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Fertility of frozen sex-sorted sperm at 4 x 10^6 sperm per dose in lactating dairy cows in seasonal-calving pasture-based herds

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ABSTRACT

The objective was to evaluate the reproductive performance of frozen sex-sorted sperm at 4 × 10^6 sperm per dose (SexedULTRA 4M, Sexing Technologies, Navasota, TX) relative to frozen conventional sperm in seasonal-calving pasture-based dairy cows. Semen from Holstein-Friesian (n = 8) and Jersey (n = 2) bulls was used. Four of the Holstein bulls used were resident at or near a sex-sorting laboratory (Cogent, UK, or ST Benelux, the Netherlands). The remaining 6 bulls were located at studs in Ireland. For these 6 bulls, ejaculates were collected, diluted with transport medium, and couriered to Cogent in parcel shippers. Transit time from ejaculation to arrival at the sorting laboratory was 6 to 7 h. For all bulls, ejaculates were split and processed to provide frozen conventional sperm (CONV) at 15 × 10^6 sperm per straw and frozen sex-sorted (SS) sperm at 4 × 10^6 sperm per straw and used to inseminate lactating dairy cows after spontaneous estrus. Pregnancy diagnosis was performed by ultrasound scanning (n = 7,246 records available for analysis). Generalized linear mixed models were used to examine effects on pregnancy per AI (P/AI) at first artificial insemination, with sperm treatment (CONV vs. SS), bull (n = 10), and treatment × bull interaction as the fixed effects, and herd (n = 142) as a random effect. Overall, P/AI was greater for cows inseminated with CONV than for those inseminated with SS (59.9% vs. 45.5%; 76.0% relative to CONV). This study was not designed to compare resident bulls vs. shipped ejaculates, but the magnitude of the difference between P/AI achieved by CONV and SS was apparently less for resident bulls (60.3% vs. 50.2%) than for shipped ejaculates (58.6% vs. 40.7%). We discovered a treatment × bull interaction for shipped ejaculates (P/AI ranged from 45 to 86% relative to CONV) but not for the resident bulls (P/AI ranged from 81 to 87% relative to CONV). Relative P/AI of SS compared with CONV was greater in cows with high or average fertility potential (76.1% and 78.3%, respectively) than in cows with low fertility potential (58.1%). In 33.1% of the enrolled herds, the P/AI achieved with SS was 90% or more of the P/AI achieved with CONV; this was mainly explained by herds in which SS performed exceptionally well but CONV performed poorly. In conclusion, SS had lower overall P/AI compared with CONV; however, P/AI achieved with SS was dependent on the bull, fertility potential of the cow, and herd. Strategies to improve the P/AI with SS in seasonal-calving pasture-based lactating dairy cows require further research.

Key words: sex-sorted sperm, pasture-based system, dairy, fertility

INTRODUCTION

In cattle, as in other placental mammals, the female produces oocytes that bear an X chromosome, whereas the male produces sperm that bear an X or a Y chromosome. The sex of the offspring is determined by the sperm that successfully fertilizes the oocyte, because diploid cells carry 2 X chromosomes (XX) in females and 1 X and 1 Y chromosome (XY) in males. Bull ejaculates contain approximately equal numbers of each type of sperm, as has been demonstrated by flow cytometry (Garner et al., 1983), fluorescence in situ hybridization (Di Berardino et al., 2004; Habermann et al., 2005), and PCR (Parati et al., 2006). Therefore, in the absence of differences between X and Y sperm in the ability to reach and fertilize the oocyte (i.e., primary sex ratio) or factors preferentially affecting the survival of embryo and fetuses of one sex over the other, one would expect approximately equal numbers of male and female offspring (i.e., secondary sex ratio) after natural mating or AI with conventional (non-sexed) sperm doses. Indeed, only slight deviations (<2%) from the 50:50 ratio have been reported, with a higher percentage of male calves born from AI than from natural

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mating (Berry and Cromie, 2007), and from AI with frozen compared with fresh sperm (Xu et al., 2000).

The Y chromosome is considerably smaller than the X chromosome (Moruzzi, 1979), which translates to a difference of approximately 4% in terms of DNA content between the X and Y sperm in cattle (Garner et al., 1983). The discovery of this difference in DNA content facilitated the development of sex-sorting by flow cytometry (Garner et al., 2013), which can produce populations of sperm enriched to contain 90% or more X sperm and, consequently, bias the female:male ratio in the offspring to 9:1. Sex-sorted sperm is of particular relevance to the dairy industry to generate female calves for replacements and for herd expansion (Holden and Butler, 2018). In addition, sex-sorted sperm can resolve the issue of unwanted male dairy calves, which have low economic value and may be subjected to poorer management practices than female calves (Renaud et al., 2017; Shivley et al., 2019), leading to increasing concerns regarding their welfare.

