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The reproductive success of bovine sperm after sex-sorting: a meta-analysis

Sven Reese[1], Miguel Camara Pirez[2], Heather Steele[2] & Sabine Kölle[2]✉

In the three decades since its inception, the sex-sorting technology has progressed significantly. However, field studies report conflicting findings regarding reproductive outcomes. Therefore, we conducted this meta-analysis of all trials published between 1999 and 2021. Non-return rates after 24 or 60 d (NRR 24/60), pregnancy, calving, abortion, and stillbirth rates were compared after AI with sex-sorted vs non-sorted sperm. Additionally, the impact of recent developments in the sex-sorting technology was assessed. Of 860 studies found, 45 studies (72 trials) provided extractable data and were included. Overall, the results of this meta-analysis provided evidence that the NRR 24/60 was diminished by 13%, pregnancy rates were reduced by 23% (25% cows, 21% heifers) and calving rates were reduced by 24% when using sex-sorted sperm. Enhancing the dosage to 4 million sex-sorted sperm/straw (including recent improvements, high vs low dose) as well as using fresh sex-sorted sperm (sorted vs non-sorted) increased pregnancy rate ratios by 7 percentage points. The refinement of the sex-sorting technology after 2015 resulted in a lowered reduction of pregnancy and calving rate of 19% and 23%, respectively. Whereas abortion rates were similar, the stillbirth of male calves was increased by 6.3%.

Sex-sorting of bovine spermatozoa was established through the development of flow cytometric sorting in the late 1980s with the first live calf being born in 1993[1]. In the three decades since the development of sex-sorted sperm, its use has been integrated into many farming systems globally. As both the dairy and beef industries face mounting pressure to increase farm efficiency with less available agricultural land[2], the potential for greater integration across all farming systems of sex-sorted sperm for AI is promising. Furthermore, the increased focus on animal welfare highlights the necessity to reduce the surplus of male calves in the dairy industry[3].

The technology for sex-sorting sperm was developed at the Lawrence Livermore National Laboratory (LLNL, CA) which established the technology for orientation of the sperm enabling precise DNA content recordings[4]. In collaboration with USDA, Oklahoma State University, and LLNL the technology was further developed to precisely determine the DNA content differences between X and Y bearing sperm for cattle, pigs, sheep and rabbits[5]. Maintaining the viability of sperm was achieved by labelling with the dye Hoechst 33342 instead of DAPI[5,6].

The combination of this labelling method and the sorting technology from LLNL at the USDA Beltsville Agricultural research centre led to the establishment of an early sex-sorting protocol. A major breakthrough was the first reported live offspring born using this technology in rabbits in 1989[7]. In 1991, the methodology was patented[8]. Initially, flow cytometry limitations could only accurately resolve 350,000 sperm/hr so that standard insemination doses of bovine cryopreserved sperm of 20×10^6 were not achievable[7]. When field trials revealed that insemination doses ranging from 1×10^6 to 2.5×10^6 sperm achieved sufficient conception rates[9,10] the USDA granted a license to the Colorado State University Research Foundation, under the company XY Inc., to begin the commercialisation of this technology named Beltsville Sperm Sexing Technology. XY Inc. was acquired by Sexing Technologies (Navasota, TX, USA) in 2007[11].

In this sorting process sperm DNA is stained stoichiometrically with Hoechst 33342 before being pumped in a stream passing a laser at specific wavelengths[12]. The Hoechst 33342 stained sperm emit a bright blue fluorescence when illuminated, which is measured by a photomultiplier[13]. Using a crystal vibrator sperm are forced into individual droplets. Opposite charges are applied to droplets containing X or Y bearing sperm. The droplets then pass electrical fields which forces them into streams for collection. Droplets which remain uncharged due to inadequate sperm orientation or sperm death are discarded.

[1]School of Veterinary Medicine, Institute of Veterinary Anatomy, Histology and Embryology, LMU Munich, Munich, Germany. [2]School of Medicine, Health Sciences Centre, University College Dublin (UCD), Dublin, Ireland. ✉email: sabine.koelle@ucd.ie

In the following decades, the technology underwent further improvements. As the low sorting throughput rate was a main barrier to commercial success, the flow cytometric system, the MoFlo™ cytometer, first underwent modifications to its nozzle so that an increased number of sperm were orientated correctly by the fluidic system pressure[13]. Further improvements of the nozzle led to an increased analytic capacity exceeding 20,000 sperm/s and sorting up to 6,000 of each X and Y bearing sperm with 90% accuracy[14]. Reduction in fluidic pressure from 50 to 40 psi resulted in an increased number of recoverable viable sperm[15]. The addition of further photodetectors, (at the angles of 45° and 135° relative to the detector at 0°) enabled to measure diagonally orientated sperm[16]. For improving the accuracy of determining the X and Y bearing sperm gas-based argon ion lasers were replaced by diode-pumped solid-state systems[16]. Moreover, alternative gating systems were implemented resulting in 98% female calves (published in patent US7371317B2, 2008). The most recent improvement was the development of SexedULTRA™ technology which minimizes stress on spermatozoa due to the sorting fluctuations in pH, tonicity, and temperature[17,18]. Moreover, sperm sorted using SexedULTRA™ were recently packaged in doses of 4×10^6 sperm per insemination as compared to 2×10^6 sperm per insemination which was used previously[19].

Overall, the sorting process, which includes mechanical stress, staining with a fluorescent dye and increased handling time, is associated with molecular alterations in sex-sorted bovine sperm. Thus, bull sperm reveal decreased motility and longevity after sorting as well as decreased amounts of acrosome-intact sperm, reduced stability of the plasma membrane, mitochondrial damage, and impaired sperm-oviduct interactions[20–23]. In regard to in vitro embryo production (IVP) the use of sex-sorted bovine sperm has been reported to decrease in vitro embryo production yields[24–30], and to cause aberrant embryo development[31] and phenotypic alterations of calves born[32,33].

To date, numerous field studies have been published regarding the reproductive outcome when using sex-sorted bovine sperm for artificial insemination (AI). The results of these studies are inconclusive. Conception rates using sex-sorted sperm for AI have either been reported to be similar[34,35], or significantly reduced in heifers[9,10,36,37] and lactating cows[18,19,38–41] when using sex-sorted sperm for AI. Further to that, the effects of refinements of the sex-sorting technology such as the introduction of SexedULTRA™[17,18,35], have not been fully elucidated. Thus, this study is the first to perform a comprehensive analysis of the reproductive performance of bovine sex-sorted sperm, covering all studies performed from the beginning of the commercialization until to date, spanning from 1999 to 2021 (22 years). For this aim we set out to perform a meta-analysis on NRR 24/60, conception rate, pregnancy rate, and calving rate as well as on the number of abortions and stillbirths in heifers and cows inseminated with sex-sorted sperm compared to conventional sperm.

Material and methods

Data sources and search strategy. A systemic search of the literature was conducted using Scopus (1999–2021) with the following databases included in the search: Web of Science Core Collection, BIOSIS Citation Index, BIOSIS Previews, Current Contents Connect, Derwent Innovations Index, KCI-Korean Journal Database, MEDLINE (including PubMed), Russian Science Citation Index, SciELO Citation Index. The following search strategy was implemented into Scopus: "cow" OR "cattle" OR "heifer" OR "heifers" OR "bull" OR "bovine" AND "sex-sorted" OR "sexed" OR "sexing" AND "sperm". The reference lists of relevant articles were also searched for eligible studies that were absent from the electronic search. The systematic review and meta-analysis checklist PRISMA were used for this meta-analysis[42]. A total of 3136 results were retrieved following the search. Duplicate studies were excluded leaving a total number of 860 studies.

Inclusion and exclusion criteria. Articles and reviews were included in the meta-analysis if: (1) The research was conducted in a species of domestic cattle (*Bos taurus* or *Bos indicus*). (2) The study conducted the analyses of fertility in vivo. Investigations utilising IVF and embryo transfer were excluded. (3) The sex sorting of sperm was accomplished via flow cytometry. Investigations utilising sexed sperm generated using Percoll density gradient centrifugation or the swim-up procedure were excluded. (4) Cows/heifers were artificially inseminated with sex-sorted sperm and compared to cows/heifers inseminated with conventional sperm. (5) The semen of more than one bull was used for the study. (6) The study numerically compared the reproductive performance of conventional (non-sorted sperm) and sex-sorted sperm (sperm sorted into discreet X or Y populations by flow cytometry) in at least one of the fertility measures of interest. The fertility measures of interest included non-return rates 24/60 (the proportion of females not subsequently rebred within 24 or 60 days following insemination), pregnancy rate (the percentage of cows eligible to become pregnant in a given time frame), calving rate (the percentage of cows eligible to calve within a given time frame), rate of abortions (percentage of non-viable calves produced between 50 and 270 days gestation) and stillbirths (percentage of calves born deceased or died within 24 h after birth). Additionally, timing of pregnancy detection was also evaluated, where early detection of pregnancy referred to pregnancies confirmed before 55 days and late detection of pregnancy referred to pregnancies confirmed on day 56 or later. Following these criteria, a total of 45 studies with 72 trials were included in the meta-analysis.

Data extraction. For each study, which passed the inclusion criteria, the following categorical information was extracted: first author's name, year of publication, study population (breed of cattle used, type of use (dairy or beef), reproductive age (heifers or cows), herd management (insemination during natural estrus or after synchronization), as well as the amount and type of semen used for insemination (fresh or frozen, less or more than 2.5 million sex-sorted sperm per straw). The following numerical data were extracted from the 72 trials where available: number of inseminated animals, non-return rate (NRR, 24 or 60 days after insemination), pregnancy rate, calving rates, occurrence of abortions and stillbirths including the discrimination between male and female calves.

Statistical analyses. The meta-analysis was performed by using the software Review Manager (RevMan), Version 5.4.1, The Cochrane Collaboration 2020. MedCalc® Statistical Software version 19.6.4 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021) was also used. For additional statistical analyses IBM SPSS 26.0 was used. In a first step, the data were checked for publication bias by analysing the asymmetry of funnel plots according to Sterne and Egger (2001)[43] and by applying the Begg's test. According to Hooijmans et al. (2014)[44] a random-effects model was chosen because of the high heterogeneity of the trials included in the meta-analysis. Heterogeneity I^2 was mostly more than 50% up to more than 75% which means a substantial and considerable heterogeneity, respectively[45]. For the comparison of the non-return rates (NRR24/60), pregnancy rates (PR), calving rates (CR) the impact of sex-sorting was estimated as the relative effect measures rate ratio (RR). According to Deeks and Higgins (2010)[46] we used the DerSimonian and Laird random-effects model based on the Mantel–Haenszel methods for combining results across studies with additional weighting of each study effect. For comparison of the abortion rate and stillbirth rate, the odds ratio (OR) was calculated. The reliability of the effective measures was described by the 95% confidence interval (CI 95). In order to estimate the between-study variance tau^2 was calculated[47,48]. The significance of the effective measures was tested using the Cochran–Mantel–Haenszel test (CMH). To quantify the heterogeneity of the effective measures, the I^2 Index was calculated and tested for significance using the chi-square test. In view of the large heterogeneity, subgroup analyses were performed using the chi-square test. The subgroup analyses included the effects of the type of cow (dairy/beef), of the reproductive age (heifer/cow), of the type of semen (fresh/frozen), of the sperm dosage (more or less than 2.5 million sex-sorted sperm per straw), of the ULTRA sexing technology, and of herd management (insemination during natural estrus or after synchronization). Further to that, the reliability of early and late detection of pregnancy, as well as of rectal palpation and sonography, and the relationship between geographical distribution and pregnancy rate were compared by using the Cochran–Mantel–Haenszel test. The Bonferroni-Holm correction for multiple comparisons was used for pairwise comparison of the pregnancy rate ratios in geographical regions. Subgroup analyses were not performed if the total number of trials was smaller than 10. For comparison of non-return rates, pregnancy rates, calving rate, stillbirth, and abortion rates a t-test was used after the normal distribution had been confirmed by a Shapiro–Wilk test. If the data were not normally distributed, the Mann–Whitney *U* test was applied. Differences in the variance were tested for significance by using the Levene's test. The results of the meta-analysis were visualized as forest plots. The Spearman's rank correlation (Spearman *rho*) was used to analyse the rank correlation between publication year and the effective measures. If p was < 0.05, results were considered significant.

Results

Systematic search, selection, and data extraction. The electronic search of Scopus returned 3,136 hits, with an additional 10 identified through the screening of reference lists. Following the removal of duplicates, 860 studies were assessed for eligibility under the inclusion criteria. After reading the titles and abstracts, 75 studies were found to directly compare conventional and sexed sperm in at least one of the fertility measures of interest. Following the inclusion criteria, 72 trials across 45 studies were eligible for inclusion in the meta-analysis (Fig. 1). Table 1 provides the descriptive data of all studies for the meta-analysis highlighting the characteristics and reproductive outcome for each trial.

