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Effect of insemination-related factors on pregnancy rate using sexed semen in Holstein heifers

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ABSTRACT: The objectives were to determine the effects of insemination with sexed semen at spontaneous and induced estrus, fixed-time insemination at synchronized estrus, the deposition site, estrous intensity, housing, age, body weight, and bull on the pregnancy rate in Holstein heifers, and to compare the quality traits of sexed sperm with those of unsexed semen. The study was conducted on 3206 heifers, housed in three free-stall barns and in four tie-stall facilities. After synchronization by two prostaglandin F2α (PGF2α) treatments 14 days apart, 281 heifers were inseminated conventionally and 118 intracornually with sexed semen, and 532 and 148 heifers, respectively, with unsexed semen 80–82 h after the second treatment. At spontaneous estrus, 1129 heifers were inseminated with sexed and 529 with unsexed semen, and at estrus induced by a single PGF2α treatment 185 heifers were inseminated with sexed and 284 with unsexed semen. Heifers were inseminated conventionally with sexed semen 12 h after detection of estrus, and with unsexed semen according to the a.m.–p.m rule. Sexed and unsexed semen doses from five bulls were evaluated for motility, morphology, membrane integrity, and chromatin stability. Overall pregnancy rate with sexed semen (41.7%) was 80.8% of that with unsexed semen (51.6%) and was lower than with unsexed semen irrespective of the type and intensity of estrus, and deposition site. Insemination at spontaneous estrus resulted in a higher pregnancy rate (53.4%) than at induced (41.9%) or synchronized (44.7%) estrus. Pregnancy rates did not differ after intracornual (44.9%) or conventional insemination (48.4%). Strong estrus resulted in higher pregnancy rate (by 14.4% points) compared to weak estrus. The type of housing, age, and weight of heifers had no effect irrespective of the type of semen. The total, progressive and linear motility, and membrane integrity were lower and proportions of immotile sperm greater, for sexed than for unsexed semen.

Keywords: Holstein; sex-sorted semen; estrus; sperm quality; intracornual insemination; fertility

INTRODUCTION

The invasive nature of flow cytometry exerts detrimental effects on the viability and quality of sex-sorted sperm resulting in lowered conception rates compared with unsexed semen. For that reason heifers are mainly used for insemination with sexed semen, as their fertility potential is higher than that of lactating cows (Schenk et al. 2009; DeJarnette et al. 2011).

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Apart from the small number of sperm per dose and reduced fertility of sex-sorted sperm, there are numerous factors which may affect the success of insemination. According to Hunter and Greve (1998), the decline in conception rates due to small sperm numbers could be avoided by fixed-time intracornual deposition at synchronized estrus, although other relevant studies on sexed semen have reported contradictory results (An et al. 2010). The sorting procedure and subsequent freezing-thawing were found to accelerate the acrosome reaction and capacitation, reducing the lifespan of sperms in the genital tract (Moce et al. 2006), thus timing of insemination with sexed semen relative to ovulation could be more critical compared to unsexed semen (Schenk et al. 2009). Insemination with sexed semen could be carried out 12 h later compared to the recommended insemination time when using unsexed semen (Schenk et al. 2009). The type of estrus (Abdel-Azim 2010) and an evidence and intensity of the estrous signs (DeJarnette et al. 2010) appeared to be related with the pregnancy rate when sexed semen was used. Different results have been reported concerning insemination with sexed semen in the studies that examined the interaction effect of the bull and the type of estrus on the pregnancy rate (Abdel-Azim 2010; Sa Filho et al. 2010). Considering the complex nature of the fertility of semen, estimation of the functional and structural traits of sex-sorted sperm could be useful when evaluating the fertility potential of sexed semen. Although potentially viable sperm are selected by detecting membrane integrity, flow-sorting may cause unrecognizable damage leading to decreased motility (Sartory et al. 2004). Membrane and chromatin integrity damage can be caused by the exposure of sperm to pressure, ultraviolet radiation, and Hoechst/laser interaction (Boe-Hansen et al. 2005; Garner 2006; Gosalvez et al. 2011).

Consequently, further information concerning the effect of the insemination-related factors and those related to the fertility of semen on pregnancy rate is required as it would facilitate the use of sexed semen for insemination in dairy herds.