Sex-sorting of sperm involves multiple steps (Seidel and Garner, 2002; Garner et al., 2013), several of which can potentially cause cell damage, including dilution (Garner et al., 1997), centrifugation (Shekarriz et al., 1995), DNA staining (Garner and Seidel, 2008), laser exposure (Garner, 2006), and mechanical pressures (Suh et al., 2005). In addition, more than 75% of sperm are typically discarded during the sex-sorting process (Seidel, 2014). As a consequence, commercially available sex-sorted doses contain fewer sperm per straw compared with straws of conventional sperm. Historically, frozen sex-sorted straws typically contained 2×10^6 sperm, whereas frozen conventional straws typically contain 12 to 20×10^6 sperm. The pregnancy per AI (**P/AI**) achieved with frozen sex-sorted sperm has historically been approximately 75 to 80% of that achieved with frozen conventional sperm (reviewed by Butler et al., 2014). It has been hypothesized that the low sperm concentration and cell damage during the sorting and subsequent freezing processes could contribute to this lower fertility (Frijters et al., 2009), although others have reported that increasing the concentration of sex-sorted sperm per straw did not result in a corresponding increase in P/AI (DeJarnette et al., 2011). The technology has continued to evolve from the original XY technology to a newer product, SexedULTRA (Sexing Technologies, Navasota, TX; reviewed by Vishwanath and Moreno, 2018). Xu (2014) reported that New Zealand dairy cows inseminated with fresh SexedULTRA at 1×10^6 sperm per straw had 24-d nonreturn rates greater than 95% of those achieved with fresh conventional sperm. In a large-scale controlled field study in Ireland, frozen SexedULTRA at 2×10^6 sperm per straw achieved P/AI of 84.7% and 86.7% relative to conventional sperm in lactating cows and heifers, respectively (Maicas et al., 2019).

Recent preliminary reports have indicated that P/AI was improved when dairy heifers were inseminated with increasing sperm concentration per straw (Lenz et al., 2016; Utt et al., 2017), leading to the launch of a new product with 4×10^6 sperm per straw (SexedULTRA 4M) in 2017 (Vishwanath and Moreno, 2018). Since the introduction of this product, initial field data have suggested that that the gap in fertility between sex-sorted and conventional sperm has been reduced in lactating dairy cows (Vishwanath and Moreno, 2018). Given the importance of compact calving in a seasonal system, and the significant economic consequences of low fertility, the objective of this study was to evaluate the reproductive performance of frozen-thawed sex-sorted sperm at 4×10^6 sperm per straw (SexedULTRA 4M) compared with conventional frozen sperm at 15×10^6 sperm per straw in seasonal-calving, pasture-based, lactating dairy cows.

MATERIALS AND METHODS

Semen Collection and Transportation

Semen from Holstein-Friesian (n = 8) and Jersey (n = 2) bulls was used in this trial. Jersey bulls were included in the study because of the growing demand for Jersey × Holstein-Friesian crossbred cows in Ireland due to their favorable production, health, fertility, and longevity characteristics. Ejaculates were collected using an artificial vagina at 1 of 4 EU-approved (Directive 88/407/EEC) semen collection centers (National Cattle Breeding Centre, Enfield, Ireland; Dovea Genetics, Thurles, Ireland; Cogent Breeding Ltd., Chester, United Kingdom; and ST Benelux, Deventer, Netherlands). Each ejaculate processed was from a single collection (or jump). Mean number of ejaculates processed per bull was 3.2 (range 2 to 6).

Resident Bulls. Four of the Holstein bulls were resident at or near a sex-sorting laboratory (Cogent, UK, or ST Benelux, Netherlands). Ejaculates from these bulls were collected and immediately delivered to the nearby sex-sorting laboratory for processing.

Shipped Ejaculates. The ejaculates of 6 bulls residing at the 2 Irish AI centers were collected and shipped to the sex-sorting laboratory operated by Cogent Breeding Ltd. (UK) for processing. From Feb. 12 to Mar. 8, 2018, the ejaculates of 3 bulls were collected in one of the Irish AI centers each morning. After collection, ejaculates were kept at room temperature (18 to 20°C) and evaluated under a microscope. The following standards were required for approval: sperm motility 65% or higher, sperm primary defects 15% or less, sperm secondary defects 25% or less, sperm total defects 25% or less, and sperm concentration of 500 × 10^6 cells/mL or higher. Approved ejaculates were gently diluted (1:1) in polypropylene tubes with transport medium (proprietary medium supplied by Sexing Technologies) at room temperature (18 to 20°C), sealed by a veterinarian from the Irish Department of Agriculture, Food and the Marine, inserted in foil pouches, and placed in parcel shippers (Crēdo Cube, Pelican BioThermal, Plymouth, MN). Ejaculates were couriered from the Irish AI centers to the Cogent sex-sorting laboratory, with an average transport time of approximately 6 to 7 h. Although no differences were found in sperm chromatin integrity or in vitro embryo development after X-ray screening of bovine frozen straws at carry-on-luggage exposure doses (Hendricks et al., 2010), long-term genetic and epigenetic effects have not been studied yet. Therefore, an exemption from X-ray screening of the ejaculates during airport security control was provided by the Irish Aviation Authority. The parcel shippers containing the ejaculates were carried in the cabin of the plane to ensure stable temperature and motion conditions. On arrival at the Cogent sex-sorting laboratory, the mean (±SD) temperature of the ejaculates was 11.1 ± 0.8°C (range: 9.8 to 14.4°C).

Semen Processing

Upon arrival at the laboratory, ejaculates were subjected to quality control tests, where sperm concentration, subjective motility, and sperm morphology were assessed. Ejaculates containing fewer than 1 × 10^9 sperm/mL or fewer than 5 × 10^9 total sperm were rejected. Minimum values of 65% motile sperm and 75% morphologically normal sperm were required for processing. Each ejaculate was split into 2 aliquots: 1 to generate frozen sex-sorted (**SS**) sperm (approximately 90% X chromosome–bearing sperm) at 4 × 10^6 sperm per straw using the SexedULTRA Genesis III sorting technology (Sexing Technologies) described by Vishwanath and Moreno (2018), and the other processed as frozen conventional (**CONV**, unsorted) sperm at 15 × 10^6 sperm per straw. Both SS and CONV were packaged in 0.25-mL straws. Media used during the processing of CONV and SS were proprietary media from Sexing Technologies. All sperm batches underwent post-thaw quality control tests, including subjective assessment of sperm motility and acrosome integrity after 3 h of incubation at 35°C. Analysis of purity was also performed for the sex-sorted straws (i.e., X chromosome–bearing sperm population ≥ 87%; Garner et al., 2013; Vishwanath and Moreno, 2018).