Non-return rates (NRR 24/60). The NRR 24/60 was investigated in 6 trials in 3 publications (Fig. 2). The NNR 24/60 was significantly reduced from 70.7% (CI 95: 66.1–75.3 54.7–68.7) to 61.7% (CI 95: 54.7–68.7) ($p < 0.001$, Cochran–Mantel–Haenszel test) when using sex-sorted sperm for insemination compared to conventional sperm. The rate ratio was 0.87 (CI 95:0.81–0.94) indicating a 13% reduction in the occurrence of a successful early pregnancy 24 – 60 days after AI with sex-sorted sperm as compared to 24–60 days after AI with conventional sperm ($p < 0.001$, Cochran–Mantel–Haenszel test, Fig. 2). There was a statistically significant heterogeneity between trials (I^2: 98%, tau^2: 0.01, $p < 0.001$, chi-square test).

Pregnancy rates. Pregnancy rates were investigated in 67 different trials. The overall pregnancy rates in all cows were significantly reduced from 56.1% to 43.9% when using sex-sorted sperm ($p < 0.001$, Cochran–Mantel–Haenszel test, Table 2). The rate ratio was 0.77 pointing to a reduction of 23% in pregnancy rates after sex-sorting (CI 95: 0.75–0.8, $p < 0.001$, Cochran–Mantel–Haenszel test, Table 2, Fig. 3). The heterogeneity was statistically significant ($I^2 = 93\%$, $tau^2 = 0.01$, $p < 0.001$, chi-square test) confirming the use of a random-effects model and pointing to the necessity of subgroup analyses.

When comparing dairy and beef cows, pregnancy rates were significantly higher (6.7–8.5 percentage points) in beef cows compared to dairy cows irrespective of using conventional or sex-sorted sperm ($p = 0.024$ and $p = 0.009$, respectively, t-test, Table 2). Overall, the negative effect of sex-sorting on pregnancy rates was not significantly different in dairy and beef cows ($p = 0.450$, Cochran–Mantel–Haenszel test, Table 2).

When comparing heifers and cows, both in heifers and cows pregnancy rates were significantly reduced ($p < 0.001$ and $p < 0.001$, respectively, Cochran–Mantel–Haenszel test) when using sex-sorted sperm. In heifers, the pregnancy rate was reduced from 60.2% (CI 95: 58.4–62.6) to 48.4% (CI 95: 45.0–51.8, Table 2). The RR was 0.79 (CI 95: 0.75–0.82) pointing to a reduction in pregnancy rates of 21% in heifers. In cows, pregnancy rates were reduced from 46.2% (CI 95: 39.3–53.2) to 34.4% (CI 95: 28.8–39.9), The RR was 0.75 (CI 95: 0.72–0.80) indicating a decrease of pregnancy rates of 25% in cows. The heterogeneity was higher in heifers than in cows (I^2: 93%, $p < 0.001$ in heifers, I^2: 81%, $p < 0.001$ in cows, tau^2: 0.01, chi-square test, Table 2). The subgroup comparison between heifers and cows revealed that the negative impact of sperm sexing on pregnancy rate was similar in cows and heifers ($p = 0.240$, Cochran–Mantel–Haenszel test, Table 2).

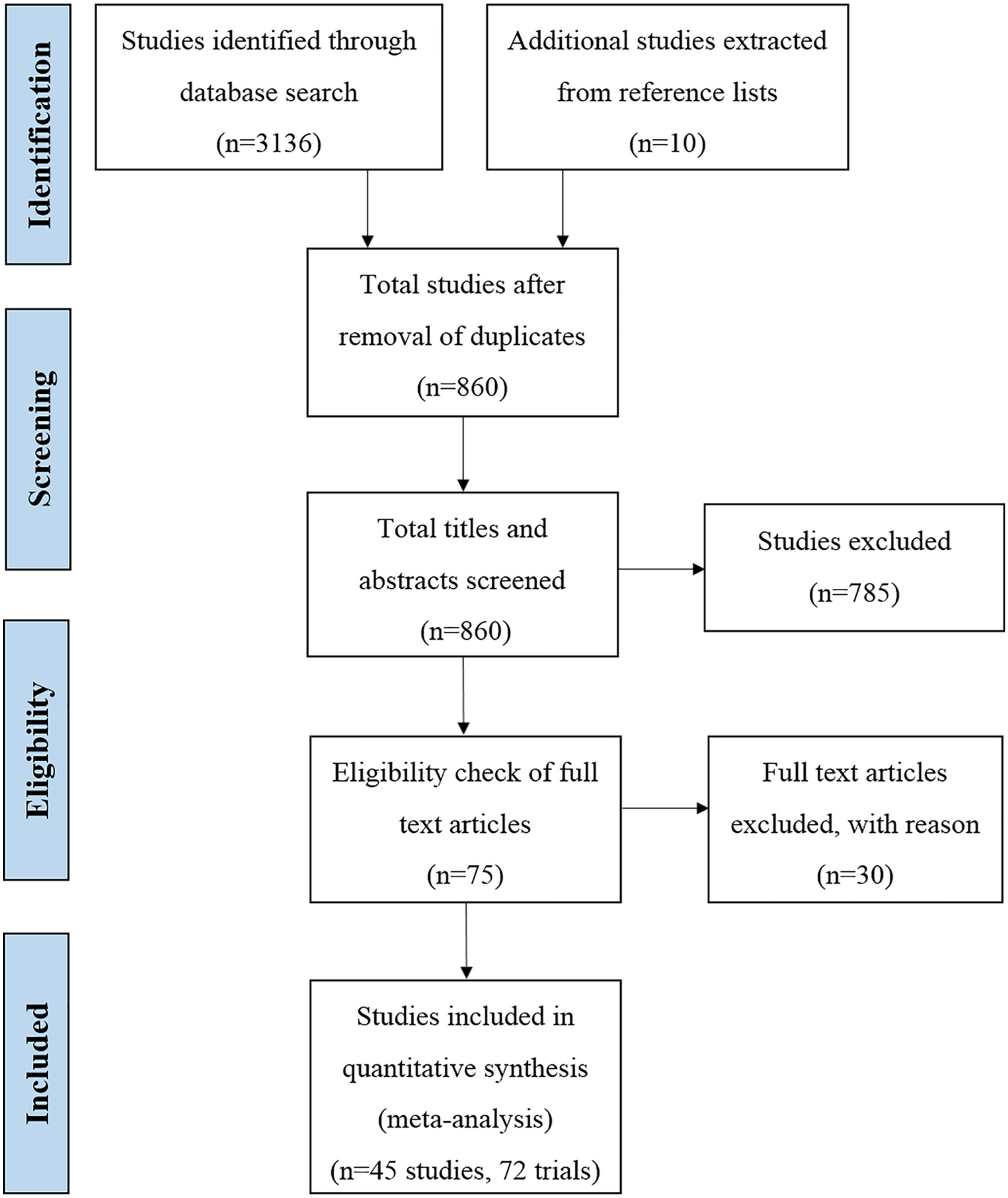


Figure 1. Flow diagram of search and selection strategy in the systematic review and meta-analysis of the reproductive success of bovine semen after sex-sorting.

The reproductive success of frozen and fresh semen was analysed in 49 and 7 different trials, respectively. Pregnancy rates after sex-sorting were significantly decreased after freezing and thawing ($p = 0.008$, Cochran–Mantel–Haenszel test). The rate ratio was 0.77 (CI 95: 0.75–0.80) for frozen semen and 0.84 (CI 95: 0.80–0.88) for fresh semen indicating a decrease of 7 percentage points in pregnancy rate ratios when using frozen semen (Table 2). The heterogeneity was high in frozen sperm (I^2: 77%, tau^2: 0.01, $p < 0.001$, chi-square test, Table 2) and low in fresh sperm (I^2: 12%, tau^2: 0.01, $p = 0.340$, chi-square test, Table 2).

The effects of an increased sperm dosage (mostly 4 million sperm/straw) were investigated in 16 trials whereas 44 trials used a sperm concentration of 2.5 million or less. Overall, the increase of sperm dosage resulted in significantly higher pregnancy rates both in conventional sperm ($p = 0.004$, t-test) and in sex-sorted sperm ($p < 0.001$, t-test, Table 2). The increase in pregnancy rates was 14.1 percentage points (PR: 41.4% and 55.5% for the increased concentration) in sex-sorted sperm and 8.3 percentage points in conventional sperm (PR 55.2% and 63.5%, for the increased concentration). For 2.5 million sperm per straw the RR was 0.76 (CI 95: 0.73–0.79)