The objectives of this study were to determine the effects of insemination with sexed semen at spontaneous and induced estrus, fixed-time insemination at synchronized estrus, the deposition site, estrous intensity, housing, age, body weight, and bull on the pregnancy rate in Holstein heifers, and to estimate the structural and functional quality traits of sex-sorted sperm in comparison with those of unsexed semen.

MATERIAL AND METHODS

Animals, semen, and inseminations. The study was conducted on 3206 heifers of the Holstein breed (mean age 478.5 ± 71.0 days, and mean weight 418.0 ± 40.4 kg) from seven dairy herds. The heifers were housed in three free-stall barns and in four individual tie-stall facilities. Commercial frozen semen doses from 10 Holstein bulls, containing flow-sorted 2.1×10^6 X-chromosome bearing sperm, or 15×10^6 unsorted sperm in 0.25 ml straws (Cogent Ltd., Chester, UK and Select Sires Inc., Plain City, USA) were used. Due to the genetic requirements of the on-farm breeding programs, the bulls were not balanced within and across farms. Only first service inseminations with a single dose of sexed or unsexed semen were used.

For insemination at synchronized estrus, 1079 heifers were treated twice with 25 mg of prostaglandin F2α (PGF2α) (Dinolytic®; Pharmacia N.V./S.A., Puurs, Belgium) 14 days apart. Intracornual and conventional inseminations were performed with sexed or unsexed semen, respectively, 80–82 h after the second PGF2α treatment. A total of 281 heifers were inseminated conventionally and 118 intracornually using sexed semen, while 532 heifers were inseminated conventionally and 148 intracornually with unsexed semen. At intracornual insemination, semen was deposited near the tip of the uterine horn, ipsilaterally to the ovary bearing the largest, presumed to be ovulatory follicle, identified by transrectal ultrasonography (HS-1500V, equipped with a 7.5 MHz linear-array transducer; Honda Electronics Co., Toyohashi City, Japan). Prior to insemination, the intensity of estrus was evaluated. The presence of mucous discharge, vulvar edema, hyperemia of vulvar mucosa, and relaxation of the cervix (ease to pass through with a catheter) were recorded. Estrus was considered "strong" if at least three of the signs were well expressed, and catheter passed easily through the cervix. In the absence of at least two of these signs, and if a difficulty passing through the cervix was encountered, the estrus was considered "weak". Evaluations of the estrus, ultrasonography as well as conventional and intracornual inseminations were performed by an experienced veterinarian. The inseminations were performed on all the seven farms, and eight bulls were used.

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A total of 2127 heifers which displayed estrus spontaneously (n = 1658) or following a single treatment with PGF2α (n = 469) were inseminated upon detection of estrus. Observations for the detection of estrus were performed four times a day by on-farm personnel. At spontaneous estrus, 1129 heifers were inseminated with sexed, and 529 with unsexed semen. At induced estrus, detected after a single treatment with PGF2α (Dinolytic®; Pharmacia N.V./S.A.), 185 heifers were inseminated with sexed, and 284 with unsexed semen. Insemination with sexed semen was performed 12 h after detection of spontaneous or induced estrus (Schenk et al. 2009), and unsexed semen was used according to the a.m.–p.m. rule. Prior to insemination, the intensity of estrus was evaluated as described above and recorded. Observations for the detection of estrus and inseminations were carried out by on-farm technicians. The deposition site of both types of semen was the uterine body at both spontaneous and induced estrus. The inseminations were performed on six farms using semen from eight bulls (six of the bulls overlapped with those used for insemination at synchronized estrus).

The pregnancy status of heifers was diagnosed by rectal palpation of the uterus 45–60 days after insemination.

Assessment of sperm quality. The sexed and unsexed semen doses of the same batches from the five bulls that produced a large difference in pregnancy rates for sexed semen (35.2–60.3%) were evaluated for motility, morphology, membrane integrity, and chromatin stability. Unless otherwise specified, all chemicals were purchased from Becton Dickinson (San Jose, USA) and Sigma Aldrich (St. Louis, USA).