Distribution, Insemination, and Recording of Straws

After 30-d quarantine (required by EU directive), AI straws were shipped to Ireland in liquid nitrogen and used to inseminate lactating cows after spontaneous estrus during the first 3 weeks of the breeding season in 142 participating herds. Each herd was allocated up to 60 straws (30 SS and 30 CONV), comprising 12 straws from each of 5 bulls, and these straws were randomly used on cows that were enrolled in the study. The AI technicians and the farmers were blind to the sperm treatment. The participating farmers were instructed to use study straws at the first insemination of the breeding season, in younger cows (parity ≤ 3) that were 60 d or more calved (DIM) and in good body condition (BCS ≥ 3.0).

Each insemination was recorded on a handheld computer and exported to the Irish Cattle Breeding Federation (ICBF) database, which is the national repository for cattle data in Ireland. Insemination data and other animal information, including date of previous calving, parity, economic breeding index (**EBI**), and PTA values for genetic traits of interest were retrieved from the ICBF database. The EBI is a single-figure profit index used in Ireland to identify animals with superior genetic merit (Teagasc, 2014) and is composed of 7 subindexes with different weights (in parentheses): fertility (35%), milk production (33%), calving performance (9%), beef performance (9%), cow maintenance (7%), cow management (4%), and health (3%).

Ultrasound Scanning

Ultrasound scanning for pregnancy diagnosis was performed by 6 experienced veterinarians on the cows enrolled in the study during the breeding season. Pregnancies resulting from the first AI were distinguished from those resulting from later services based on insemination dates and fetal crow-rump length. Body condition score data were recorded at pregnancy diagnosis on 7,238 cows; however, because BCS was recorded by several different veterinarians and the measurement was conducted several weeks after the insemination event, it was not further considered in the analysis.

Statistical Analysis

Only scanned cows with a study straw used at the first AI of the breeding season (n = 7,246) were included in the final analysis. Cow was considered the experimental unit. Generalized linear mixed models (PROC GLIMMIX, SAS 9.4; SAS Institute Inc., Cary, NC) for data with a binary distribution, with residual pseudo-likelihood as estimation technique and containment method for calculation of the degrees of freedom, were used to evaluate the effect of sperm treatment and other variables on P/AI. Several covariates and interactions were tested for inclusion and retained as fixed effects for the final model when $P \leq 0.25$; use of a relaxed P-value cutoff point is recommended (Mickey and Greenland, 1989; Sperandei, 2014) during preselection because it minimizes type II error and is less likely to overlook contributing covariates than lower significance levels for entry such as the traditional $P \leq 0.05$. In every model, comparisons of least squares means (LSM) for P/AI between levels of the categorical variables of interest (e.g., sperm treatment, bulls, quintiles for fertility subindex of EBI, categories of fertility potential) and interactions between them were performed using the Tukey-Kramer adjustment for multiple comparisons. A P-value < 0.05 was considered significant. Results are reported as statistic ± standard error of the statistic (e.g., LSM ± SEM).

Model for the Effect of Sperm Treatment and Other Variables on P/AI. The final model included the following variables as fixed effects: sperm treatment (CONV vs. SS), bull (n = 10), parity, fertility subindex of EBI, DIM at AI, and 2-way interactions between sperm treatment and bull, fertility subindex, and DIM. The model was first run with the fertility subindex of the EBI as a continuous variable and, second, as a categorical variable after being stratified into quintiles (≤28, 29 to 44, 45 to 55, 56 to 69, and > €69). Herd (n = 142) was included as a random effect.

Models for Resident Bulls and Shipped Ejaculates. Bull was confounded with location (i.e., straws were from resident bulls or from shipped ejaculates, and no bull had straws from both locations). As a result, it was not possible to include bull and location in the same model, and 2 separate models were run: one for resident bulls and another for bulls with shipped ejaculates.

Model for the Effect of Fertility Potential on P/AI. To incorporate additional factors known to affect fertility performance, cows were classified into 3 categories: (1) high fertility potential (n = 813): cows that were ≥70 DIM at AI, in parity ≤2, and with an EBI fertility subindex > €60; (2) low fertility potential (n = 718): cows that were <70 DIM at AI, in parity >2, and with an EBI fertility subindex ≤ €60; and (3) average fertility potential (n = 5,715): cows meeting any of the other possible combinations of DIM at AI, parity, and EBI fertility subindex. The final model included the following variables as fixed effects: sperm treatment, bull, fertility potential (high, average, or low), and the 2-way interaction between sperm treatment and bull, and fertility potential. Herd was included as a random effect.

Model for the Effect of Selected Sperm Pack on P/AI. Farmers chose to use sperm packs composed of straws only from Holstein-Friesian bulls (black and white, **BW** pack), or sperm packs composed of straws from both Holstein-Friesian and Jersey bulls (crossbred, **XB** pack). More than 75% of the herds (n = 109) used a BW pack, whereas 33 herds used a XB pack. Therefore, the sperm pack selected affected the bull allocation to herds (i.e., straws from Jersey bulls were used only on the 33 herds that signed up for the XB pack, but straws from Holstein-Friesian bulls were used on all herds). A model was run to evaluate the effect of sperm pack on P/AI. The final model included the following variables as fixed effects: sperm treatment, bull, sperm pack (BW vs. XB), and sperm treatment × sperm pack interaction. Herd was included as a random effect. The bull with the greatest reduction in fertility after sorting was a Jersey (bull 10). As this bull contributed to the greater relative difference between the CONV and SS inseminations for the XB pack, the model was rerun without Jersey bulls (bulls 9 and 10), to test the P/AI of SS relative to CONV on herds designated BW or XB, but using only the bulls that were used in both BW and XB herds.

