5%), or Ayrshire (0.5%). Each herd received 100 straws (approximately two-thirds SS and one-third CONV), and the proportion of CONV and SS straws from each bull was identical for each herd.

Prebreeding Ultrasound Scanning, Synchronization, and Experimental Treatments

Transrectal ultrasonography to assess uterine health and ovarian status was performed on eligible cows by

experienced veterinary practitioners. The number of corpora lutea (CL) was recorded, and a 4-point ultrasound reproductive tract score (URTS) scale adapted from Mee et al. (2009) was used to assess uterine health status. Only cows with a URTS ≤ 2 were enrolled in the study.

A modified 10-d Progesterone-Ovsynch protocol adapted from Herlihy et al. (2011) was used (Figure 1). On d -10 relative to the farm mating start date, a 2-mL i.m. injection of GnRH analog (Ovarelin, 100 μ g of gonadorelin diacetate tetrahydrate; Ceva Santé Animale, Libourne, France) was administered, and a progesterone-releasing intravaginal device (PRID; Ceva Santé Animale) was inserted. On d -3, a 5-mL i.m. injection of PGF_{2 α} (Enzaprost, 25 mg of dinoprost trometamol; Ceva Santé Animale) was administered. On d -2, a second 5-mL i.m. of PGF_{2 α} was administered and the PRID was removed. On d -1 (32 h after PRID removal) a second i.m. injection of GnRH was administered.

The experiment was a completely randomized block design. Within each herd, synchronized cows were

stratified based on parity and DIM, and randomly assigned to 1 of 3 treatments: (1) TAI 16 h after the second GnRH injection with a CONV straw (CONV), (2) TAI 16 h after the second GnRH injection with a SS straw (SS-16), or (3) TAI 22 h after the second GnRH injection with a SS straw (SS-22). One of 3 different oil-based tail paint colors (Tell Tail; FIL, Mount Maunganui, New Zealand) that corresponded to the cow treatment was used to make a visible strip across the middle of the back of every cow on the day of synchronization protocol initiation. This served 2 purposes: (1) aid identification of the specific cows within the herd that were enrolled in the study and would require hormonal interventions and (2) aid identification of cows that required AI at 16 (and whether SS or CONV semen) or 22 h (SS only) after the final GnRH injection. The initiation of the protocol on d -10 was implemented by a veterinary practitioner, and the remaining interventions were undertaken by the herdowner or farm staff. On the day of protocol initiation, all herdowners were given their farm-specific timetable of the remaining interventions and the exact

Figure 1. Schematic diagram summarizing the synchronization protocol used and timing of AI. On d -10 (i.e., 10 d before AI date), cows were scanned to verify suitability, an injection of GnRH was administered, and a progesterone-releasing intravaginal device (PRID) was inserted. On d -3, an injection of PGF_{2 α} (PGF) was administered. On d -2 a second injection of PGF was administered and the PRID was removed at 0900 h. On d -1, an injection of GnRH was administered at 1700 h. On d 0, timed AI (TAI) was conducted at either 0900 h (16 h after second GnRH; CONV and SS-16) or 1500 h (22 h after second GnRH; SS-22). At 35 to 40 d after TAI, pregnancy diagnosis (PD) was conducted using transrectal ultrasound.

supply of required hormones, syringes, and needles. In addition, each herdowner received communication from a member of the research team the day before every intervention with a reminder of the specific intervention needed and the time that the intervention should be undertaken. Finally, all herdowners were provided with print-out sheets of the list of cows in each treatment, indicating which cows needed to be made available for AI at 16 and 22 h after GnRH. This sheet also ranked the bulls in order of suitability to breed each cow (and minimize inbreeding) based on sire mating advice software developed by the ICBF.

All inseminations were recorded on a hand-held device by a professional AI technician, and data were subsequently uploaded via an application program interface link to the ICBF database (www.icbf.com). Upon completion of all inseminations, data including cow identification number, treatment straw, bull code, cow parity, calving date, and breed were extracted from the ICBF database.

Pregnancy was diagnosed by transrectal ultrasound scanning of the uterus 35 to 40 d after AI. All pregnancy diagnosis results were compiled into a single database and merged with insemination and cow information data. Data from a total of 2,175 cows were available for analysis. The average number of cows per herd was 91 (range 62–100). The number of inseminations per bull ranged from 579 to 892.