The total, progressive, linear and non-linear motility, immotility, average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), wobble (WOB, %; VAP/VCL × 100), straight line velocity (VSL, μm/s), lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) of sperm were assessed using computer assisted semen analysis (CASA, Sperm Vision; Minitüb GmbH & Co., Tiefenbach b. Landshut, Germany). Samples of 3 μl were placed in 20-μl deep disposable four-chamber Leja® slides (IMV, Maple Grove, USA), and about 400 sperm were tracked and assessed at 200× under a phase contrast microscope Olympus CX 31 (Olympus Corp., Tokyo, Japan).

The morphology of sperm was evaluated in air-dried smears stained with Spermac stain (Stain Enterprises Inc., Wellington, USA). A total of 100 sperm cells were counted on duplicate slides at 1000×. The frequencies of sperm with abnormal acrosomes, mid-pieces, head shapes, and coiled or missing tails were recorded as a percentage of the number of sperm evaluated. The membrane integrity was assessed by the hypoosmotic swelling test (HOST). After incubation of 20 μl of unsexed semen and 40 μl (due to the low concentration of sperm) of sexed semen with 0.5 ml of a 150 mOsm/kg hypoosmotic solution for 60 min, 100 sperm were assessed in two replicates by two operators at 1000×. The average percentage of sperm with swollen tails was calculated.

The susceptibility of DNA to acid-induced denaturation was measured using a method that is based on the ability of acridine orange (AO) to metachromatically shift from green (double-stranded DNA) to red (denaturized, single-stranded DNA) fluorescence (Evenson et al. 1980). Denaturation was expressed as a function of α_t ratio of red to red + green (total sperm DNA) fluorescence intensity. In the samples, α_t was calculated for each spermatozoon, and expressed as the percentage of sperm with high α_t values (excess of single-stranded DNA, or DNA fragmentation index (DFI)). Samples were diluted to $1–2 \times 10^6$/ml in TNE buffer (0.01M TRIS, 0.15M NaCl, 1mM EDTA, pH 7.4). After 60 s, 200 μl of semen was mixed with 400 μl of acid-detergent solution (0.15M NaCl, 0.08 N HCl, 0.17% Triton-X100, pH 1.2). After 30 s, the samples were stained with 1.2 ml of the AO solution (0.2M Na_2HPO_4, 1mM EDTA, 0.15M NaCl, 0.1M citric acid, 6 μg/ml AO, pH 6.0; Merck, Kebo Lab, Stockholm, Sweden). The flow-cytometry was started 3 min after the acidic treatment (FACSStar Plus; Becton Dickinson). AO was excited with an argon ion laser Innova 90 (Coherent, Santa Clara, USA) at 488 nm, and running at 200 mW. In association with double-stranded DNA, AO fluorescence is green (530 ± 30 nm, detected with the FL 1 detector), while it is red with single-stranded DNA (> 660 ± 20 nm, detected with the FL 3 detector). Data acquisition was performed by using the CellQuest 3.1 software (Becton Dickinson), and stopped after 10 000 events were recorded. The resulting list-mode files were processed using FCS Express Version 2 (De Novo Software, Thornhill, Canada) for calculation of DFI.

Statistical analysis. As the number of inseminations was different at different factor levels and combinations of levels, the multifactorial generalized linear model with logit link function was used instead of the simple univariate analysis to compare the pregnancy rates. The following factors were considered simultaneously: type of

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semen (sexed and unsexed), deposition site (uterine horn and uterine body), type of estrus (synchronized, spontaneous, and induced), intensity of estrus (strong and weak), bull, type of housing (tie- and free-stall), and the farm nested to housing type. The effect of inseminator was covered by the effect of the farm, while the effects of heifer age and body weight were not considered since the effect of these factors on pregnancy rate was not statistically significant ($P > 0.05$). To determine the interaction effects of the deposition site, type of estrus, intensity of estrus, donor bull, and farm by the type of sperm, the corresponding interaction effects were included in the model. As simultaneous estimation of all the interaction effects was impossible, a separate model was fitted for each interaction. The non-significant effects of deposition site and type of housing were excluded to guarantee the estimability of interactions. In addition to the logistic regression model parameters, the adjusted pregnancy rates and adjusted odds ratios were estimated. For all the factors studied, the base level was that with the lowest pregnancy rate. Modelling was performed using the GLIMMIX procedure of the SAS software (Statistical Analysis System, Version 9.1, 2006).