Publications	Population [cow/heifer (breed), inseminations management)]	Semen	Outcome (%)	
			Conventional	Sexed
1) Abdalla et al. 2014[49]	Heifers (H), $n_c = 325$, $n_s = 426$, *E*	FZ	PR 62, CR 51, AR 11	PR 34, CR 29, AR 8
2) An et al. 2010[50]	Heifers (H), $n_c = 26$, $n_s = 36$, *E*	FZ	PR 58	PR 53
3) Andersson et al. 2006[41]	Cows (HF), $n_c = 149$, $n_s = 157$, *E*	FZ	PR 46, CR 44, SBR 5	PR 21, CR 20, SBR 6
4) Bodmer et al. 2005a[51]	Cows (BS + RH), $n_c = 64$, $n_s = 105$, *E*	FZ	PR 28, CR 25, AR 6	PR 28, CR 22, AR 17
5) Bodmer et al. 2005b[51]	Heifers (BS + RH), $n_c = 27$, $n_s = 27$, *E*	FZ	PR 59, CR 58, AR 0	PR 33, CR 30, AR 11
6) Borchersen et al. 2009a[52]	Heifers (DRD), $n_c = 153$, $n_s = 530$, *E*	FZ	NRR 76, PR 65, CR 63, SBR 5, SBR-M 12, SBR-F 0,	NRR 67, PR 60, CR 56, SBR 6, SBR-M 21, SBR-F 4
7) Borchersen et al. 2009b[52]	Heifers (H), $n_c = 181$, $n_s = 554$, *E*	FZ	NRR 74, PR 62, CR 57, SBR 16, SBR-M 20, SBR-F 12	NRR 59, PR 49, CR 46, SBR 10, SBR-M 10, SBR-F 10
8) Borchersen et al. 2009c[52]	Heifers (J), $n_c = 165$, $n_s = 504$, *E*	FZ	NRR 69, PR 54, CR 50, AR 7, SBR 2, SBR-M 2, SBR-F 2	NRR 56, PR 47, CR 42, AR 11, SBR 3, SBR-M 7, SBR-F 7
9) Chebel et al. 2010[53]	Heifers (H), $n_c = 1028$, $n_s = 343$, *S*	ND	PR 52, CR 38, AR 27, SBR 3, SBR-M 5, SBR-F 1	PR 40, CR 27, AR 34, SBR 9, SBR-M 15, SBR-F 8
10) Chebel et al. 2020[54]	Heifers (H), $n_c = 390$, $n_s = 415$, *S*	ND	PR 67, CR 57, AR 15, SBR 9, SBR-M 20, SBR-F 3	PR 45, CR 40, AR 11, SBR 5, SBR-M 6, SBR-F 0
11) Colazo et al. 2017[55]	Heifers (H), $n_c = 107$, $n_s = 117$, *S*	FZ	PR 69, CR 64, AR 7	PR 64, CR 62, AR 3
12) Cooke et al. 2014[56]	Heifers and cows (HAC), $n_c = 454$, $n_s = 439$, *S*	FZ	PR 56	PR 34
13) Crites et al. 2018[35]	Heifers and cows (ND), $n_c = 201$, $n_s = 193$, *S*	US	PR 57	PR 49
14) Dawod and Elbaz 2020[36]	Heifers (H), $n_c = 122$, $n_s = 346$, *S*	US	PR 61	PR 51
15) DeJarnette et al. 2009[57]	Heifers (H), $n_c = 53\,718$, $n_s = 39\,763$, *E*	FZ	PR 56, SBR-M 13, SBR-F 11	PR 45, SBR-M 21, SBR-F 9
16) DeJarnette et al. 2010a[58]	Heifers (H), $n_c = 2\,089$, $n_s = 2\,089$, *E*	FZ	PR 61	PR 44
17) DeJarnette et al. 2010b[58]	Cows (H), $n_c = 1\,822$, $n_s = 1\,822$, *E*	FZ	PR 32	PR 23
18) DeJarnette et al. 2011[59]	Heifers (H), $n_c = 2\,292$, $n_s = 2\,319$, *E* or *S*	FZ	PR 60	PR 38
19) Djedovic et al. 2016[60]	Heifers (BPL), $n_c = 2\,115$, $n_s = 1\,205$, *E*	FZ	PR 55, CR 52, SBR 7	PR 44, CR 41, SBR 8
20) Dominguez et al. 2012[61]	Heifers and cows (N), $n_c = 325$, $n_s = 338$, *S*	FZ	PR 58	PR 39
21) Drake et al. 2020[39]	Heifers and cows (HF + J), $n_c = 722$ ns = 1 442, *S*	US	PR 62	PR 51
22) Duarte et al. 2007a[62]	Heifers (N), $n_c = 83$, $n_s = 61$, *E*	FZ	PR 70	PR 67
23) Duarte et al. 2007b[62]	Heifers (N), $n_c = 103$, $n_s = 180$, *S*	FZ	PR 50	PR 46
24) Frijters et al. 2009[63]	ND, $n_c = 64\,985$, $n_s = 2\,036$, *ND*	ND	NRR 66	NRR 53
25) Healy et al. 2013[64]	Heifers (H), ND, *S*	ND	AR 6, SBR 12, SBR-M 14, SBR-F 9	AR 6, SBR 13, SBR-M 16, SBR-F 13
26) Holden et al. 2017[65]	Heifers and cows (ND), $n_c = 39\,366$, $n_s = 1\,486$, *ND*	FZ	PR 54	PR 48
27) Joezy-Shekalgorabi et al. 2017[66]	Heifers (H), $n_c = 2\,419$, $n_s = 1\,154$, *E*	ND	PR 64, CR 60, AR 6, SBR 5, SBR-M 5, SBR-F 4	PR 48, CR 43, AR 11, SBR 5,SBR-M 6, SBR-F 5
28) Karakaya et al. 2014a[40]	Heifers (HF), $n_c = 66$, $n_s = 60$, *S*	FZ	PR 53	PR 42
29) Karakaya et al. 2014b[40]	Cows (HF), $n_c = 88$, $n_s = 88$, *S*	FZ	PR 32	PR 25
30) Ketchum et al. 2021[67]	Heifers (A), $n_c = 404$, $n_s = 390$, *S*	US	PR 59	PR 48
31) Klinc et al. 2007[68]	Heifers (HF), $n_c = 24$, $n_s = 22$, *E*	F	PR 67	PR 55
32) Kurykin et al. 2016[69]	Heifers (H), $n_c = 1\,493$, $n_s = 1\,713$, *E* or *S*	FZ	PR 52	PR 42
33) Lenz et al. 2016[34]	ND, $n_c = 62\,398$, $n_s = 1\,890$, *ND*	US	NRR 66	NRR 67
34) Maicas et al. 2019 SS-1M[37]	Cows (HF), $n_c = 1\,593$, $n_s = 1\,299$, *E*	F	PR 48	PR 38
35) Maicas et al. 2019 SS-2M[37]	Cows (HF), $n_c = 1\,593$, $n_s = 1\,428$, *E*	F	PR 48	PR 39
36) Maicas et al. 2019 SS-FRZ[37]	Cows (HF), $n_c = 1\,593$, $n_s = 1\,173$, *E*	US	PR 48	PR 41
37) Maicas et al. 2019 SS-1M[37]	Heifers (HF), $n_c = 865$, $n_s = 811$, *E*	F	PR 61	PR 54
38) Maicas et al. 2019 SS-2M[37]	Heifers (HF), $n_c = 865$, $n_s = 726$, *E*	F	PR 61	PR 53
39) Maicas et al. 2019 SS-FRZ[37]	Heifers (HF), $n_c = 865$, $n_s = 812$, *E*	US	PR 61	PR 53
40) Maicas et al. 2020[19]	Cows (ND), $n_c = 3\,666$, $n_s = 3\,580$, *E*	US	PR 60	PR 46
41) Mallory et al. 2013[70]	Heifers (H), $n_c = 120$, $n_s = 120$, *S*	ND	PR 68	PR 38
42) Mellado et al. 2010[71]	Heifers and cows (HGC), $n_c = 426$, $n_s = 223$, *E* or *S*	FZ	PR 38	PR 23
43) Mellado et al. 2014a[72]	Heifers (H), $n_c = 6\,816$, $n_s = 15\,497$, *S*	ND	PR 52	PR 42
44) Mellado et al. 2014b[72]	Cows (H), $n_c = 28\,779$, $n_s = 13\,574$, *S*	ND	PR 24	PR 17
45) Naniwa et al. 2017a[73]	Heifers (H), $n_c = 219$, $n_s = 524$, *ND*	ND	PR 58	PR 46
46) Naniwa et al. 2017b[73]	Cows (H), $n_c = 65$, $n_s = 214$, *ND*	ND	PR 40	PR 34
47) Noonan et al. 2016[74]	Heifers (H), $n_c = 398$, $n_s = 379$, *S*	FZ	PR 60	PR 46
48) Norman et al. 2010a[75]	Heifers (H), $n_c = 1\,171\,188$, $n_s = 128\,702$, *ND*	ND	PR 56 SBR 10, SBR-M 11, SBR-F 10	PR 39, SBR 11, SBR-M 16, SBR-F 11
Continued				

Publications	Population [cow/heifer (breed), inseminations management)]	Semen	Outcome (%) Conventional	Sexed
49) Norman et al. 2010b[75]	Cows (H), n_c = 10 784 793, n_s = 25 910, *ND*	ND	PR 30, SBR 4, SBR-M 4, SBR-F 4	PR 25, SBR 3, SBR-M 3, SBR-F 3
50) Sá Filho et al. 2012[76]	Cows (N), n_c = 245, n_s = 246, *S*	FZ	PR 55	PR 46
51) Sales et al. 2011a[77]	Heifers (J), n_c = 112, n_s = 102, *S*	FZ	PR 52	PR 31
52) Sales et al. 2011b[77]	Cows (N), n_c = 193, n_s = 196, *S*	FZ	PR 52	PR 42
53) Schenk et al. 2009[78]	Cows (H), n_c = 58, n_s = 57, *S*	FZ	PR 55	PR 40
54) Schenk et al. 2009[78]	Cows (H), n_c = 713, n_s = 708, *S*	FZ	PR 38	PR 25
55) Seidel et al. 1999a[79]	Heifers (H), n_c = 118, n_s = 114, *S*	FZ	PR 74, CR 69, AR 6	PR 51, CR 46, AR 9
56) Seidel et al. 1999b[79]	Heifers (HAC), n_c = 35, n_s = 86, *S*	FZ	PR 51, CR 51, AR 0	PR 40, CR 40, AR 0
57) Seidel et al. 1999c[79]	Heifers (RA), n_c = 30, n_s = 14, *S*	FZ	PR 70	PR 86
58) Seidel et al. 1999d[79]	Heifers (A), n_c = 28, n_s = 45, *S*	F	PR 54, CR 32, AR 40	PR 44, CR 42, AR 5
59) Seidel et al. 1999e[79]	Heifers (AC), n_c = 58, n_s = 51, *S*	F	PR 47, AR 11	PR 33, AR 6
60) Seidel et al. 1999f[79]	Heifers (A), n_c = 37, n_s = 35, *S*	FZ	PR 73, CR 73, AR 0	PR 51, CR 51, AR 0
61) Seidel et al. 1999g[79]	Heifers (A), n_c = 35, n_s = 43, *S*	FZ	PR 57	PR 53
62) Seidel et al. 2008a[80]	Heifers (H), n_c = 263, n_s = 288, *E*	FZ	PR 62	PR 43
63) Seidel et al. 2008b[80]	Heifers (A), n_c = 126, n_s = 123, *S*	FZ	PR 67	PR 54
64) Seidel et al. 2008c[80]	Heifers (A), n_c = 40, n_s = 38, *S*	FZ	PR 73, CR 68, AR 7	PR 47, CR 42, AR 11
65) Seidel et al. 2008d[80]	Heifers (H), n_c = 124, n_s = 121, *S*	FZ	PR 60, AR 8	PR 47, AR 7
66) Seidel et al. 2008e[80]	Cows (A), n_c = 21, n_s = 42, *S*	FZ	PR 76, CR 71, AR 6	PR 57, CR 55, AR 4
67) Seidel et al. 2008f[80]	Heifers (RA), n_c = 30, n_s = 30, *S*	FZ	PR 70	PR 80
68) Thomas et al. 2014[81]	Cows (ND), n_c = 429, n_s = 422, *S*	FZ	PR 56	PR 26
69) Thomas et al. 2017[82]	Heifers (ND), n_c = 218, n_s = 217, *S*	US	PR 60	PR 52
70) Thomas et al. 2019[18]	Cows (ND), n_c = 812, n_s = 808, *S*	US	PR 65	PR 48
71) Tubman et al. 2004[83]	Heifers and cows (A + H), n_c = 787, n_s = 1 158, *S*	FZ + F	AR 5, SBR 4	AR 4, SBR 4
72) Xu 2014[84]	Cows (HF), nc = 57 085, n_s = 51 712, *E*	FZ	NRR 73, CR 53	NRR 69, CR 50

Table 1. Descriptive data of trials included in meta-analysis. Semen: FZ: Frozen. F Fresh, US UltraSexed (frozen). ND: not determined.n_c: number of inseminations with conventional sperm, n_s: number of inseminations with sex-sorted sperm, Breeds: (A) Angus. (AC) Angus crossbreed. (BPL) Black Pied Lowland. (BS) Brown Swiss. (DRD) Danish Red Dairy. (H) Holstein. (HAC) Herford-Angus crossbreed. (HGC) Holstein-Gyr crossbreed. (HF) Holstein Friesian. (J) Jersey. (N) Nelore. (RA) Red Angus. (RH) Red Holstein. Management: *E* natural estrus, *S* synchronized. Outcome: PR Pregnancy rate (pregnancies/insemination), CR calving rate (births/insemination), AR Abortion rate (abortions/pregnancy), NRR Non-return rate 24–60 days after insemination, SBR Stillbirth rate (stillbirths/birth), SBR-M Stillbirth rate in male calves, SBR-F Stillbirth rate in female calves.

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Risk Ratio M-H, Random, 95% CI	Year
Borchersen et al. a 2009	1423	2136	4395	5750	16.8%	0.87 [0.84, 0.90]	2009
Borchersen et al. b 2009	2076	3506	57043	77189	16.9%	0.80 [0.78, 0.82]	2009
Borchersen et al. c 2009	579	1036	3621	5256	15.8%	0.81 [0.77, 0.86]	2009
Frijters et al. 2009	1075	2036	43150	64985	16.5%	0.80 [0.76, 0.83]	2009
Xu 2014	35733	51712	41729	57085	17.3%	0.95 [0.94, 0.95]	2014
Lenz et al. 2016	1261	1890	40971	62398	16.8%	1.02 [0.98, 1.05]	2016
Total (95% CI)		**62316**		**272663**	**100.0%**	**0.87 [0.81, 0.94]**	
Total events	42147		190909				

Heterogeneity: Tau² = 0.01; Chi² = 254.10, df = 5 (P < 0.00001); I² = 98%
Test for overall effect: Z = 3.49 (P = 0.0005)

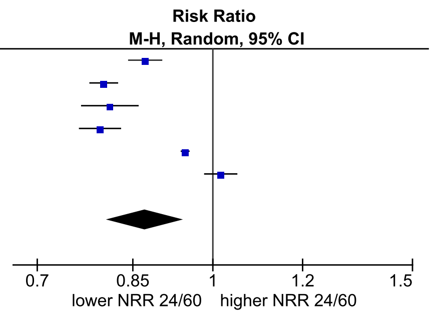


Figure 2. Forest plot of the non-return rates (NRR) 24/60 after the use of sex-sorted sperm compared to conventional sperm.

and for the increased concentration the RR was 0.83 (CI 95: 0.78–0.88) indicating a disproportionately higher degree of improvement of pregnancy rates by 7 percentage points (p = 0.010, Cochran–Mantel–Haenszel test) in sex-sorted sperm compared to conventional sperm when increasing sperm dosage. The use of increased sperm concentration resulted in a decrease of heterogeneity (I^2: 79%, tau^2: 0.01, p < 0.001 in 2.5 mill/straw and I^2: 52%, tau^2: 0.01, p = 0.009 in > 2.5 mill/straw sperm concentration, chi-square test, Table 2).