Data Handling and Statistical Analysis

Cows were removed from the data set for the following reasons: lost PRID ($n = 30$), PRID removal at time of AI ($n = 1$), inseminated with nontrial bull ($n = 23$), missing record of insemination event ($n = 21$), incorrect sperm treatment recorded ($n = 13$), ovarian cyst ($n = 5$), uterine disease (i.e., URTS ≥ 3 ; $n = 1$), ovarian tumor ($n = 1$), and lameness ($n = 1$). The variable DIM was divided into 3 categories: 50 to 70, 71 to 79, and ≥ 80 (each DIM category had an approximately equal number of cows). Parity was divided into 4 categories: 1, 2, 3, and 4 (Table 1). The number of CL visible on the ovary on the day of initiation of synchronization was categorized as 0 or ≥ 1 . A generalized linear mixed model (PROC GLIMMIX, SAS 9.4, SAS Institute Inc., Cary, NC) was used to evaluate the effect of treatment on P/AI. The experimental unit was considered to be cow, and fixed effects in the model included treatment ($n = 3$), bull ($n = 3$), treatment by bull interaction, parity ($n = 4$), DIM category ($n = 3$), and treatment by DIM category. Herd ($n = 24$) was included as a random effect.

Herd mean P/AI for each treatment was calculated, and these data were used to create a box-and-whiskers

plot to visualize the between-herd variation in P/AI for each treatment. As the mean P/AI was similar and the between-herd variation for P/AI was large for both SS-16 and SS-22, these 2 SS treatments were combined to form a single SS treatment. Generalized linear mixed models were then used to evaluate herd and AI technician effects in separate models. Fixed effects in the models included treatment ($n = 3$), bull ($n = 3$), treatment by bull interaction, herd (or AI technician), and treatment by herd (or AI technician) interaction. The SLICE statement was used to perform a partitioned analysis of the least squares means for the treatment by herd (or AI technician) interaction.

The relative P/AI was calculated as $[(SS \text{ P/AI} \div CONV \text{ P/AI}) \times 100]$. A relative P/AI of $<100\%$, 100% , or $>100\%$ indicated that the P/AI achieved by SS was less than, equal to, or greater than the P/AI for CONV, respectively.

RESULTS

Factors Affecting P/AI

Treatment had an effect on P/AI ($P < 0.001$; Figure 2). Both SS-16 and SS-22 resulted in lesser P/AI compared with CONV, and did not differ from each other. The relative P/AI for SS-16 and SS-22 was 80.2% and 84.0%, respectively. The presence or absence of a CL, the URTS score at the start of the synchronization protocol, parity, and DIM category did not affect P/AI

Table 1. Characteristics of cows enrolled on the trial

Variable	n	%
Parity ¹		
1	843	38.8
2	556	25.6
3	423	19.5
4	353	16.2
DIM ²		
50–70	707	32.5
71–79	743	34.2
≥ 80	725	33.3
CL ³		
0	328	15.6
1	1,606	76.6
2	161	7.7
3	3	0.1
URTS ⁴		
1	1,635	78.0
2	461	22.0

¹Parity = lactation number.

²DIM on the day of timed AI.

³CL = number of corpora lutea present on the day of synchronization protocol initiation.

⁴URTS = uterine ultrasound reproductive tract score on the day of synchronization protocol initiation.

Figure 2. The effect of sperm treatment and timing of AI on pregnancy per AI (P/AI) in lactating dairy cows. There was a treatment effect on P/AI ($P < 0.001$). The values in the black box indicate the relative P/AI of the sex-sorted (SS) treatments compared with conventional (CONV). Treatments with different letters (a,b) differ $P < 0.001$. Error bars denote lower and upper limits of 95% confidence intervals. The numbers of pregnant and total cows for each treatment are indicated at the base of each bar. CONV = timed AI 16 h after the second GnRH injection with a conventional semen straw; SS-16 = timed AI 16 h after the second GnRH injection with a sexed semen straw; SS-22 = timed AI 22 h after the second GnRH injection with a SS straw.

(all $P > 0.1$). The herd-to-herd variation in P/AI was considerably greater in both SS treatments compared with the CONV treatment (Figure 3).