Semen quality analysis was performed using the data obtained from ten observations (five bulls, two treatments), the pairwise *t*-test was used to test the effect of treatment, and the relationships between sperm quality and pregnancy rate were estimated using the Pearson's correlation analysis. The results were considered to be significant at $P < 0.05$.

RESULTS

Factors affecting pregnancy rate in heifers. The adjusted pregnancy rate for heifers inseminated with sexed semen at spontaneous, PGF2α induced, and synchronized estrus (Table 1) was 9.9% points lower than that with unsexed semen (41.7% and 51.6%, $P < 0.001$, respectively) across the entire experiment.

The adjusted pregnancy rate at spontaneous estrus was 11.5% points higher than that at induced estrus ($P < 0.001$), and by 8.7% points higher compared to that at synchronized estrus ($P < 0.001$). The pregnancy rates obtained at synchronized and induced estrus did not differ ($P = 0.545$). At strong estrus, the adjusted pregnancy rate was by 14.4% points higher than that at weak estrus ($P < 0.001$).

Table 1. Estimates of simultaneously considered multivariate logistic model parameters, adjusted pregnancy rates (aPR), and adjusted odds ratios (aOR)

Effects[1]	No. of heifers	Estimate (± SE)	aPR (%)	aOR	*P*-value
Type of semen					
Unsexed	1493	0.399 ± 0.094	51.6	1.49	< 0.001
Sexed	1713	0	41.7	1	
Deposition site					
Uterine body	2940	0.139 ± 0.173	48.4	1.15	0.423
Uterine horn	266	0	44.9	1	
Type of estrus					
Spontaneous	1658	0.462 ± 0.144	53.4	1.59	< 0.001
Synchronized	1079	0.112 ± 0.186	44.7	1.12	0.545
Induced	469	0	41.9	1	
Intensity of estrus					
Strong	2074	0.584 ± 0.079	53.9	1.79	< 0.001
Weak	1132	0	39.5	1	
Housing					
Tie-stall	2300	0.150 ± 0.119	48.3	1.16	0.208
Free-stall	906	0	44.5	1	

[1]the number of inseminated heifers per farm varied between 59 and 1530, and the adjusted pregnancy rates among tie-stall farms ranged from 39.5 to 63.9% and among free-stall farms from 30.6 to 54.4% ($P < 0.001$); the number of inseminated heifers per bull varied between 100 and 684, and the adjusted pregnancy rates among bulls ranged from 32.1 to 64.0% ($P < 0.001$)

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Table 2. Quality traits of sperm in sexed and unsexed semen across bulls (CDt–HMs)