Regarding the method of sex-sorting the Ultra sexing technology proved to achieve a significantly higher pregnancy rate compared to the conventional sexing method (p = 0.047, Mann–Whitney *U* test). The conventional sexing technology was used in 54 trials, the Ultra sexing method was applied in 13 trials. The RR was 0.82 (CI 95: 0.79–0.86) in the Ultra sexing technology compared to 0.76 (CI 95: 0.73–0.78) in the conventional

Subgroups	Trials (n)	Sexed sperm (mean, CI 95)	P^1	conv. Sperm (mean, CI 95)	P^2	Rate ratio	P^3	Heterogenity I^2/tau^2	P^4	P^5 rate ratio subgroups
Total	67	43.9% (40.9–47.0)		56.1% (53.4–58.8)		0.77 (0.75–0.80	< 0.001	93%/0.01	< 0.001	
Dairy	45	41.2% (37.9–44.4)	0.009	54.0% (50.4–57.6)	0.024	0.77 (0.74–0.79)	< 0.001	94%/0.01	< 0.001	0.450
Beef	21	49.7% (43.2–56.2)		60.7% (56.7–64.6		0.79 (0.73–0.86)	< 0.001	65%/0.02	< 0.001	
Heifers	43	48.4% (45.0–51.8)	< 0.001	60.2% (58.4–62.6)	0.001	0.79 (0.75–0.82	< 0.001	93%/0.01	< 0.001	0.240
Cows	18	34.4% (28.8–39.9)		46.2% (39.3–53.2)		0.75 (0.72–0.80)	< 0.001	81%/0.01	< 0.001	
Frozen semen	49	45.1% (41.3–48.9)	0.843	57.3% (54.2–60.4)	0.393	0.77 (0.75–0.80)	< 0.001	77%/0.01	< 0.001	0.008
Fresh semen	7	45.2% (37.0–53.5)		55.0% (47.6–62.3)		0.84 (0.80–0.88)	< 0.001	12%/ < 0.01	0.340	
Sperm dosage ≤ 2.5mill. per 0.25 cc straw	44	41.4% (38.4–44.4)	< 0.001	55.2% (52.0–58.3)	0.004	0.76 (0.73–0.79)	< 0.001	79%/0.01	< 0.001	0.010
Sperm dosage > 2.5mill. per 0.25 cc straw	16	55.5% (49.1–61.8)		63.5% (59.8–67.3)		0.83 (0.78–0.88)	< 0.001	52%/0.01	0.009	
conv sexing method	54	42.9% (39.3–46.7)	0.047	55.7% (52.4–59.0)	0.590	0.76 (0.73–0.78)	< 0.001	93%/0.01	< 0.001	0.002
Ultra sexing method	13	47.9% (44.5–51.3)		57.7% (54.2–61.2)		0.82 (0.79–0.86)	< 0.001	55%/ < 0.01	0.009	
Estrus	22	44.3% (39.1–49.4)	0.919	55.7% (51.0–60.4)	0.551	0.80 (0.77–0.83)	< 0.001	74%/ < 0.01	< 0.001	0.160
Synchronisation	37	45.3% (40.8–49.7)		58.0% (54.2–61.7)		0.76 (0.73–0.80)	< 0.001	66%/0.01	< 0.001	
Before 2015	45	42.3% (38.1–46.6)	0.034	55.3% (51.5–59.1)	0.496	0.74 (0.71–0.78)	< 0.001	94%/0.01	< 0.001	< 0.001
2015 or later	22	47.2% (44.3–50.2)		57.7% (54.6–60.8)		0.81 (0.79–0.84)	< 0.001	59%/ < 0.01	< 0.001	
Early detection	34	40.6% (37.2–44.1)	0.010	53.7% (49.8–57.6)	0.031	0.75 (0.72–0.79)	< 0.001	75%/0.01	< 0.001	0.130
Late detection	24	49.5% (43.0–56.0)		60.5% (56.2–64.8)		0.80 (0.75–0.85)	< 0.001	72%/0.01	< 0.001	
Rectal palpation	13	39.8% (31.4–48.2)	0.319	50.9% (43.6% (58.2)	0.050	0.77 (0.73–0.82)	< 0.001	76%/0.01	< 0.001	0.740
Sonography	46	45.91% (42.2–49.6)		58.0% (54.9–61.1)		0.78 (0.75–0.82)	< 0.001	76%/0.01	< 0.001	

Table 2. Summary of the results of the meta-analysis of pregnancy rates after the use of sex-sorted and conventional sperm with a special focus on the effects of cow type, age, sperm freezing, sperm concentration, sperm sexing technology, timepoint of publication, and herd management. p: determination of statistical significances of: 1: the differences between two subgroups of cows inseminated with sex-sorted sperm using the t-test or Mann–Whitney *U* test. 2: the differences between two subgroups with conventional semen using the t-test or Mann–Whitney *U* test. 3: the determined rate ratio and odds ratio, respectively, using the Cochran–Mantel–Haenszel test (CMH). 4: the heterogeneity using the chi-square test. 5: the differences between subgroups inseminated with sex-sorted and conventional sperm using the CMH.

method indicating a 6 percentage points increase in pregnancy rate ratio when using this technology ($p = 0.002$, Cochran–Mantel–Haenszel test). Further to that the heterogeneity was higher in the conventional sexing technology (I^2: 93%, tau^2: 0.01, $p < 0.001$, chi-square test, Table 2) compared to the Ultra sexing technology (I^2: 55%, tau^2: 0.01, $p = 0.009$, chi-square test, Table 2). In line with this, the sex-sorted sperm used in the trials published in the years 2016–2020 achieved significantly higher pregnancy rates compared to the trials published between 1999 and 2015 ($p = 0.034$, Mann–Whitney *U* test, Table 2). Further to that, the heterogeneity was significantly higher ($p = 0.003$, Levene's test) in the trials published before 2015 (I^2: 94%, tau^2: 0.01, $p < 0.001$, chi-square test, Table 2) (in 2015 no respective studies were published) compared to those published after 2015 (I^2: 59%, tau^2: 0.01, $p < 0.001$, chi-square test, Table 2). When analysing the combined effects of the use of sex-sorted sperm after freezing in heifers and after 2015 (8 trials), the overall reduction of pregnancy rates was 17.7% (CI 95: 12.3–22.8). The combination of frozen sperm sorted by the SexedUltra™ Technology in heifers (2 trials) resulted in a reduction of pregnancy rates of 13.5% as compared to AI with conventional sperm (CI 95: 7.2–19.9).

When correlating the pregnancy rates with the publication year there was no correlation in the years 1999–2020 (Spearman *rho* = 0.078, $p = 0.532$, number of trials: 67). However, there was a correlation in the years 1999–2015 (Spearman rho = -0.305, $p = 0.042$, number of trials: 45). In the years 2016–2020 there was no correlation between pregnancy rate and publication year (Spearman rho = -0.085, $p = 0.707$, number of trials: 22). As shown in Fig. 4a, the pregnancy rate ratios show a high variation before 2015 whereas the data after 2016 reveal much less heterogeneity and an increased number of studies near the regression line (Fig. 4a).

In a last step the impact of herd management on the reproductive success was investigated. When comparing pregnancy rates after insemination during natural estrus (22 trials) or after synchronization (37 trials) pregnancy rates were not significantly different irrespective of the use of conventional or sex-sorted sperm ($p = 0.551$ and 0.919, respectively, Mann–Whitney *U* test, Table 2). Thus, the negative impact of sex-sorting on pregnancy rates was similar in inseminations during estrus and after synchronisation ($p = 0.160$, Cochran–Mantel–Haenszel test). The RR was 0.8 (CI 95: 0.77–0.83) after insemination during estrus and 0.76 (CI 95: 0.73–0.80) after synchronisation. The heterogeneity was substantial both in estrus and after synchronization (I^2: 74% and 66%, respectively, tau^2: 0.01, $p < 0.001$, chi-square test, Table 2). Regarding early and late detection of pregnancy, the late detection was significantly more reliable to detect pregnancy both after the use of conventional and sex-sorted sperm ($p = 0.031$ and $p = 0.010$, t-test, respectively, Table 2). The impact of sex-sorting on pregnancy rates was not affected by the timepoint of pregnancy detection ($p = 0.130$, Cochran–Mantel–Haenszel test, Table 2). When

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Risk Ratio M-H, Random, 95% CI	Year
1.1.1 all							
Seidel et al. g 1999	23	43	20	35	0.5%	0.94 [0.63, 1.40]	1999
Seidel et al. f 1999	18	35	27	37	0.6%	0.70 [0.48, 1.03]	1999
Seidel et al. a 1999	58	114	87	118	1.2%	0.69 [0.56, 0.85]	1999
Seidel et al. d 1999	20	45	15	28	0.4%	0.83 [0.52, 1.33]	1999
Seidel et al. e 1999	17	51	27	58	0.4%	0.72 [0.44, 1.15]	1999
Seidel et al. b 1999	34	86	18	35	0.5%	0.77 [0.51, 1.16]	1999
Seidel et al. c 1999	12	14	21	30	0.7%	1.22 [0.89, 1.68]	1999
Bodmer et al. a 2005	29	105	18	64	0.4%	0.98 [0.60, 1.62]	2005
Bodmer et al. b 2005	9	27	16	27	0.2%	0.56 [0.30, 1.04]	2005
Andersson et al. 2006	33	157	69	149	0.6%	0.45 [0.32, 0.64]	2006
Duarte et al. b 2007	83	180	51	103	1.0%	0.93 [0.72, 1.20]	2007
Klinc et al. 2007	12	22	16	24	0.4%	0.82 [0.51, 1.32]	2007
Duarte et al. a 2007	41	61	58	83	1.2%	0.96 [0.77, 1.20]	2007
Seidel et al. c 2008	18	38	29	40	0.5%	0.65 [0.44, 0.96]	2008
Seidel et al. b 2008	67	123	85	126	1.3%	0.81 [0.66, 0.99]	2008
Seidel et al. e 2008	24	42	16	21	0.6%	0.75 [0.53, 1.07]	2008
Seidel et al. d 2008	57	121	74	124	1.1%	0.79 [0.62, 1.00]	2008
Seidel et al. a 2008	124	288	163	263	1.6%	0.69 [0.59, 0.82]	2008
Seidel et al. f 2008	24	30	21	30	0.8%	1.14 [0.85, 1.53]	2008
Schenk et al. a 2009	23	57	32	58	0.5%	0.73 [0.49, 1.08]	2009
DeJarnette et al. 2009	17893	39763	30082	53718	2.7%	0.80 [0.79, 0.81]	2009
Borchersen et al. b 2009	273	554	112	181	1.8%	0.80 [0.69, 0.92]	2009
Borchersen et al. c 2009	235	504	89	165	1.5%	0.86 [0.73, 1.02]	2009
Borchersen et al. a 2009	319	530	100	153	1.8%	0.92 [0.80, 1.05]	2009
Schenk et al. b 2009	177	708	269	713	1.6%	0.66 [0.57, 0.78]	2009
DeJarnette et al. a 2010	917	2089	1268	2089	2.5%	0.72 [0.68, 0.77]	2010
Norman et al. b 2010	6478	25910	3235438	10784793	2.6%	0.83 [0.82, 0.85]	2010
Mellado et al. 2010	51	223	161	426	0.9%	0.61 [0.46, 0.79]	2010
Norman et al. a 2010	50194	128702	655865	1171188	2.7%	0.70 [0.69, 0.70]	2010
Chebel et al. 2010	138	343	533	1028	1.8%	0.78 [0.67, 0.89]	2010
DeJarnette et al. b 2010	419	1822	574	1822	2.1%	0.73 [0.66, 0.81]	2010
An et al. 2010	19	36	15	26	0.4%	0.91 [0.58, 1.44]	2010
Sales et al. a 2011	32	102	58	112	0.7%	0.61 [0.43, 0.85]	2011
Dominguez et al. 2011	131	338	188	325	1.6%	0.67 [0.57, 0.79]	2011
DeJarnette et al. 2011	881	2319	1375	2292	2.4%	0.63 [0.60, 0.67]	2011
Sales et al. b 2011	82	196	100	193	1.2%	0.81 [0.65, 1.00]	2011
Sá Filho et al. 2012	113	246	134	245	1.5%	0.84 [0.70, 1.00]	2012
Mallory et al. 2013	45	120	82	120	1.0%	0.55 [0.42, 0.71]	2013
Abdalla et al. 2014	145	426	203	325	1.6%	0.54 [0.47, 0.64]	2014
Karakaya et al. a 2014	25	60	35	66	0.6%	0.79 [0.54, 1.14]	2014
Karakaya et al. b 2014	22	88	28	88	0.4%	0.79 [0.49, 1.26]	2014
Mellado et al. b 2014	2321	13574	6878	28779	2.6%	0.72 [0.69, 0.75]	2014
Mellado et al. a 2014	6524	15497	3551	6816	2.6%	0.81 [0.78, 0.83]	2014
Cooke et al. 2014	149	439	252	454	1.7%	0.61 [0.52, 0.71]	2014
Thomas et al. 2014	56	217	123	218	1.0%	0.46 [0.35, 0.59]	2014
Holden et al. 2016	710	1486	21336	39366	2.5%	0.88 [0.84, 0.93]	2016
Djedovic et al. 2016	530	1205	1163	2115	2.3%	0.80 [0.74, 0.86]	2016
Noonan et al. 2016	173	379	239	398	1.8%	0.76 [0.66, 0.87]	2016
Kurykin et al. 2016	714	1713	770	1493	2.3%	0.81 [0.75, 0.87]	2016
Joezy-Shekalgorabi 2017	557	1154	1543	2419	2.4%	0.76 [0.71, 0.81]	2017
Colazo et al. 2017	75	117	74	107	1.4%	0.93 [0.77, 1.12]	2017
Thomas. et al. 2017	218	422	257	429	1.9%	0.86 [0.76, 0.97]	2017
Naniwa et al. b 2018	72	214	26	65	0.6%	0.84 [0.59, 1.20]	2018
Crites et al. 2018	95	193	114	201	1.4%	0.87 [0.72, 1.05]	2018
Naniwa et al. a 2018	242	524	128	219	1.7%	0.79 [0.68, 0.91]	2018
Thomas et al. 2018	387	808	525	812	2.2%	0.74 [0.68, 0.81]	2018
Maicas et al. b SS_FRZ 2019	429	812	527	865	2.3%	0.87 [0.80, 0.94]	2019
Maicas et al. b SS-2M 2019	388	726	527	865	2.2%	0.88 [0.80, 0.96]	2019
Maicas et al. a SS-2M 2019	555	1428	765	1593	2.3%	0.81 [0.75, 0.88]	2019
Maicas et al. a SS-1M 2019	488	1299	765	1593	2.2%	0.78 [0.72, 0.85]	2019
Maicas et al. b SS-1M 2019	440	811	527	865	2.3%	0.89 [0.82, 0.97]	2019
Maicas et al. a SS-FRZ 2019	476	1173	765	1593	2.2%	0.85 [0.78, 0.92]	2019
Maicas et al. 2020	1629	3580	2196	3666	2.5%	0.76 [0.73, 0.79]	2020
Chebel et al. 2020	188	415	262	390	1.9%	0.67 [0.59, 0.77]	2020
Dawod and Elbaz 2020	178	346	75	122	1.5%	0.84 [0.70, 1.00]	2020
Drake et al. 2020	742	1442	445	722	2.3%	0.83 [0.77, 0.90]	2020
Ketchum et al. 2020	187	390	240	404	1.8%	0.81 [0.71, 0.92]	2020
Subtotal (95% CI)		**257082**		**12118090**	**100.0%**	**0.77 [0.75, 0.80]**	
Total events	97838		3971645				