Model for the Effect of Herd on P/AI. A generalized linear mixed model (PROC GLIMMIX, SAS 9.4) for data with a binary distribution was used to evaluate the effect of herd on P/AI of the scanned cows at first AI. In this case, herd was considered as fixed factor. Herds were ranked in uppermost, middle, and lowest tertiles (groups that contain a third of the samples) defined by P/AI achieved by CONV, by SS, or by relative P/AI [(P/AI for SS ÷ P/AI for CONV) × 100]. Then, herds with relative P/AI >90% were examined.

RESULTS

Characteristics of the Animals Used in the Study

The average number of inseminations per bull was 725 (range 204 to 1,521). The characteristics of the 10 bulls included in the final analysis are summarized in Table 1. An average of 51 cows (range 14 to 61) were inseminated and scanned on each farm. Of the scanned cows (n = 7,238), 52.3% had BCS ≥ 3.00. Of the cows with known parity (n = 6,754), 77.1% were in parity 1, 2, or 3. Of the cows with a known calving date (n = 6,726), 81.8% were ≥60 DIM at the time of AI.

Conventional vs. Sex-Sorted Sperm

The results are presented as P/AI recorded for both the CONV and SS treatments and also as the relative performance of SS compared with CONV [relative P/

Table 1. Characteristics of the bulls used in the study; bulls are sorted according to the pregnancy per AI (P/AI) at first AI in lactating cows obtained in the study for both treatments, conventional sperm (CONV) and sex-sorted sperm (SS), combined[1]

Bull	Location	DOB	Main breed	Number of AI (CONV + SS)
1	R	Jan-16	HO	861 (430 + 431)
2	R	Jan-15	HO	870 (445 + 425)
3	R	Jan-16	HO	974 (477 + 497)
4	R	Jan-15	HO	1,521 (773 + 748)
5	S	Jan-13	HO	723 (379 + 344)
6	S	Feb-15	HO	1,076 (540 + 536)
7	S	Feb-15	HO	254 (131 + 123)
8	S	Feb-15	HO	204 (105 + 99)
9	S	Sep-14	JE	383 (197 + 186)
10	S	Feb-14	JE	380 (189 + 191)
Total				7,246 (3,666 + 3,580)

[1]Frozen CONV at 15 × 106 sperm/dose and frozen SS at 4 × 106 sperm/dose. R = resident bull, S = shipped ejaculate, DOB = date of birth (mo-d), HO = Holstein, JE = Jersey.

AI = (P/AI for SS ÷ P/AI for CONV) × 100]. Overall, sperm treatment had an effect on P/AI ($P < 0.001$). Cows inseminated with CONV had a greater ($P < 0.001$) P/AI than those inseminated with SS (59.9 ± 1.2% vs. 45.5 ± 1.3%). Therefore, the overall relative P/AI was 76.0%. Bull affected P/AI ($P < 0.001$; Figure 1a), with greater variation observed for bulls with shipped ejaculates (range 43.9% to 55.4%) than for resident bulls (range 52.6% to 58.4%). We found an interaction between bull and sperm treatment ($P < 0.01$; Figure 1b), with greater differences between CONV and SS treatments noted for bulls with shipped ejaculates. All 4 resident bulls and 1 of the bulls with shipped ejaculates (bull 5) achieved relative P/AI that exceeded 80%; the other 5 bulls with shipped ejaculates achieved relative P/AI ≤75%. Although P/AI achieved by CONV did not differ ($P > 0.05$) between bulls (range 53.2 to 64.2%), SS from bull 10 achieved lower P/AI (27.5 ± 3.4%; $P < 0.001$) than did SS from bulls 1 to 6. Bull 10 also exhibited the greatest difference ($P < 0.001$) between CONV and SS treatments, a decrease of 34.1 percentage points in P/AI.

The P/AI decreased with increasing parity ($P < 0.05$) but increased with increasing DIM ($P < 0.001$) and with increasing EBI fertility subindex ($P < 0.001$). We discovered no interaction effects ($P > 0.05$). The relative P/AI for EBI fertility subindex quintiles were 73.1, 71.0, 80.1, 77.2, and 75.3% (from lowest to highest fertility subindex quintiles, respectively).

Resident Bulls vs. Bulls with Shipped Ejaculates

Ejaculates from resident bulls and shipped ejaculates accounted for 58.3 and 41.7% of the total number of inseminations, respectively. Both resident bulls and shipped ejaculates had greater P/AI for CONV than for SS (60.3 ± 1.3% vs. 50.2 ± 1.4%, and 58.6 ± 1.8% vs. 40.7 ± 1.8%, respectively; both $P < 0.001$). The relative P/AI was apparently better for resident bulls than for shipped ejaculates (83.3 and 69.5%, respectively). An interaction between sperm treatment and bull was detected for shipped ejaculates ($P < 0.01$) but not for the resident bulls ($P = 0.70$). The interaction between sperm treatment and bull for shipped ejaculates was primarily due to the large divergence in the P/AI achieved for CONV and SS for bull 10 (Figure 1b).