Traits	Sexed semen						Unsexed semen						*P*-value
	CDt	DDr	ECt	FDx	HMs	means ± SD	CDt	DDr	ECt	FDx	HMs	means ± SD	
Motility (%)													
Total	28.6	26.2	56.3	53.4	64.2	45.7 ± 17.2	42.1	48.5	63.1	79.1	85.4	63.6 ± 18.7	< 0.01
Progressive	17.1	13.9	49.3	41.6	58.2	36.0 ± 19.7	41.1	47.0	56.8	75.2	80.8	60.2 ± 17.3	< 0.01
Linear	11.4	7.6	36.6	34.6	47.8	27.6 ± 17.3	28.6	42.1	50.6	51.7	85.7	51.7 ± 21.1	< 0.01
Non-linear	0	1.5	2.8	4.4	1.5	2.0 ± 1.6	0	0	0	3.5	0	0.7 ± 1.5	< 0.05
Immotility	71.4	73.8	43.7	46.6	35.8	54.3 ± 17.2	57.9	51.5	36.9	20.1	14.6	36.2 ± 18.9	< 0.01
VAP (μm/s)	65.2	62.7	70.4	56.3	69.9	64.9 ± 5.8	55.8	75.0	52.3	43.1	52.1	55.7 ± 11.8	0.22
VCL (μm/s)	126.8	120.8	118.6	94.1	124.0	116.9 ± 13.1	116.0	134.8	84.6	76.2	88.0	99.9 ± 24.6	0.14
WOB (%)	48.0	56.0	62.0	56.0	59.0	56.2 ± 4.7	51.0	52.0	59.0	60.0	56.0	55.6 ± 3.6	0.74
VSL (μm/s)	53.6	48.5	55.9	47.1	60.1	53.0 ± 5.3	47.1	64.2	42.0	35.2	44.7	46.6 ± 10.8	0.33
ALH (μm)	2.8	2.8	2.9	2.6	2.6	2.7 ± 0.1	2.7	3.3	2.2	2.0	2.2	2.5 ± 0.5	0.29
BCF (Hz)	31.3	30.5	29.9	31.0	28.4	30.2 ± 1.2	30.4	31.8	29.7	28.7	29.7	30.0 ± 1.1	0.81
Morphology (%)													
Acrosomal abnormality	2	1	0	0	0	0.6 ± 0.9	2	2	1	2	1	1.6 ± 0.5	< 0.05
Abnormal mid-pieces	0	1	0	0	0	0.2 ± 0.4	0	7	4	1	7	3.6 ± 3.3	0.06
HOST (%)	7.5	13.0	8.5	22.0	17.5	13.7 ± 6.1	49.0	48.5	43.0	62.5	60.0	52.6 ± 8.3	< 0.001
DFI (%)	4.5	4.7	3.8	3.0	4.2	4.0 ± 0.7	2.1	2.5	4.9	3.9	3.7	3.4 ± 1.1	0.44

VAP = average path velocity, VCL = curvilinear velocity, WOB = wobble, VSL = straight line velocity, ALH = lateral head displacement, BCF = beat cross frequency, HOST = hypo-osmotic swelling test, DFI = DNA fragmentation index

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No statistically significant difference in pregnancy rate was found due to deposition site ($P = 0.423$). Also, none of the interaction effects between semen and the deposition site, type of estrus, and intensity of estrus were statistically significant ($P = 0.243$, $P = 0.819$, and $P = 0.979$, respectively), which indicates that the difference in pregnancy rate between sexed and unsexed semen did not depend on other insemination-related factors.

The adjusted pregnancy rates varied between 30.6 and 63.9% among farms ($P < 0.001$). However, there was no statistically significant difference between tie-stall and free-stall farms ($P = 0.208$). Furthermore, the interaction effects between farm and type of semen were not statistically significant ($P = 0.133$), although there were two farms where the adjusted pregnancy rate with sexed semen was even higher than that with unsexed semen.

The adjusted pregnancy rates ranged from 32.1 to 64.0% ($P < 0.001$) among bulls, however the interaction effect of the bull and the type of semen on pregnancy rate was not statistically significant ($P = 0.828$). Other interaction effects between bulls and insemination-related factors were not statistically significant ($P > 0.05$), albeit the type of estrus and bull tended to interact ($P = 0.067$).

The mean age and body weight of heifers that conceived with sexed semen (482.9 days and 415.0 kg) did not differ from those of heifers that did not conceive (470.1 days and 414.4 kg), which was also the case with unsexed semen (483.4 days and 419.9 kg vs 471.8 days and 431.2 kg, respectively; all $P > 0.05$, t-test).

Semen quality traits and the relationship between semen quality and pregnancy rate. Table 2 outlines the quality traits of semen of the five bulls studied. The total, progressive, and linear motility of sexed sperm were lower ($P < 0.01$, all values) than those of unsexed semen, and greater proportions of non-linear and immotile sperm were observed ($P < 0.05$ and $P < 0.01$, respectively). The total motility of sexed sperm tended to correlate positively with pregnancy rate ($r = 0.82$, $P = 0.09$). The proportion of sperm with intact membrane was lower in sexed semen compared to unsexed semen ($P < 0.001$). Chromatin stability tests revealed no differences between sexed and unsexed sperm ($P > 0.05$).