Bull had an effect on P/AI ($P = 0.047$). Across all treatments, the P/AI for bulls 1, 2, and 3 were 55.2%, 49.7%, and 56.6% respectively. A bull by treatment interaction ($P < 0.05$) was also detected (Figure 4). Both SS treatments resulted in lesser ($P < 0.05$) P/AI compared with CONV for bull 1. For bull 2, SS-16 resulted in lesser ($P < 0.05$) P/AI compared with CONV and SS-22, whereas P/AI for SS-22 did not differ from CONV. There was no difference between treatments for bull 3.

Herd Variation in Relative P/AI

There was considerable variation in P/AI between herds. When the 2 SS treatments were combined, a treatment effect on P/AI was also detected (61.1 vs. 50.9%; $P < 0.001$). Herds were ranked in order of decreasing relative P/AI (Figure 5). The mean relative P/AI was 83.3%, with a range from 48 to 121% between herds. The best tertile, intermediate tertile, and poorest tertile of herds ($n = 8$ herds per tertile) achieved mean (and range) relative P/AI of 100% (91–121%), 84% (78–89%), and 67% (48–77%), respectively. Of note, in the tertile of herds with the poorest relative P/AI, the P/AI achieved with CONV was greater than

both the intermediate tertile and best tertile (66.0%, 59.0%, and 59.6%, respectively).

Technician Variation and P/AI

Eighteen technicians were involved in the study. Technicians were ranked based on the relative P/AI calculated using the combined SS treatments (Figure 6). Technicians 1 and 2 were assigned to 2 herds and 4 herds, respectively, while all other technicians were assigned to a single herd. There was no effect of technician on P/AI with CONV ($P = 0.99$), but there was an effect of technician on P/AI with SS ($P = 0.052$). The P/AI for SS achieved by technicians 16, 17, and 18 was poorer ($P < 0.05$) than that achieved by technicians 1, 2, and 3, but P/AI for CONV did not differ (Figure 6).

DISCUSSION

This study used an ovulation synchronization protocol to evaluate the effect of timing of AI with SS on P/AI. The main findings were that P/AI did not differ between SS treatments (SS-16 and SS-22), and both SS treatments were poorer than CONV. Similar to previous studies, bull and bull by treatment interaction effects were detected. Of particular note, herd-to-herd variation in P/AI was markedly greater for both SS treatments compared with the CONV treatment.

Figure 3. Box and whisker plot indicating the interquartile ranges in herd mean pregnancy per AI (P/AI) for each treatment. The ends of the whiskers represent the maximum and minimum P/AI for an individual herd. The upper and lower quartiles make up the boundaries of the box. The height of the box represents the interquartile range, and the median is indicated by the horizontal line within the box. The arithmetic mean P/AI for each treatment is indicated by the diamond. For SS-22, one herd was identified as an outlier (open circle). Greater variation is evident for the 2 sex-sorted (SS) treatments compared with the conventional (CONV) treatment. CONV = timed AI 16 h after the second GnRH injection with a conventional semen straw; SS-16 = timed AI 16 h after the second GnRH injection with a sexed semen straw; SS-22 = timed AI 22 h after the second GnRH injection with a SS straw.

Overall, P/AI for both SS and CONV treatments were comparable with previous studies, where CONV was reported to have a better field fertility performance compared with SS (Schenk et al., 2009; Karakaya et al., 2014). Maicas et al. (2020) recently reported P/AI results in lactating dairy cows following insemination with CONV or SS (Sexed ULTRA 4M) after detected estrus (59.9% and 45.5%, respectively). No differences were observed between the 2 SS treatments in the current study, failing to support the original hypothesis that delaying timing of AI would benefit P/AI with SS. This agrees with a recent report by Chebel and Cunha (2020), who treated heifers with a 5-d CoSynch protocol and used activity monitors to detect onset of estrus during a 72-h period after PGF_{2α} administration. Heifers that exhibited estrus were assigned to receive AI either immediately after onset of estrus (CONV or **SSEarly**) or 12 h after onset of estrus (**SSLate**). Heifers that did not exhibit estrus by 72 h after PGF_{2α} were administered GnRH and assigned to receive AI either immediately after GnRH (CONV or **SSEarly**) or 12 h after GnRH (**SSLate**). The authors reported that P/AI