Fertility Potential (High vs. Average vs. Low) of the Cows

Fertility potential had an effect on P/AI ($P < 0.001$). We discovered a tendency for an interaction between fertility potential and sperm treatment ($P = 0.059$). Cows with high and average fertility potentials had greater P/AI than cows with low fertility potential for both CONV (65.3 ± 2.5% and 60.2 ± 1.3%, vs. 52.0 ± 2.8%, respectively; $P < 0.05$) and SS (49.7 ± 2.7% and 47.1 ± 1.4%, vs. 30.2 ± 2.6%, respectively; $P < 0.001$). Therefore, the relative P/AI results were similar for cows with a high or average fertility potential (76.1% and 78.3%, respectively) but markedly poorer for cows with low fertility potential (58.1%).

Selected Sperm Pack (BW vs. XB Pack)

When every bull was included in the analysis, the sperm pack chosen tended to affect P/AI ($P = 0.089$), but an interaction occurred between sperm treatment and sperm pack ($P < 0.05$); when CONV was used, P/AI was greater ($P < 0.05$) with the XB pack than with the BW pack (63.8 ± 2.3% vs. 56.6 ± 1.4%, respectively),

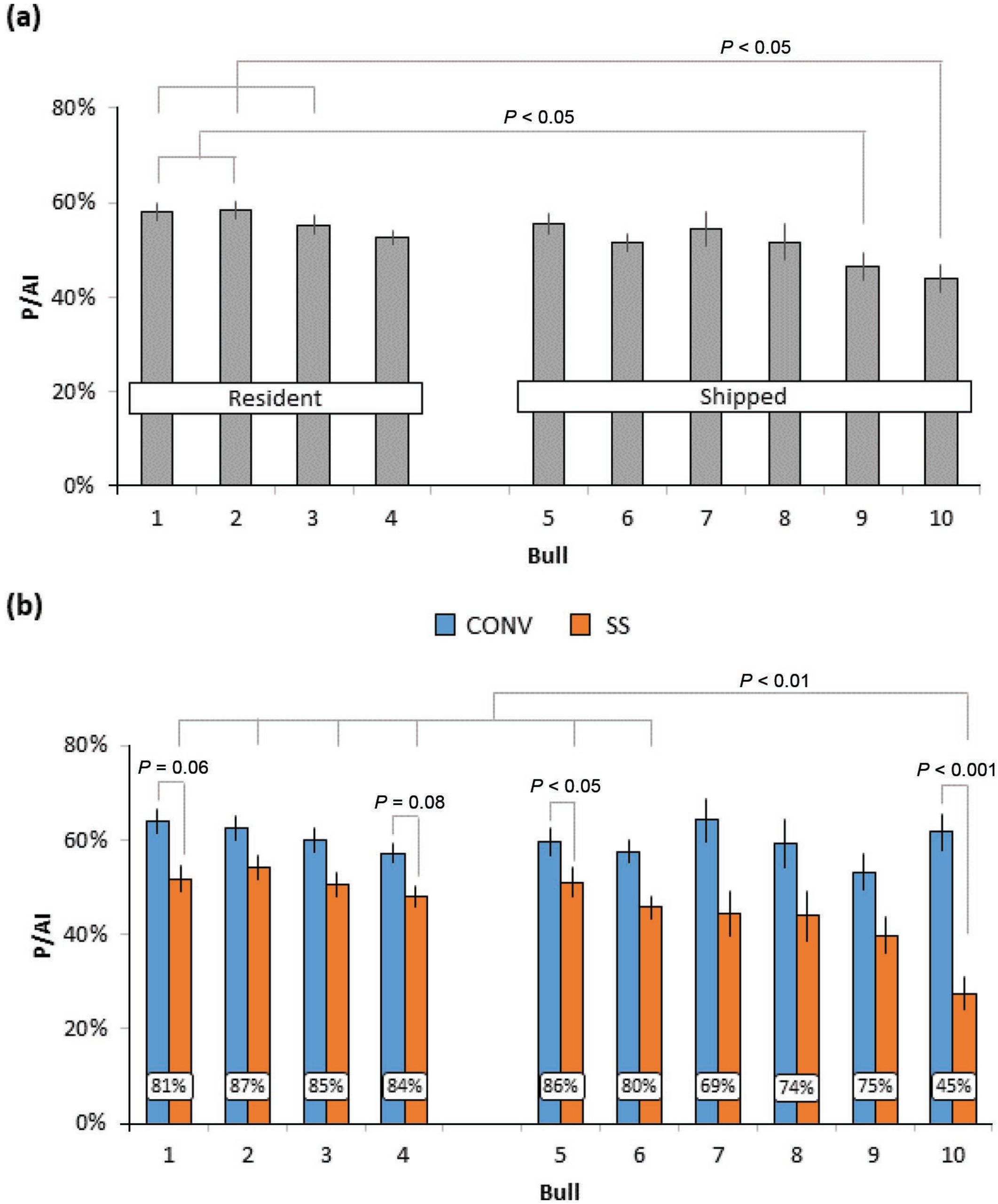

Figure 1. Effect of bull and treatment × bull interaction on pregnancy per AI (P/AI) at first insemination. (a) Mean P/AI per bull; and (b) mean P/AI for each treatment within bull. Data reported as LSM ± SEM (error bars). Bulls were sorted by decreasing P/AI separately for the resident bulls and the shipped ejaculates. Sperm treatments were frozen conventional sperm at 15×10^6 sperm/dose (CONV) and frozen sex-sorted sperm at 4×10^6 sperm/dose (SS). Relative P/AI values [(P/AI for SS ÷ P/AI for CONV) × 100] for each bull are displayed in white boxes at the base of the bars. P/AI achieved by CONV did not differ ($P > 0.05$) between bulls.

whereas there was no difference between packs when SS was used (47.5 ± 2.5% vs. 46.4 ± 1.4%, respectively). Therefore, SS had a lower relative P/AI for XB herds compared with the BW herds (74.3% vs. 81.8%). The 2 Jersey bulls used on the study had the lowest P/AI for sex-sorted sperm (bulls 9 and 10, Figure 1b). When the 2 Jersey bulls were omitted from the analysis, the sperm pack chosen did not affect P/AI (CONV: 63.1 ± 2.7% vs. 58.8 ± 1.3%, $P > 0.05$; SS: 51.4 ± 2.9% vs. 48.9 ± 1.3%, $P > 0.05$; XB vs. BW, respectively), and we found no interaction between sperm treatment and sperm pack ($P > 0.05$). The relative P/AI was similar for XB herds and BW herds (81.5% vs. 83.2%).