DISCUSSION

The pregnancy rate for sexed semen from this trial is comparable to that previously reported for inseminations at spontaneous (Cerchiaro et al. 2007; Frijters et al. 2009) and synchronized estrus (Seidel and Schenk, 2008; An et al. 2010), but higher than that reported by Bodmer et al. (2005) and Mallory et al. (2013). Intracornual deposition of sexed semen did not prevent decline in pregnancies. This is in agreement with the findings by Seidel and Schenk (2008), whereas An et al. (2010) reported similar pregnancy rates after intracornual deposition of sexed (52.8%) and unsexed semen (59.6%). The sample sizes in that study were possibly too small ($n = 36$ and 47, respectively) to reach significance. Despite the intracornual deposition of sexed semen, the proportion of unfertilized ova in embryo donors was higher than in unsexed controls (Sartory et al. 2004; Peippo et al. 2009). The percentage of transferable embryos from the total embryos and ova recovered was 45% using sexed semen and intracornual deposition, whereas it was 70% using conventional semen, suggesting that sperm numbers were more important than insemination site (Kaimio et al. 2013).

The type of estrus affected pregnancy rates. Despite synchronization of estrus with PGF2α, the interval between the onset of estrus and ovulation can vary from 28 to 61 h depending on the maturity of the dominant follicle (Saumande and Humblot 2005). A combination of variable times of ovulation after synchronized estrus with reduced sexed sperm motility and viability may compromise chances of conception. Induction of estrus by a single PGF2α treatment at a random stage in the estrous cycle may initiate ovulation of immature follicles, and cause higher incidence of fertilization failure (Dorsey et al. 2011). The higher pregnancy rate that was observed for inseminations at spontaneous estrus, compared to that at induced or synchronized estrus, was probably due to the larger number of ovulations that occurred within the time appropriate for sexed sperm. The tendency towards interaction between the bull and the type of estrus is in accordance with the decreased conception rates related to the use of sexed semen for synchronized services, compared to non-synchronized services (Abdel-Azim 2010). With regards to sperm longevity, individual bulls can maintain high fertility rates over a broad range of time relative to ovulation (Dorsey et al. 2011).

Regardless of the type of semen and the type of estrus, pregnancy rate was by 14.4% points higher

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at the presence of strong estrous signs, compared to the absence of signs, whereas Seidel et al. (1999) did not find this tendency when using sexed semen. Mallory et al. (2013) reported pregnancy rate with sexed semen in heifers that displayed estrus after synchronization by 20% points higher compared to those that did not express apparent signs. Unlike strong estrus, weak estrus has been associated with elevated progesterone levels (Schopper et al. 1993) that exert a suppressive effect on the amplitude and the frequency of LH pulses in the pre-ovulatory period (Savio et al. 1993). A shortened duration of estrus in heifers with weak signs in connection with improper timing of insemination can be related to fertilization failures (Yoshida and Nakao 2005).

Results from the studies on the use of conventional (Donovan et al. 2003) and sexed (Cerchiaro et al. 2007) semen indicate that neither age nor weight influence pregnancy rate in heifers. Brickell et al. (2009) reported a 30 kg higher weight of heifers that did not conceive after being served, compared to those that conceived, but no effect of age was found. The failure to conceive has been associated with high growth rates during the rearing period, which may result in physiological immaturity at the first service. The differences in pregnancy rates between farms were greater than those due to the type of semen, housing, and estrous intensity. Abdel-Azim (2010) found that the herds that contributed most to variation in fertility showed less variations between bulls than between technicians. Improper semen handling and inability to maintain sperm viability throughout the insemination process may result in a low conception rate, which can be expressed as "herd effect" (DeJarnette et al. 2011).

Compared to unsorted sperm, the motility traits of sex-sorted sperm were lower across the bulls along with the increase in nonlinearity and immotility. Sperm motility and pregnancy rate that showed a substantial variation between bulls indicate that the response to sorting is individual-specific. Reduced mitochondrial activity was found in oocytes exposed to the dye and laser light (Smith 1993), which could be linked to decreased motility in sex-sorted sperm, as mitochondria produce adenosine triphosphate as a source of energy (Mannella 2000). High dilution rates of semen causing a fall in the concentration of protective seminal lipids and proteins (Maxwell et al. 1997) may lead to greater nonlinearity and immotility. Lipids and phospholipids that are in the form of polyunsaturated fatty acids in the membrane are susceptible to oxidation by reactive oxygen species. Oxidative stress damages membranes and decreases motility in frozen-thawed sperm (Chatterjee and Gagnon 2001). It may occur to a greater extent in sexed semen due to the high susceptibility of sex-sorted sperm to cryopreservation stressors (Garner 2006). A combination of these factors in conjunction with the small semen doses that are currently commonly used, may lead to a sub-optimal functional sperm concentration for some individuals. Frijters et al. (2009) reported the effect of a low dose and sorting on the decline in the 56-day non-return rate for four bulls, and that of just sorting for two bulls.