Heterogeneity: Tau² = 0.01; Chi² = 873.11, df = 66 (P < 0.00001); I² = 92%
Test for overall effect: Z = 15.70 (P < 0.00001)

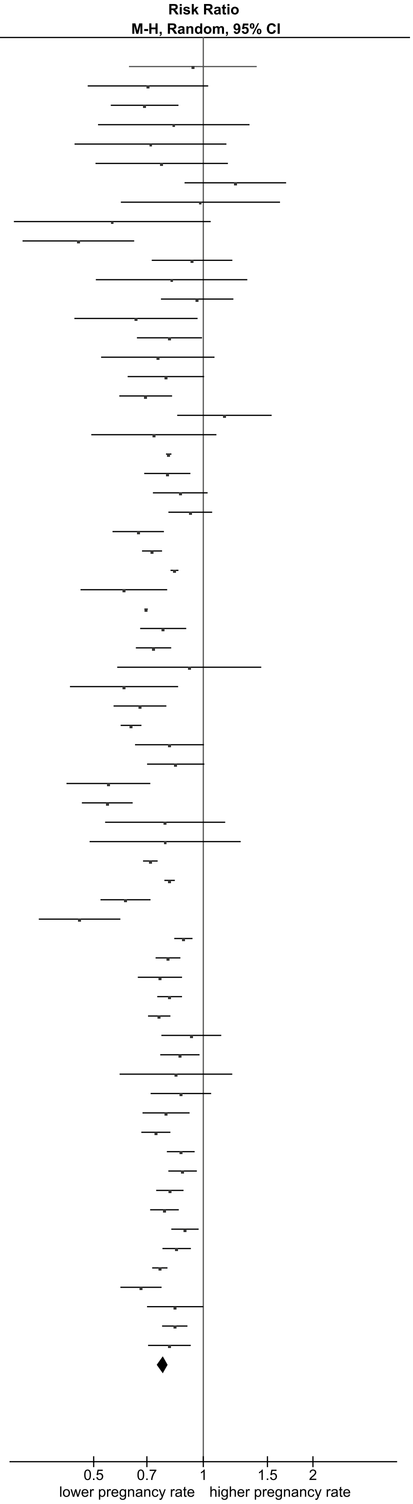


Figure 3. Forest plot of the pregnancy rates after the use of sex-sorted sperm compared to conventional sperm.

comparing rectal palpation and sonography as method for the diagnosis of pregnancy sonography was more reliable to detect pregnancy rates both after insemination with conventional and sex-sorted sperm (p = 0.050 and p = 0.319, Mann–Whitney *U* test, respectively). The impact of sperm sexing was not associated with the method of pregnancy detection (p = 0.74, Table 2). The heterogeneity was the same in both methods (I^2: 76%, tau^2: 0.01, p < 0.001, chi-square test, Table 2).

Further to that the effects of the geographical location of the trials on the pregnancy rate were analysed. The 67 studies were performed in 20 countries of 6 regions (Africa: 1; RR 0.84; Asia: 7, RR 0.73 (CI 95: 0.64–0.84);

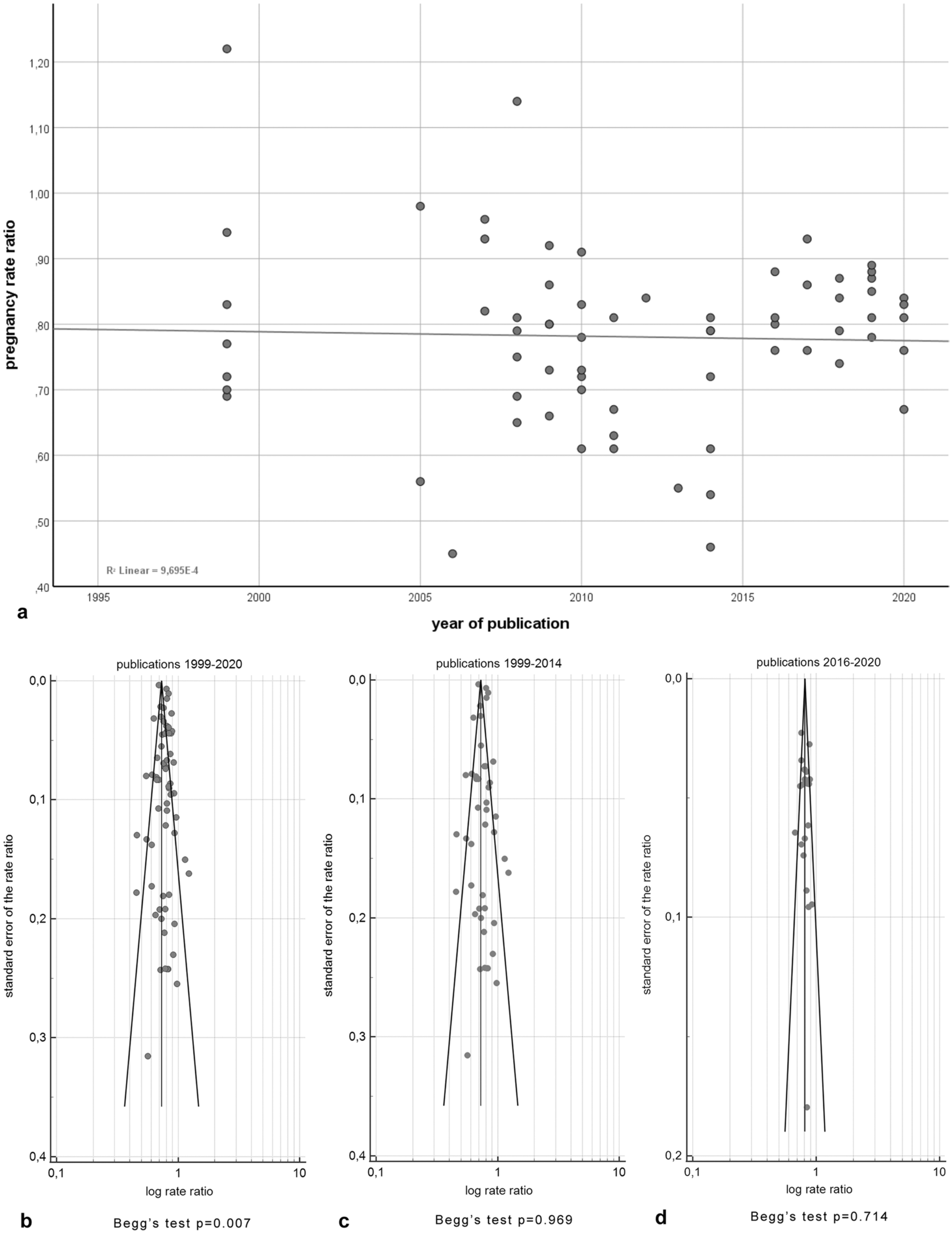


Figure 4. Pregnancy rate ratios in relation to the year of publication and funnel plots of studies evaluating publication bias in the reproductive success of bovine sperm after sex-sorting. (**a**) The pregnancy rate ratios show a high variation before 2015 whereas the data after 2015 reveal much less heterogeneity and an increased number of studies near the regression line. (**b**) The funnel plot of the studies published between 1999 and 2020 reveal asymmetry with outliers located besides the lines marking the 95% confidence limits. The Begg's test reveals a significant p value of 0.007. (**c**) In the funnel plot of the studies between 1999 and 2015 there is no publication bias (p = 0.969, Begg's test). (**d**) In the funnel plot of the publications between 2016 and 2020 the majority of values are within the 95% confidence limits and there is no publication bias (p = 0.714, Begg's test).

Subgroups	Trials (n)	Sexed sperm (mean, CI 95)	P^1	conv. Sperm (mean, CI 95)	P^2	Rate ratio	P^3	Heterogenity I^2/tau^2	P^4	p^5 rate ratio subgroups
Total	19	41.3% (35.8–46.9)		54.6% (48.4–60.9)		0.76 (0.69–0.83)	< 0.001	89%/0.03	< 0.001	
Dairy	14	39.6.2% (32.4–46.9)	0.301	53.1% (46.4–59.7)	0.388	0.75 (0.68–0.84)	< 0.001	91%/0.03	< 0.001	0.970
Beef	5	46.0% (37.7–54.3)		59.1% (37.6–80.6)		0.76 (0.63–0.92)	0.004	0%/ < 0.01	0.430	
Heifers	15	42.5% (37.1–47.8)	0.665	56.3% (50.1–62.5)	0.469	0.75 (0.70–0.81)	< 0.001	57%/0.01	0.004	0.940
Cows	4	36.9% (8.0–65.7)		48.4% (17.9–79.0)		0.74 (0.52–1.06)	0.100	81%/0.09	0.001	
Fresh semen	2	50.2%	0.686	53.2%	0.305	0.95 (0.87–1.03)	0.220	3%/ < 0.01	0.310	< 0.001
Frozen semen	13	41.7% (33.7–49.7)		57.4% (49.4–65.4)		0.77 (0.68–0.86	< 0.001	63%/0.03	0.001	
Sperm dosage ≤ 2.5mio per 0.25 cc straw	16	40.2% (33.8–46.6)	0.209	52.9% (46.0–59.7)	0.052	0.76 (0.68–0.85)	< 0.001	89%/0.03	< 0.001	0.450
Sperm dosage > 2.5mio per 0.25 cc straw	2	50.6% (0.0–100.0		70.5% (58.2–82.8		0.69 (0.57–0.85)	0.001	0%/ < 0.01	0.550	
Before 2015	15	39.9% (33.4–46.4)	0.596	53.7% (45.7–61.6)	0.596	0.74 (0.66–0.84)-	< 0.001	82%/0.04	< 0.001	0.690
2015 or later	4	46.5% (29.6–63.5)		58.3% (49.7–66.9)		0.77 (0.69–0.85)	< 0.001	71%/0.01	0.020	
Estrus	10	37.9% (29.3–46.5)	0.183	51.4% (43.7–59.1)	0.261	0.75 (0.66–0.86)	< 0.001	93%/0.03	< 0.001	0.990
Synchronisation	9	45.1% (37.2–53.0)		58.3% (46.8–69.8)		0.75 (0.69–0.84)	< 0.001	38%/0.01	0.140	

Table 3. Summary of the results of the meta-analysis of calving rates after the use of sex-sorted and conventional sperm with a special focus on the effects of cow type, age, sperm freezing, sperm concentration, timepoint of publication, and cow management. p: determination of statistical significances of: 1: the differences between two subgroups of cows inseminated with sex-sorted sperm using the t-test or Mann–Whitney *U* Test. 2: the differences between two subgroups with conventional semen using the t-test or Mann–Whitney *U* Test. 3: the determined rate ratio and odds ratio, respectively, using the Cochran–Mantel–Haenszel test (CMH). 4: the heterogeneity using the chi-square test. 5: the differences between subgroups inseminated with sex-sorted and conventional sperm using the CMH.