Herd-to-Herd Variation in Relative P/AI

Herd had an effect on P/AI ($P < 0.001$); mean herd P/AI ranged from 24% to 79%. Of the 142 herds, 101 (71.1%) had mean P/AI ≥ 50% (for both sperm treatments combined). Mean herd P/AI with the CONV inseminations only explained 7.7% of variation in mean herd P/AI with the SS (Figure 2a). In 47 of the 142 herds (33.1%), the relative P/AI was >90% (Figure 2b). The majority of these herds (70.8%) were in the tertile with the greatest P/AI (i.e., best tertile) for SS, but only 16.7% were also in the tertile with the greatest P/AI for CONV (Figure 2b, pie charts). Hence, herds with relative P/AI > 90% were primarily a reflection of herds that had excellent performance with SS but performed relatively poorly with CONV. Cow characteristics and sperm source for the herds with relative P/AI > 90% were similar to the cow characteristics and sperm source for all herds (Table 2).

DISCUSSION

This study used split ejaculates to compare P/AI at spontaneous estrus in pasture-based dairy cows following insemination with either frozen sex-sorted sperm at 4×10^6 sperm per straw (SexedUltra 4M) or frozen conventional sperm at 15×10^6 sperm per straw. The main findings were: (1) overall, SS achieved 76.0% relative P/AI; (2) the effect of sex-sorting on P/AI was highly variable between bulls, with greater variation among bulls whose ejaculates were shipped to the sorting center compared with resident bulls; (3) SS resulted in greater relative P/AI in cows with high and average fertility potential than in cows with low fertility potential; and (4) the relative P/AI was >90% in a third of the enrolled herds.

Lactating cows inseminated with frozen SexedULTRA product at 2×10^6 sperm per straw had a relative P/AI of 84.7% when compared with fresh conventional sperm in a previous study from our group (Maicas et al., 2019). SexedULTRA 4M (SS in this study) has double the sperm dosage of its predecessor SexedULTRA 2M, as well as optimized medium and process conditions that have been reported to result in improved in vitro sperm quality (González-Marín et al., 2018) and conception rates for both heifers and cows in field data from commercial farms in the United States (Vishwanath and Moreno, 2018). In the current study, P/AI results for both CONV (59.9%) and SS (45.5%) were better than those achieved in our previous study (48.0% vs. 40.6%, respectively; Maicas et al., 2019), but the relative P/AI was not improved, either across all bulls or when the analysis was restricted to resident bulls alone. A preliminary report from Xu et al. (2018) indicated comparable results in a similar field trial with lactating cows in New Zealand (54.2% vs. 40.9% P/AI for CONV vs. SS, respectively; relative P/AI = 75.5%). The effect of increasing the sperm dose from 2 to 4 million cannot be quantified, because none of these recent studies were designed to answer a dose-response question. Nevertheless, Utt et al. (2017) reported that SS at 4 million sperm per dose was superior to SS at 2 million sperm per dose but failed to achieve P/AI equivalent to CONV. The split-ejaculate technique (i.e., SS and CONV derived from the same ejaculate) was used in the current study and in 3 other recent studies (Utt et al., 2017; Xu et al., 2018; Maicas et al., 2019). Collectively, these results demonstrate nonequivalence in the P/AI achieved with SS versus CONV and indicate that this is due either to the sex-sorting process or to the lower final sperm dose, or both, and is not due to ejaculate-to-ejaculate variation.

The detrimental effect of the sex-sorting process varied between bulls, but this was evident only for the bulls with shipped ejaculates. The shipped ejaculates were exempted from X-ray airport controls, transported in insulated parcel shippers using a rapid courier (average transport time approx. 6 to 7 h) to reduce the risk of sperm damage during shipping. Every ejaculate passed minimum pre-sorting and post-thaw quality control criteria. Despite this, the decrease in P/AI due to sex-sorting varied depending on the bull, in agreement with previous studies (Frijters et al., 2009; DeJarnette et al., 2010; Maicas et al., 2019; Thomas et al., 2019). It is unclear from the current study why this was the case, but there are some plausible potential explanations: (1) transport conditions used for the shipped ejaculates (medium, temperature, door-to-door transit time) were not optimal to achieve high fertility performance, especially for sperm that go through the sorting process; (2) it was simply a bull effect, whereby the bulls that had ejaculates shipped had inherently poorer fertility or were inherently less suited to sex-sorting (or both) compared with the resident bulls; or (3) some bulls had a low number of inseminations, and the estimate for P/AI in these bulls is potentially inaccurate.