was not different between heifers that received **SSEarly** or **SSLate**, and both had poorer P/AI compared with CONV. In a study using the Double Ovsynch protocol with primiparous dairy cows, Lauber et al. (2020) reported that delaying timing of AI with SS from 16 to 24 h after the final GnRH reduced P/AI in primiparous dairy cows. However, in contrast to the current study, Lauber et al. (2020) fixed the time of AI for all cows (48 h after final PGF_{2α}) and varied the interval from the last PGF_{2α} to GnRH2 (24 vs. 32 h), and thereby time from GnRH2 to TAI (24 vs. 16 h). Thus, timing of AI was likely confounded with differences in preovulatory follicle size, stage of follicle development, and circulating progesterone and estradiol concentrations when GnRH2 was administered to induce ovulation. Nevertheless, the collective observations from both the current study and the available literature indicate that 16 to 22 h after the second GnRH injection likely encompasses the optimum window for timing of AI with SS in lactating dairy cows.

Several studies have used synchrony protocols that did not use GnRH before AI to induce ovulation. Sales

et al. (2011) observed greater P/AI with SS when TAI was performed between 0 to 12 h before ovulation compared with 12 to 14 h before ovulation. This is consistent with the observations in the current study, as timing of AI for both SS-16 and SS-22 should have occurred between 0 and 12 h before ovulation (expected time of ovulation after final GnRH is approximately 28 h later; Herlihy et al., 2012). Schenk et al. (2009) inseminated Angus heifers with SS at either 55 to 56 h or 67 to 68 h after progesterone device-removal and PGF_{2α} administration, and reported that delaying timing of AI improved P/AI with SS (49% with delayed TAI vs. 34% with standard TAI). Conversely, administering GnRH at the time of AI, Hall et al. (2017) reported no difference in P/AI in beef cows receiving TAI with SS at 80 versus 72 h after the first PGF_{2α} injection in a CoSynch TAI protocol.

In agreement with previous studies, bull had a significant effect on P/AI in the current study (Frijters et al., 2009; Sá Filho et al., 2013; Hall et al., 2017; Thomas et al., 2019; Maicas et al., 2019). Ejaculates from each bull on the trial were subjected to industry-standard sperm-quality evaluation assessments including concentration, morphology, and motility assessments. Unsatisfactory field fertility with SS is still a barrier to widespread commercial utilization, particularly in compact calving systems. Holden et al. (2017) suggested that a suite of custom in vitro assessment techniques for different

sperm treatments (CONV vs. SS), rather than general quality assessments, may help to identify and eliminate bulls whose sperm characteristics are not suited to the sex-sorting process. Split ejaculates (CONV and SS straws originated from same ejaculate) were used in the current study to eliminate possible effects of ejaculate variation on P/AI, similar to previous studies (Xu et al., 2018; Bo et al., 2019; Maicas et al., 2019, 2020).

A bull by treatment interaction on P/AI was observed in the current study. This occurred because bull 2 had an improvement in P/AI when SS was used at the later time (SS-22) compared with the normal time (SS-16), which provided partial support for the hypothesis that delaying timing of AI closer to the time of ovulation would improve P/AI for SS. This finding perhaps indicated that the timing of AI with SS is more critical for some bulls than for others. Further research is needed to identify tests that accurately identify individual bulls that are more sensitive to timing of AI with SS.

The variation between herds in mean P/AI was small for CONV, but large for both SS treatments. All herds achieved acceptable P/AI with CONV, where the target in seasonal-calving herds is $\geq 60\%$ (Burke et al., 2007; Butler, 2014). Of note, in the third of herds with the poorest relative P/AI, P/AI with CONV was better than both the intermediate and best third of herds. This indicated that the cows in these herds were fertile, the synchronization protocol was correctly implemented, a high fertility ovulation event was induced, and AI was conducted at the appropriate time. In the third of the herds with the best relative P/AI, the P/AI results achieved with SS were similar to CONV. This indicated that neither the SS itself nor the times chosen for TAI were the primary causes of compromised fertility performance in the herds that had poor P/AI with SS. Accuracy of heat detection and timing of AI have been highlighted previously as possible reasons for herd-to-herd variability in P/AI with SS (Arruda et al. 2012; Thomas et al. 2014). In the current study, however, heat detection was eliminated as a source of variation due to the implementation of a synchronization protocol to control timing of ovulation, thereby allowing TAI. Herd-to-herd variation was also observed by Maicas et al. (2019), whereby approximately 33% of enrolled herds achieved a relative P/AI of $\geq 90\%$, with the majority of that subset of herds achieving a relative P/AI of $\geq 100\%$. Herd management factors may account for some of this variation, with farms achieving excellent P/AI results with SS when providing conditions suited to SS usage, but the exact contributory factors remain undefined. In the current study, within each herd, treatments were balanced for parity and DIM, and cows within each herd were exposed to the same nutrition and herd management conditions. The