Australia/New Zealand: 1, RR 0.76; Europe:18, RR 0.83 (CI 95: 0.80–0.86); North America: 31, RR 0.74 (CI 95: 0.71–0.78); South America: 9, RR 0.77 (CI 95: 0.71–0.83). There were significant differences related to the geographic location (Cochran–Mantel–Haenszel test., p = 0.01). When applying pairwise comparisons with Bonferroni-Holm correction for multiple testing, Europe had a significantly higher pregnancy rates ratio compared to North America (Europe vs North America: p = 0.003, Europe vs South America: p = 0.400, Europe vs Australia p = 0.480, Europe vs Asia: p = 0.400, Europe vs Africa: p = 0.900). In Europe the pregnancy rate ratio was increased by 9 percentage points compared to North America.

In order to analyse publication bias, funnel plots were calculated for the overall time period of the meta-analysis (1999–2020) as well as for the time periods 1999–2015 and 2016–2020. The funnel plot of the studies published between 1999 and 2020 revealed asymmetry with outliers located right beside the lines marking the 95% confidence limits (Fig. 4b). In the funnel plot of the studies between 1999 and 2015 (Fig. 4c) as well as in the funnel plot of the publications between 2016 and 2020 there was no asymmetry, and most values were within the 95% confidence limits (Fig. 4d). Accordingly, the Begg's test for analysis of publication bias in the 67 trials between 1999–2020 resulted in p = 0.007 (< 0.05 is an indication for publication bias). When analysing the trials between 1999 and 2015, p was 0.969 (number of trials: 45). In the time period between 2016 and 2020, p was 0.714 (22 trials).

Calving rates. Calving rates were compared in 19 trials. The overall calving rates in all cows were significantly reduced from 54.6% to 41.3% when using sex-sorted sperm (p < 0.001, Cochran–Mantel–Haenszel test, Table 3). The rate ratio was 0.76 indicating a significant decrease of 24% in calving rates after sex-sorting (CI 95: 0.69–0.83, p < 0.001, Cochran–Mantel–Haenszel test, Table 3, Fig. 5). The heterogeneity was statistically significant (I^2 = 89%, tau^2 = 0.03, p < 0.001, chi-square test) indicating the necessity of subgroup analyses (Table 3).

The comparison of the reproductive success in dairy (14 trials) and beef cows (5 trials) showed that the calving rates were higher (6.0–6.4 percentage points) in beef cows compared to dairy cows irrespective of using conventional or sex-sorted sperm. In view of the small and unbalanced number of trials this effect was not significant (p = 0.388 and p = 0.301, respectively, t-test, Table 3). Overall, the negative effect of sex-sorting on calving rates were similar in dairy and beef cows (p = 0.970, Cochran–Mantel–Haenszel test Table 3).

Regarding heifers and cows, calving rate ratios were not significantly different when using sex-sorted sperm (p = 0.940, Cochran–Mantel–Haenszel test, Table 3). In heifers (15 trials), the calving rate was significantly decreased from 56.3% (CI 95: 50.1–62.5) to 42.5% (CI 95: 37.1–47.8, p < 0.001, Cochran–Mantel–Haenszel test, Table 3) after sex-sorting. The RR was 0.75 (CI 95: 0.70–0.81, p < 0.001) pointing to a significant reduction in calving rates of 25% in heifers (p < 0.001, Cochran–Mantel–Haenszel test). In cows, calving rates were reduced from 48.4% (CI 95: 17.9–79.0) to 36.9% (CI 95: 8.0–65.7). The high range of the CI 95 and the lack of significance was due to the small number of 4 trials and to the high heterogeneity of the results of these trials. The RR was 0.74 (CI 95: 0.52–1.06) indicating a decrease of calving rates of 26% in cows. The heterogeneity in cows was considerably higher than in heifers (I^2: 57%, p = 0.004 in heifers, I^2: 81%, p = 0.001 in cows, tau^2: 0.01, chi-square test, Table 3).

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Risk Ratio M-H, Random, 95% CI	Year
1.7.1 all							
Seidel et al. d 1999	19	45	9	28	1.8%	1.31 [0.69, 2.49]	1999
Seidel et al. a 1999	53	114	82	118	5.7%	0.67 [0.53, 0.84]	1999
Seidel et al. b 1999	34	86	18	35	3.3%	0.77 [0.51, 1.16]	1999
Seidel et al. f 1999	18	35	27	37	3.7%	0.70 [0.48, 1.03]	1999
Bodmer et al. a 2005	23	104	16	63	2.3%	0.87 [0.50, 1.52]	2005
Bodmer et al. b 2005	8	27	15	26	1.7%	0.51 [0.26, 1.00]	2005
Andersson et al. 2006	32	157	65	149	3.9%	0.47 [0.33, 0.67]	2006
Seidel et al. e 2008	23	42	15	21	3.6%	0.77 [0.52, 1.13]	2008
Seidel et al. c 2008	16	38	27	40	3.2%	0.62 [0.41, 0.96]	2008
Borchersen et al. c 2009	210	504	83	165	6.5%	0.83 [0.69, 1.00]	2009
Borchersen et al. a 2009	298	530	97	153	7.1%	0.89 [0.77, 1.02]	2009
Borchersen et al. b 2009	255	554	104	181	6.9%	0.80 [0.69, 0.93]	2009
Chebel et al. 2010	91	343	388	1028	6.3%	0.70 [0.58, 0.85]	2010
Abdalla et al. 2014	125	426	166	325	6.5%	0.57 [0.48, 0.69]	2014
Xu 2014	22736	45290	25890	48666	8.3%	0.94 [0.93, 0.96]	2014
Djedovic et al. 2016	490	1205	1091	2115	7.9%	0.79 [0.73, 0.85]	2015
Joezy-Shekalgorabi 2017	494	1154	1453	2419	8.0%	0.71 [0.66, 0.77]	2017
Colazo et al. 2017	73	117	69	107	6.2%	0.97 [0.79, 1.18]	2017
Chebel et al. 2020	167	415	223	390	7.0%	0.70 [0.61, 0.81]	2020
Subtotal (95% CI)		**51186**		**56066**	**100.0%**	**0.76 [0.69, 0.83]**	
Total events	25165		29838				

Heterogeneity: Tau² = 0.03; Chi² = 158.78, df = 18 (P < 0.00001); I² = 89%
Test for overall effect: Z = 5.62 (P < 0.00001)

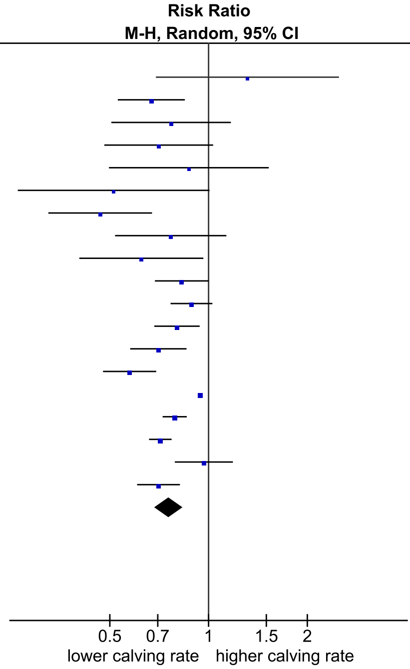


Figure 5. Forest plot of the calving rates after the use of sex-sorted sperm compared to conventional sperm.

The effects of frozen and fresh semen on the calving rates were analysed in 13 and 2 trials, respectively. Calving rates after sex-sorting were significantly decreased ($p<0.001$) after freezing and thawing. The rate ratio was 0.77 (CI 95: 0.68–0.86) for frozen semen and 0.95 (CI 95: 0.87–1.03) for fresh semen pointing to a reduction of 18 percentage points in calving caused by the freezing of the sex-sorted sperm ($p<0.001$, Cochran–Mantel–Haenszel test Table 3). The heterogeneity was high in frozen sperm (I^2: 63%, tau^2: 0.03, $p<0.001$, chi-square test, Table 3) whereas the heterogeneity was low in fresh sperm (I^2: 3%, $tau^2<0.01$, $p=0.31$, chi-square test, Table 3).

The effect of a sperm dosage with more than 2.5 million sperm per straw (4 million) was only investigated in 2 trials. The effects of 2.5 million sperm or less on the calving rate were investigated in 16 trials. Overall, there was no significant impact of the sperm dose on the calving rate ratio ($p=0.450$, Cochran–Mantel–Haenszel test, Table 3). For 2.5 million sperm (or less) per straw the RR was 0.76 (CI 95: 0.68–0.85) and for the increased concentration > 2.5 million sperm the RR was 0.69 (CI 95: 0.57–0.85, $p<0.001$ for ≤ 2.5 million, $p=0.001$ for > 2.5 million sperm per straw) (Table 3).

When comparing the effects of sperm sexing on calving rates in the trials published between 1999–2015 and in the trials published between 2016–2020 calving rates increased from 39.9% (CI 95: 33.4–46.4) to 46.5% (CI 95: 29.6–63.5) in sex-sorted sperm and from 53.7% (CI 95: 45.7–61.6) to 58.3% (CI 95: 49.7–66.9) in conventional sperm (Table 3). The RR increased from 0.74 to 0.77 ($p=0.69$, Cochran–Mantel–Haenszel test, Table 3) pointing to an improvement of calving rates after sex-sorting of 3 percentage points after 2015 (Table 3). Further to that, the heterogeneity was higher in the trials published before 2015 (I^2: 82%, tau^2: 0.04, $p<0.001$, chi-square test Table 3) compared to those published 2015 or later (I^2: 71%, tau^2: 0.01, $p=0.020$, chi-square test, Table 3).

When correlating the calving rates with the publication year over the whole period (1999–2020) no significant relationship was found (Spearman rho = −0.116, $p=0.637$, number of trials: 19).

In a last step the impact of herd management on calving rates was investigated. When comparing calving rates after insemination during natural estrus (10 trials) and after synchronization (9 trials) calving rates were not significantly different irrespective of the use of conventional or sex-sorted sperm ($p=0.183$ and 0.261, respectively, t-test, Table 3). The negative impact of sex-sorting on calving rates was similar after inseminations during estrus or after synchronisation ($p=0.990$, Cochran–Mantel–Haenszel test). Consequently, the RR was the same (0.75) after insemination during estrus and after synchronisation (CI: 0.66–0.86 and 0.69–0.84, respectively, $p<0.001$, Cochran–Mantel–Haenszel test). The heterogeneity was reduced after synchronization (I^2: 93% for insemination during estrus and 38%, for insemination after synchronisation, tau^2: 0.03 and 0,01, respectively, $p<0.001$, Table 3). Publication bias was not detected in the 19 trials analysed in the meta-analysis (Begg's test, $p=0.087$).

A summary of the impact of sperm sexing on reproductive success is provided in Fig. 6. The comparison of NRR 24/60, pregnancy rates and calving rates after insemination with sex-sorted and conventional sperm showed that all these rates were significantly reduced after sex-sorting (Fig. 6). Overall, sex-sorting of sperm resulted in a 13% (9 percentage points) decrease of the NRR, a 23% (12.3 percentage points) decrease of pregnancy rate and a 24% (13.3 percentage points) decrease of calving rate (Fig. 6).