Bulls can differ in susceptibility to stressing procedures (e.g., incubation time in transport medium, sex-sorting, and freezing), and therefore no conclusions can be drawn from this study about the effect of shipping on P/AI of SS. Nonetheless, our results indicate that sex-sorting and subsequent freezing had a more adverse effect on P/AI for most of the shipped ejaculates than for ejaculates from resident bulls. This may indicate that sperm from certain bulls were more affected by the prolonged incubation time in transport medium, becoming even more susceptible to the detrimental effects of sex-sorting and subsequent freezing. It is note-

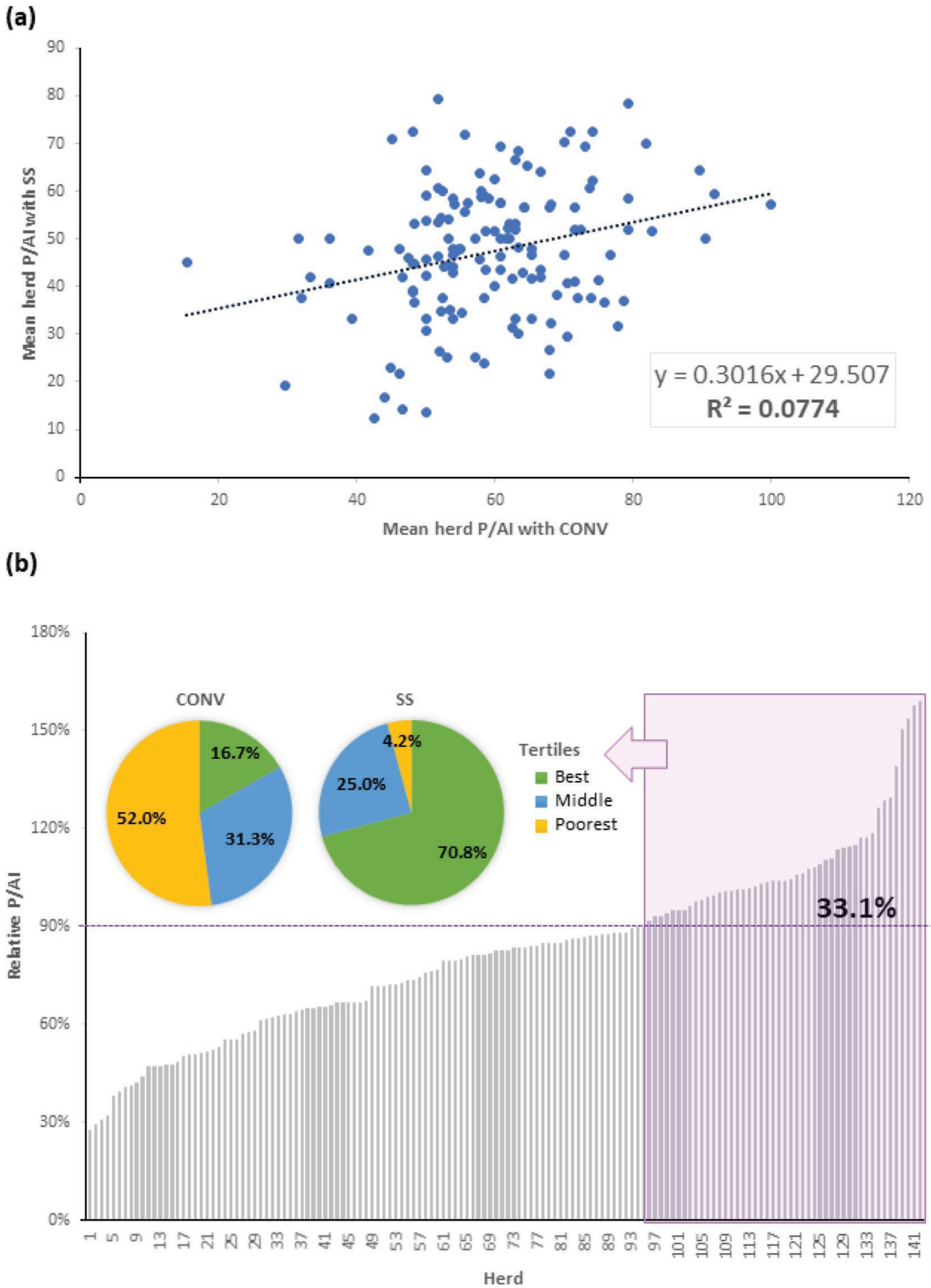

Figure 2. Effect of herd on pregnancy per AI (P/AI). (a) Scatter plot of mean herd P/AI achieved using frozen sex-sorted sperm (SS; y-axis) versus mean herd P/AI achieved via frozen conventional sperm (CONV; x-axis), with regression line and coefficient of determination. (b) Bar chart shows the relative P/AI for each of the 142 participant herds, sorted from lowest to highest. Herds with a relative P/AI >90% are highlighted by the rectangle. Of the herds with relative P/AI >90%, the proportion of cows in the best, middle, and poorest tertiles for P/AI following AI with SS or CONV are depicted in pie charts (top left); most of these herds had excellent performance with SS (70.8% were in the best tertile, shown in green) but relatively poor with CONV [83.3% (52.0 + 31.3%) were in the poorest and middle tertiles, shown in yellow and blue, respectively].

worthy that neither the detrimental effect caused by the incubation period during shipping, nor that caused by incubation periods associated with sex-sorting itself, were detected by quality controls routinely conducted with sex-sorted sperm. However, these controls rely on subjective assessment of sperm motility and acrosome integrity. Premature sperm capacitation-like modifications could be responsible for these detrimental effects (Lu and Seidel, 2004; Bucci et al., 2012; Umezu et al., 2017). The inclusion during the post-thaw quality control of assays monitoring sperm capacitation-like modifications, such as flow cytometric analysis of plasma membrane stability (Merocyanine 540 dye, Molecular Probes, Eugene, OR; Hallap et al., 2006) or image-based flow cytometric analysis of zinc signature (Kerns et al., 2018), may increase the ability to detect and discard affected ejaculates and bulls. In addition, further research on sperm function changes due to sex-sorting could also discover currently unknown differences between conventional and sex-sorted sperm, which ultimately would help to improve routine quality controls.