Figure 4. Illustration of the interaction between bull and treatment on pregnancy per AI (P/AI). There was a treatment by bull interaction ($P < 0.001$). The upper value within each bar is the model adjusted mean P/AI for each treatment and bull combination. The lower value within each bar indicates the number of inseminations for each treatment and bull combination. Within each bull, treatment means with different letters (a,b) differ ($P < 0.05$). Error bars denote lower and upper limits of the 95% confidence interval. CONV = conventional treatment; SS = sex-sorted treatment. CONV = timed AI 16 h after the second GnRH injection with a conventional semen straw; SS-16 = timed AI 16 h after the second GnRH injection with a sexed semen straw; SS-22 = timed AI 22 h after the second GnRH injection with a SS straw.

Figure 5. Mean pregnancy per AI (P/AI) for conventional (CONV) and sex-sorted (SS; SS-16 and SS-22 combined) within each study herd, ranked based on relative P/AI [(SS P/AI $\div$ CONV P/AI) $\times$ 100]. Herds were divided into tertiles (best, intermediate, and poorest based on relative P/AI). The mean (and range) relative (Rel.) P/AI for each tertile is indicated in the black boxes at the top of the figure. The type 3 test of fixed effects for treatment by herd interaction was not significant ($P = 0.66$). Partitioning the least squares means for the treatment by herd interaction using the SLICE statement indicated a significant effect for SS ($P = 0.03$) but not CONV ($P = 0.99$). $\dagger P < 0.1$; $*P < 0.05$; $**P < 0.01$. CONV = timed AI 16 h after the second GnRH injection with a conventional semen straw; SS-16 = timed AI 16 h after the second GnRH injection with a sexed semen straw; SS-22 = timed AI 22 h after the second GnRH injection with a SS straw.

Figure 6. Mean pregnancy per AI (P/AI) for conventional (CONV) and sex-sorted (SS; SS-16 and SS-22 combined) for each technician, sorted based on P/AI achieved with SS. Technicians 1 and 2 were assigned to 2 and 4 herds, respectively, and all other technicians were assigned to one herd. The type 3 test of fixed effects for treatment by herd interaction was not significant ($P = 0.59$). Partitioning the least squares means for the treatment by herd interaction using the SLICE statement indicated a significant effect for SS ($P = 0.05$) but not CONV ($P = 0.99$). $\dagger P < 0.1$; $*P < 0.05$; $**P < 0.01$. CONV = timed AI 16 h after the second GnRH injection with a conventional semen straw; SS-16 = timed AI 16 h after the second GnRH injection with a sexed semen straw; SS-22 = timed AI 22 h after the second GnRH injection with a SS straw.

current study was not designed to test the effect of AI technician, but the results suggest that technician may have an important role to play in the success of SS. The multiple steps in the sorting procedure are damaging to sperm (Vishwanath and Moreno, 2018), and hence it is likely that straw handling procedures, thawing process (including temperature and duration), and duration from thawing to deposition of sperm in the uterus may be more critical for SS compared with CONV. A retrospective analysis of on-farm records in an Australian study (9,870 inseminations on 4,456 heifers) reported that AI technician had a significant effect on P/AI, and the authors suggested that the skill and experience of the AI technician may be more important for SS than for CONV (Healy et al., 2013).

In conclusion, no significant difference in P/AI was observed when cows were inseminated with SS at 16 or 22 h after GnRH, suggesting that 16 to 22 h after GnRH likely encompasses the optimal window for timing of AI with SS. Overall, the relative P/AI was 83.5%, but considerable variation between herds and between AI technicians was observed. It is noteworthy that some herds were able to achieve exceptional results with SS, whereas other herds had relatively poor performance, the reasons for which are currently unknown and warrant further investigation.

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