Abortion rates. Abortion rates after insemination with conventional and sex-sorted sperm were compared in 18 trials. The abortion rates were similar when using sex-sorted and conventional sperm for insemination (8.8% (CI 95: 5.0–12.7) and 9.3% (CI 95: 4.4–14.2), respectively, $p=0.62$, Cochran–Mantel–Haenszel test, Fig. 7). The odds ratio was 1.08 (CI 95: 0.8–1.45) indicating that the likelihood of the occurrence of an abortion

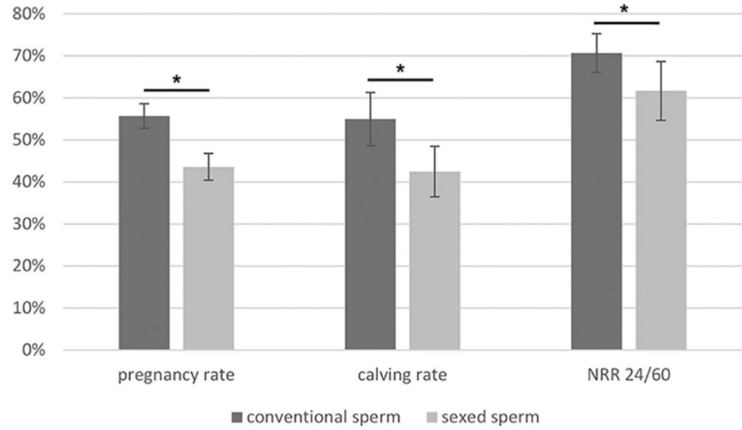


Figure 6. Comparison of Non-Return Rates (NRR) 24/60, pregnancy rates and calving rates after insemination with sex-sorted and conventional sperm. NRR, pregnancy rates and calving rates are significantly reduced after sex-sorting. The differences in the rates become more obvious with progression of pregnancy and reach the highest values in the calving rates. Overall, sex-sorting of sperm results in a 13% (9 percentage points) decrease of the NRR , a 23% (12.3 percentage unpoints) decrease of pregnancy rate and a 24% (13.3 percentage points) decrease of calving rate.

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Odds Ratio M-H, Random, 95% CI	Year
Seidel et al. e 1999	1	17	3	27	1.5%	0.50 [0.05, 5.24]	1999
Seidel et al. d 1999	1	20	6	15	1.6%	0.08 [0.01, 0.76]	1999
Seidel et al. b 1999	0	34	0	18		Not estimable	1999
Seidel et al. a 1999	5	58	5	87	4.1%	1.55 [0.43, 5.60]	1999
Seidel et al. f 1999	0	18	0	27		Not estimable	1999
Tubman et al. 2004	37	829	28	560	12.0%	0.89 [0.54, 1.47]	2004
Bodmer et al. b 2005	1	9	0	16	0.8%	5.82 [0.21, 158.82]	2005
Bodmer et al. a 2005	5	29	1	18	1.6%	3.54 [0.38, 33.11]	2005
Seidel et al. e 2008	1	24	1	16	1.0%	0.65 [0.04, 11.24]	2008
Seidel et al. d 2008	4	57	6	74	4.0%	0.86 [0.23, 3.19]	2008
Seidel et al. c 2008	2	18	2	29	1.9%	1.69 [0.22, 13.18]	2008
Borchersen et al. c 2009	25	235	6	89	6.6%	1.65 [0.65, 4.16]	2009
Chebel et al. 2010	47	138	145	533	13.7%	1.38 [0.93, 2.06]	2010
Healy et al. 2013	36	584	149	2293	14.1%	0.95 [0.65, 1.38]	2013
Abdullah et al. 2014	12	145	22	203	8.6%	0.74 [0.35, 1.55]	2014
Joezy-Shekalgorabi 2017	64	558	91	1544	14.8%	2.07 [1.48, 2.89]	2017
Colazo et al. 2017	2	75	5	74	2.7%	0.38 [0.07, 2.01]	2017
Chebel et al. 2020	21	188	39	262	11.0%	0.72 [0.41, 1.27]	2020
Total (95% CI)		**3036**		**5885**	**100.0%**	**1.08 [0.80, 1.45]**	
Total events	264		509				

Heterogeneity: Tau² = 0.13; Chi² = 29.20, df = 15 (P = 0.02); I² = 49%
Test for overall effect: Z = 0.50 (P = 0.62)

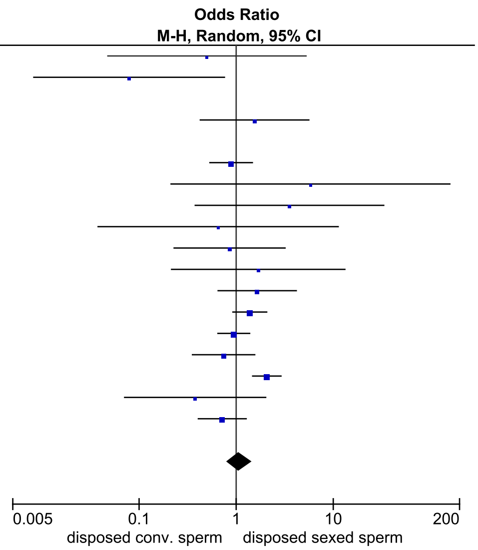


Figure 7. Forest plot of the abortion rates after the use of sex-sorted sperm compared to conventional sperm.

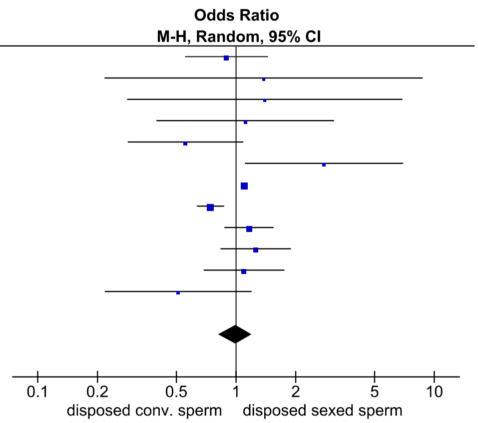
was similar when using sex-sorted and conventional sperm. The heterogeneity was moderate (I^2: 49%, tau^2: 0.13, p = 0.020, chi-square test, Fig. 7). When correlating the abortion odds ratio with the publication year there was no correlation in the years 1999–2020 (Spearman rho = −0.010, p = 0.970). Publication bias was not present in the 18 trials analysed in the meta-analysis (Begg's test, p = 0.787).

Stillbirth rates. Stillbirth rates were investigated in 12 trials with 10 trials discriminating between stillbirths of male and female calves. Stillbirth rates were 6.9% (CI 95: 4.7–9.1) when using sex-sorted sperm and 6.8% (CI 95: 4.2–9.5) when using conventional sperm for insemination. The odds ratio was 1.00 (CI 95: 0.82–1.20, p = 0.960, Cochran–Mantel–Haenszel test) indicating that the likelihood of the occurrence of a stillbirth was the same irrespective of using sex-sorted or conventional sperm. The heterogeneity was substantial (I^2: 71%, tau^2: 0.05, p < 0.001, Fig. 8a). When correlating the abortion odds ratio with the publication year there was no correlation in the years 1999–2020 (Spearman rho = −0.163, p = 0.612). Regarding the overall stillbirth rate publication bias was not present (Begg's test, p = 0.784).

When discriminating between the stillbirth rates of male and female calves, the stillbirth rate of male calves was significantly increased from 10.2% (CI 95: 6.0–14.3) to 16.5% (CI 95: 7.0–26, p = 0.003, Cochran–Mantel–Haenszel test) when using sex-sorted sperm for insemination. The odds ratio for stillbirth in male calves was 1.46 (CI 95: 1.14–1,86, p = 0.003, Cochran–Mantel–Haenszel test) indicating that it is significantly more likely to experience a stillbirth in male calves after insemination with X bearing sex-sorted sperm (Fig. 8b). The

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Odds Ratio M-H, Random, 95% CI	Year
Tubman et al. 2004	41	1158	31	787	8.9%	0.90 [0.56, 1.44]	2004
Andersson et al. 2006	2	32	3	65	1.0%	1.38 [0.22, 8.69]	2006
Borchersen et al. c 2009	7	210	2	83	1.3%	1.40 [0.28, 6.87]	2009
Borchersen et al. a 2009	17	298	5	97	2.9%	1.11 [0.40, 3.10]	2009
Borchersen et al. b 2009	25	255	17	104	5.8%	0.56 [0.29, 1.08]	2009
Chebel et al. 2010	8	91	13	388	3.6%	2.78 [1.12, 6.92]	2010
Norman et al. a 2010	5672	50194	68210	655865	20.3%	1.10 [1.07, 1.13]	2010
Norman et al. b 2010	175	6478	116476	3235438	18.1%	0.74 [0.64, 0.86]	2010
Healy et al. 2013	73	548	250	2141	14.1%	1.16 [0.88, 1.54]	2013
Djedovic et al. 2016	40	490	72	1091	10.6%	1.26 [0.84, 1.88]	2016
Joezy-Shekalgorabi 2017	26	494	70	1453	9.2%	1.10 [0.69, 1.74]	2017
Chebel et al. 2020	8	167	20	223	4.0%	0.51 [0.22, 1.19]	2020
Total (95% CI)		**60415**		**3897735**	**100.0%**	**1.00 [0.82, 1.20]**	
Total events	6094		185169				

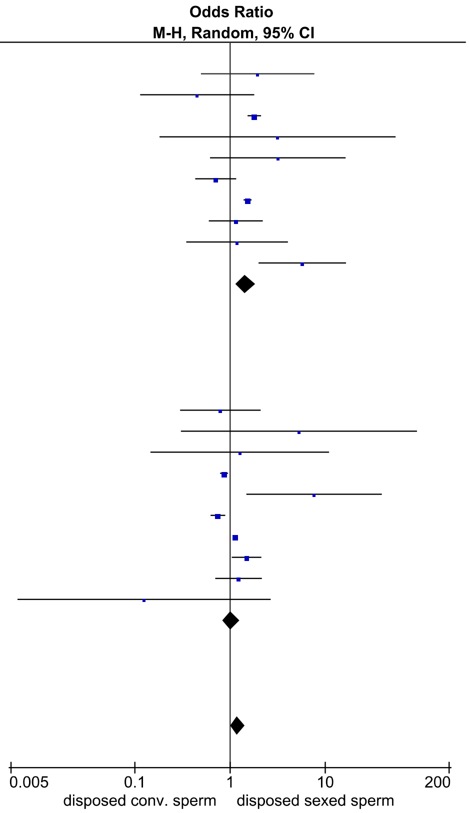
Heterogeneity: Tau² = 0.05; Chi² = 37.50, df = 11 (P < 0.0001); I² = 71%
Test for overall effect: Z = 0.05 (P = 0.96)



a

Study or Subgroup	sexed sperm Events	sexed sperm Total	conventional sperm Events	conventional sperm Total	Weight	Odds Ratio M-H, Random, 95% CI	Year
1.2.1 stillbirth male calves							
Borchersen et al. m a 2009	5	24	5	42	1.5%	1.95 [0.50, 7.57]	2009
Borchersen et al. m b 2009	3	29	11	54	1.5%	0.45 [0.12, 1.77]	2009
DeJarnette et al. m 2009	277	1318	1005	7793	11.6%	1.80 [1.55, 2.08]	2009
Borchersen et al. m c 2009	1	14	1	42	0.4%	3.15 [0.18, 54.04]	2009
Chebel et al. m 2010	2	13	11	203	1.1%	3.17 [0.63, 16.11]	2010
Norman et al. m b 2010	17	661	57306	1591835	6.6%	0.71 [0.44, 1.14]	2010
Norman et al. m a 2010	697	4467	34354	318095	12.3%	1.53 [1.41, 1.66]	2010
Healy et al. m 2013	12	76	155	1104	4.8%	1.15 [0.61, 2.18]	2013
Joezy-Shekalgorabi m 2017	3	47	38	698	1.8%	1.18 [0.35, 3.99]	2017
Chebel et al. 2020	9	18	19	128	2.4%	5.74 [2.02, 16.30]	2020
Subtotal (95% CI)		**6667**		**1919994**	**44.1%**	**1.46 [1.14, 1.86]**	
Total events	1026		92905				
Heterogeneity: Tau² = 0.05; Chi² = 25.49, df = 9 (P = 0.002); I² = 65%							
Test for overall effect: Z = 3.00 (P = 0.003)							
1.2.2 stillbirth female calves							
Borchersen et al. f b 2009	22	226	6	50	2.7%	0.79 [0.30, 2.06]	2009
Borchersen et al. f a 2009	12	274	0	55	0.4%	5.29 [0.31, 90.60]	2009
Borchersen et al. f c 2009	6	196	1	41	0.7%	1.26 [0.15, 10.78]	2009
DeJarnette et al. f 2009	1217	13228	1255	11952	12.3%	0.86 [0.79, 0.94]	2009
Chebel et al. f 2010	6	78	2	185	1.1%	7.63 [1.50, 38.66]	2010
Norman et al. f b 2010	149	5512	52880	1468889	11.4%	0.74 [0.63, 0.88]	2010
Norman et al. f a 2010	4890	45275	32128	331211	12.6%	1.13 [1.09, 1.16]	2010
Healy et al. f 2013	61	472	94	1037	8.6%	1.49 [1.06, 2.10]	2013
Joezy-Shekalgorabi f 2017	23	447	32	755	5.8%	1.23 [0.71, 2.12]	2017
Chebel et al. 2020	0	149	2	95	0.3%	0.13 [0.01, 2.63]	2020
Subtotal (95% CI)		**65857**		**1814270**	**55.9%**	**1.03 [0.84, 1.27]**	
Total events	6386		86400				
Heterogeneity: Tau² = 0.04; Chi² = 67.60, df = 9 (P < 0.00001); I² = 87%							
Test for overall effect: Z = 0.29 (P = 0.77)							
Total (95% CI)		**72524**		**3734264**	**100.0%**	**1.21 [1.01, 1.45]**	
Total events	7412		179305				

Heterogeneity: Tau² = 0.07; Chi² = 184.44, df = 19 (P < 0.00001); I² = 90%
Test for overall effect: Z = 2.09 (P = 0.04)
Test for subgroup differences: Chi² = 4.47, df = 1 (P = 0.03), I² = 77.6%



b

Figure 8. Forest plot of the stillbirth rates after the use of sex-sorted sperm compared to conventional sperm. (**a**) Forest plot including all stillbirths (**b**) Forest plot discriminating between the stillbirth of male and female calves.

stillbirth rates of female calves were similar (sex-sorted: 6.6% (CI 95: 3.6–9.5, conventional: 5.9% (2.4–9.3). The odds ratio was 1.03 (CI 95: 0.84–1.27, p = 0.77). Irrespective of the use of sex-sorted or conventional sperm the rate of stillbirths in male calves was significantly higher than in female calves (sex-sorted: p = 0.029, conventional: p = 0.043, t-test). The heterogeneity of stillbirth in female calves was higher than in male calves (female: I^2: 87%, tau^2: 0.04, p < 0.001, male: I^2: 65%, tau^2: 0.05, p = 0.002, chi-square test, Fig. 8b).