In addition to the bull effect, cow characteristics and herd management are 2 main factors that could influence the likelihood of pregnancy success when using sex-sorted sperm. Because sex-sorted sperm is expensive and usually has lower fertility than conventional sperm, more profitable breeding strategies can be achieved by targeting its use to genetically superior and more fertile female cattle (i.e., elite heifers and cows). Although use of sex-sorted sperm has traditionally been limited to heifers, more recent studies demonstrated that it can also be successfully used in lactating cows (Xu, 2014; Vishwanath and Moreno, 2018; Xu et al., 2018; Maicas et al., 2019). In a previous study (Maicas et al., 2019), we identified cow characteristics that defined elite cows among those inseminated with fresh or frozen sex-sorted sperm. Those elite cow characteristics were parity ≤ 2, BCS ≥ 3, DIM at AI > 60 d, and EBI fertility subindex $>$ €100. Using a similar approach in the current study, we identified cows of high, average, and low fertility potential. Of note, the values selected for elite cow characteristics were slightly different (parity ≤ 2, ≥ 70 DIM at AI, and EBI fertility subindex $>$ €60) and BCS was not included, reflecting differences in the cow population enrolled in the different studies and a base change in the EBI during the interval between the 2 studies. In agreement with our previous study, SS performed better in cows with high and average fertility potential than in cows with low fertility potential. Moreover, relative P/AI was greater in cows with high or average fertility potential (76.1% and 78.3%, respectively) than in cows with low fertility potential (58.1%). Thus, targeting the use of SS to elite cows based on parity, DIM at AI, and fertility breeding indexes are, once more, highlighted as suitable criteria for identifying lactating cows suitable for insemination with sex-sorted sperm.

Approximately one-third of the enrolled herds had a relative P/AI > 90%, and within this subset, most exceeded 100% (i.e., SS performed better than CONV in those herds). One possible explanation is that the cows in those herds had better reproductive characteristics, which would have benefited P/AI of SS, but a cursory examination of the cow characteristics in these herds does not support that possibility (Table 2). Interestingly, the herds that achieved high relative P/AI primarily reflect herds that had excellent performance with SS but moderate or poor performance with CONV. All of the cows enrolled in the study were identified as being in estrus and nominated for insemination by the farm owner (or farm staff), and all inseminations were conducted by professional AI technicians who were blind to the sperm treatment. It is possible that the decisions regarding identification of cows in estrus and nomination of cows for AI were particularly suited to

Table 2. Characteristics of the lactating cows used in the study from every herd vs. from herds with relative pregnancy per AI (P/AI)[1] greater than 90%[2]

	Every herd (n = 142) % AI from resident bulls = 58.3%				Herds with relative P/AI > 90% (n = 47) % AI from resident bulls = 59.8%			
Characteristic	N	Mean ± SEM	Median ± IQR	Range	N	Mean ± SEM	Median ± IQR	Range
DIM at AI	6,726	78.1 ± 0.2	82 ± 25	(2, 137)	2,128	78.2 ± 0.4	82 ± 24	(2, 132)
BCS	7,238	2.9 ± 0.0	3 ± 0.3	(0.8, 4.5)	2,404	2.9 ± 0.0	2.8 ± 0.3	(0.8, 4.5)
Parity	6,754	2.6 ± 0.0	2 ± 2	(1, 12)	2,136	2.5 ± 0.0	2 ± 2	(1, 10)
EBI (€)	6,659	125 ± 0.6	132 ± 58	(−136, 292)	2,092	126.5 ± 1.0	135 ± 58	(−136, 272)
Fert SI (€)	6,692	48 ± 0.3	50 ± 32	(−133, 175)	2,099	48.8 ± 0.6	50 ± 31	(−133, 170)
Prod SI (€)	6,662	42.7 ± 0.3	45 ± 37	(−98, 137)	2,092	43.8 ± 0.6	46 ± 39	(−67, 135)

[1]Relative P/AI = (SS P/AI ÷ CONV P/AI) × 100. CONV = conventional sperm, SS = sex-sorted sperm.

[2]IQR = interquartile range, EBI = economic breeding index, Fert SI = fertility subindex of the EBI, Prod SI = milk production subindex of the EBI.

sex-sorted sperm on some farms and particularly suited to conventional sperm on other farms (at least for the cows enrolled in the study). Capacitation-like modifications due to sex-sorting may result in reduced lifespan of the sex-sorted sperm, and hence AI closer to the time of ovulation has been proposed as potentially beneficial when using sex-sorted sperm. Indeed, improved P/AI has been reported for delayed AI when sex-sorted sperm was used in conjunction with fixed-time AI (Sales et al., 2011) and activity collars used for automated estrus detection (Bombardelli et al., 2016). This warrants further investigation in seasonally calving pasture-based dairy cows.

CONCLUSIONS

We found that SS achieved a mean relative P/AI of 76.0% compared with CONV, but the relative P/AI was apparently greater for resident bulls (83.3%) compared with shipped ejaculates (69.5%). The P/AI achieved with SS was highly dependent on the bull, herd, and fertility potential of the cow. The likelihood of successful pregnancy establishment when using SS can be increased by targeting use on early-calving young cows with the greatest fertility potential. In addition, the inclusion of an analysis of capacitation-like modifications in the post-sorting quality control may help to detect and discard affected ejaculates and bulls before distribution of the straws. Research is needed on the importance of timing of AI relative to estrus onset or expected time of ovulation with SS in lactating cows managed under seasonal-calving, pasture-based systems.

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