Hyperactivation is a movement pattern of sperm that is characterized by higher VCL, ALH, and simultaneously lower linearity and WOB (Mortimer 2000). As hyperactivation coincides with sperm capacitation, it can be used to evaluate the capacitational status in the sperm undergoing flow-sorting and freeze-thawing. No signs of hyperactivation were found in the current study except a decline in sexed semen linearity. Several authors postulate that acrosomal responsiveness and hyperactivation processes are not completely tied to each other (Suarez 2008), and that sorting-induced alterations do not necessarily indicate the capacitated status of sperm (Bucci et al. 2012). However, capacitation-like changes in the sperm of some bulls due to sorting and freezing-thawing, reducing fertilizing lifespan (Moce et al. 2006), cannot be excluded.

The damage to the acrosome and the membrane integrity of sperm was found to occur due to mechanical stress during sorting and subsequent freezing-thawing (Sartory et al. 2004; Garner 2006). Across the bulls, the proportion of sperm with damaged membranes was ~39% points higher in sex-selected semen. Carvalho et al. (2010) documented ~20% points more sperm with damaged membranes in sexed samples than in unsexed samples. The differences in pressure and the susceptibility of sperm to cryopreservation-induced damage (Garner 2006; De Graaf et al. 2009) may account for the differences in the frequency of the alterations.

Morphological examination showed quite low differences in the values, with no category exceeding 4%. Since sperm abnormalities and chromatin

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integrity are considered to be interrelated (Enciso et al. 2011), low values for DFI were expected. In the current study, the average value of DFI did not exceed 5%, which seems to be common for bulls of various breeds (Hallap et al. 2005; Christensen et al. 2011). The damage to chromatin integrity has been associated with the exposure of sperm to ultraviolet radiation (Boe-Hansen et al. 2005) and the Hoechst/laser interaction (Gosalvez et al. 2011). Although the average DFI % did not differ between two semen types, increase in the proportion of sperm with DNA fragmentation was reported in three and reduction in two bulls. Reduction in the sperm number with damaged DNA in individual bulls has been reported (Blondin et al. 2009; Gosalvez et al. 2011), suggesting a beneficial effect on semen quality. Gosalvez et al. (2011) documented that two bulls out of ten were more efficient and consistent in resisting to sperm DNA fragmentation, irrespective of the ejaculate, while the others demonstrated higher levels of damage. This can be associated with the advantage of sexed over unsexed semen in one bull (CFDx), that exhibited reduced numbers of sperm with damaged DNA. Despite the similar improvement in another bull (AECt), the fertility of sexed semen from AECt was lower than that of unsexed semen.

CONCLUSION

The pregnancy rates in heifers inseminated with sexed semen were influenced by the bull as well as the type and intensity of estrus. The effect of the deposition site was not significant. Pregnancy rates differed between farms irrespective of the type of estrus, but no effects of tie-stall and free-stall housing, heifer age or body weight were found. As regards sexed semen, insemination 12 h after detection of spontaneous estrus appeared to be more efficient compared to insemination at estrus induced by a single PGF2α treatment, or that at a fixed-time after synchronization by two PGF2α treatments. Evaluation of estrus intensity could be used to maximize the number of pregnancies, especially in regard to inseminations at induced estrus, or those at a fixed time in synchronized estrus. There is no need to deposit sexed semen near the fertilization site as similar efficiency can be achieved by conventional insemination.

Inferior quality of sexed semen compared to unsexed semen can be associated with a lower proportion of progressive and linear motility, higher rate of immotility, and impaired membrane integrity. Assessment of these traits could be indicative of the fertility potential of sexed semen.

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