Discussion

This meta-analysis is the first to systematically analyse the effects of bovine sex-sorting on the reproductive outcome by including publications over a time span of 22 years (1999–2021). It is also the first study to shed light on the effects of the numerous refinements of this technology to date. This knowledge is pivotal for insemination stations and farmers to be able to determine the best possible use of sex-sorted sperm and to maximize economic success. The sex-sorting technology is of high importance within the dairy industry as due to the increased number of female calves efficiencies are improved without farm expansion, thus leading to rising profit margins[4,76,85,86]. This enables dairy farmers to breed the required number of female calves and breed the remaining females to unsorted beef semen to produce high value dairy-beef cross calves. Sex-sorted sperm is also of value to the beef industry, where Y-sorted sperm may be used for AI to increase the number of male calves which represent a significant economic advantage over female calves. The fact that only between Jan 2020 and Jan 2021 32 studies on bovine sperm sexing have been published reflects the importance and economic role of sex-sorting in the cattle industry. It also emphasizes the need of the bovine industry to be able to avail of precise information on the actual reproductive success rate of bovine sperm after sex-sorting.

Our analyses showed that the overall pregnancy rates are reduced by 23% and the calving rates are diminished by 24% after sex-sorting. The finding that there is only 1% point difference between pregnancy and calving rate highlights that the major cause for the reduced reproductive outcome is mainly due to impact of sex-sorting sperm on the fertilization process and early embryonic development. The reasons for reduced fertilising capacity of sex-sorted sperm are only partly understood. During the sorting process, sperm undergo time, temperature, mechanical and chemical stress[16,34,87,88]. The analysis of a single sperm requires extensive dilution, post-sorting centrifugation to concentrate the highly diluted sexed sperm, incubation at 34–37 °C, nuclear staining with Hoechst 33342, high pressure passage through the flow cytometer, and exposure to UV laser light before collection at the base of the flow cytometer and being cooled to 5 °C[16,34,87,88]. Thus, sex-sorting results in numerous sperm alterations including reduced progressive motility[23,31], reduced velocity[15,31], reduced hyperactivation[31] and abnormal movement patterns[20,31]. Additionally, reduced chromatin integrity[89], increased ROS levels[90,91], increased membrane permeability and reduced intracellular ATP levels[22,23,92] as well as a shortened time to acrosome reaction have been reported[93]. Further to that, binding in the oviductal sperm reservoir is reduced[20]. Sex-sorted spermatozoa reveal deformations in the head, sharp bends in the tail and a significantly increased prevalence of damaged mitochondria[20]. These alterations are likely to be augmented by the freezing and thawing process explaining why the use of fresh sex-sorted sperm is able to achieve pregnancy rate ratios which are up to 7 percentage points higher compared to frozen sex-sorted sperm. Similarly, a significant improvement of the calving rate is seen when using fresh sex-sorted sperm. However, when interpreting this data, it has to be considered that only very few trials investigated the reproductive outcome of fresh sex-sorted sperm. Additionally, the use of fresh sex-sorted sperm is not widely applicable within the logistics of the cattle breeding industry. Interestingly, the difference in pregnancy rates was 7% points between sex-sorted and non-sorted sperm when the number of sperm per straw were increased from 2.5 million to 4 million per straw. This indicates that higher dosages of sex-sorted sperm increase the probability of the presence of an intact spermatozoon with perfect fertilizing capacity and that – within limits – the use of higher numbers of sperm is able to improve the reproductive outcome after sex-sorting. This result might also imply that some mechanical sperm defects caused by sorting may be compensable. However, it must be highlighted that only a very limited number of studies were looking at the effects of increased sperm concentrations alone. In most studies, the increased number of sperm was associated with the use of the refined SexedUltra™ technology[17–19,34]. The changes in this technology include improvements in media, reduced sorting times and incorporation of new equipment the details of which are not fully disclosed. When relating the pregnancy rates with the year of publication, it becomes clear that there is no continuous improvement of the technology and a high heterogeneity of data between 1999–2015. However, after 2015 there is rapid and highly obvious improvement in the reproductive outcome after sex-sorting. This falls in the time when SexedUltra™ technology had been developed and sperm concentrations had been increased to 4 million/straw. This rapid improvement also affects the results of the analysis of publication bias. When looking at the publication bias in all trials published between 1999 and 2020, the Begg's test indicates publication bias when pregnancy rates were determined. However, when you look separately at the results of the trials between 1999 and 2015 and between 2016 and 2020 there is no publication bias indicating that the high heterogeneity of data and the low animal numbers before 2015 might have affected the overall result of publication bias in pregnancy rates.

The subgroup analyses confirmed that pregnancy rates are significantly higher in heifers compared to cows (sex-sorted sperm: 48% vs 34%, conventional sperm: 60% vs 46%). Interestingly there is no significant effect of sex-sorting on pregnancy rate between heifers and cows. Consequently, the preferred use of heifers for artificial insemination with sex-sorted sperm is due to the decreased fertility of the multiparous cow (reviewed in Walsh et al. 2011[94]) but is not related to the sex-sorting process itself. Similarly, the subgroup analyses showed that sex-sorting sperm impacts the field fertility of dairy and beef cattle to the same extent. Further to that the results of the analyses confirmed that beef cattle have an inherent higher fertility as opposed to dairy cattle. The long-term genetic pressure on increasing milk yields in dairy cows has caused a well-documented fertility decline[95,96] and is being addressed in the actual dairy cattle breeding[97]. These results highlight that the alterations induced by the sex-sorting of sperm are the same in beef and dairy cows.

When comparing the different geographic localisations of the trials Europe had significantly higher pregnancy rate ratios compared to North America. This might be due to the lower herd numbers in Europe which is associated with a more intense individual observation and fertility management in Europe[98]. Additionally, the more moderate climate with lack of extreme temperatures in Europe might add to the higher pregnancy rate ratios, however escalating temperatures in northern latitudes may soon increase heat stress on animals thus reducing this advantage[99]. This finding highlights that sex-sorted sperm react in a more sensitive way to factors reducing the herd fertility than non-sorted sperm.

In regard to herd management, the meta-analysis confirmed the well-known facts that late detection of pregnancy is more reliable than early detection and that ultrasonography is superior to rectal palpation[100]. More interestingly, the reproductive outcome of sex-sorted sperm was not significantly different when the sex-sorted sperm was applied during natural estrus or after synchronization. Further studies with sex-sorted sperm are necessary to refine the distinctions between the different methods of estrus detection (observed heat, movement collar, and scratch sticker[101]) as well as the different methods of synchronisation and AI timing including split time-AI (STAI). Additional studies which explore the relationship between inherent herd fertility, herd management (including semen handling, semen placement during AI, nutrition, stabling) and reproductive performance of sex-sorted sperm for AI are paramount in achieving high pregnancy rates. In this context, it is important to note that the non-return rates 24/60 merely revealed a decrease of 13% after the use of sex-sorted sperm indicating that the NNR is overestimating reproductive success of sex-sorted sperm. This might be due to the fact that farmers either miss an unsuccessful insemination or do not get back to the insemination station after a failed insemination with sex-sorted sperm.

When looking at the overall abortion and stillbirth rates there is no significant impact of sex-sorting. However, when discriminating between male and female offspring, the stillbirth rates are 1.46 times higher for male calves after sex-sorting. This is supported by previous research which found a higher incidence of stillbirth in calves sexed for the wrong sex, i.e., in a male calf born after insemination with X-sexed semen[64,76]. Overall, this involves very few animals when considering that about 10% of the calves are the wrong sex with an extra 6 percentage points of deaths. The stillbirths may be caused by the increased formation of ROS, leading to DNA damage, mitochondrial dysfunction, and enzyme inactivation during embryonic development[102–104]. Stillbirths might also partly be due to trisomy as Y-sperm plus and extra autosome will look like an X sperm when measuring DNA content. Because of how sorts are gated, trisomies would be concentrated in semen sorted as X-sperm.

In summary, the inception of the SexedUltra™ technology in combination with increased sperm concentrations per straw represents a significant milestone in the improvement of the reproductive outcome after sex-sorting of bovine sperm. The optimal reproductive success can be achieved by applying sex-sorted sperm, which have been produced by the SexedUltra™ technology, in a concentration of 4 million sperm per straw, exclusively to heifers, which results in a reduction of pregnancy rates of 18% as compared to conventional sperm. This knowledge is pivotal for making deliberate decisions in insemination stations and individual cattle breeding farms regarding the use of sex-sorted sperm for contributing to animal welfare and for maximizing economic success. Bio economic modelling studies have shown that insemination with sex-sorted sperm in expanding herds can be used to increase farm profitability despite reduced fertility[105–107]. However, for the widespread adoption of these technology, further improvements before and after sex-sorting will have to be implemented, which mitigate the effects of sex-sorting on structure and function of the spermatozoon and support the maintenance of sperm fertilizing capacity.

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References

1. Cran, D. G., Johnson, L. A., Miller, N. G., Cochrane, D. & Polge, C. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilisation. *Vet. Rec.* **132**, 40–41. https://doi.org/10.1136/vr.132.2.40 (1993).
2. Zabel, F., Putzenlechner, B. & Mauser, W. Global agricultural land resources—A high resolution suitability evaluation and its perspectives until 2100 under climate change conditions (vol 9, e107522, 2014). *PLoS ONE* https://doi.org/10.1371/journal.pone.0114980 (2014).
3. Holden, S. A. & Butler, S. T. Review: Applications and benefits of sexed semen in dairy and beef herds. *Animal* **12**, s97–s103. https://doi.org/10.1017/S1751731118000721 (2018).
4. Pinkel, D. L. S., Gledhill, B. L., Van Dilla, M. A., Stephenson, D. & Watchmaker, G. High resolution DNA content measurements of mammalian sperm. *Cytometry* **3**, 1–9. https://doi.org/10.1002/cyto.990030103 (1982).
5. Garner, D. L. *et al.* Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. *Biol. Reprod.* **28**, 312–321. https://doi.org/10.1095/biolreprod28.2.312 (1983).
6. Johnson, L. A., Flook, J. P. & Look, M. V. Flow-Cytometry of X-Chromosome-Bearing and Y-Chromosome-Bearing Sperm for DNA Using an Improved Preparation Method and Staining with Hoechst-33342. *Gamete Res.* **17**, 203–212. https://doi.org/10.1002/mrd.1120170303 (1987).
7. Johnson, L. A., Flook, J. P. & Hawk, H. W. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol. Reprod.* **41**, 199–203. https://doi.org/10.1095/biolreprod41.2.199 (1989).
8. Johnson, L.A. Methods to preselect the sex offspring. US 5135759 (1992).
9. Seidel, G. E. A. C. H., Brink, Z., Holland, M. D. & Cattell, M. B. Insemination of heifers with very low numbers of frozen spermatozoa. *J. Anim. Sci.* **74**, 235 (1996).
10. Seidel, G. E. A. C. H. *et al.* Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. *Theriogenology* **48**, 1255–1264. https://doi.org/10.1016/s0093-691x(97)00368-3 (1997).
11. Seidel, G. E. Jr. Update on sexed semen technology in cattle. *Animal* **8**(Suppl 1), 160–164. https://doi.org/10.1017/S1751731114000202 (2014).
12. Johnson, L. A. Gender preselection in domestic animals using flow cytometrically sorted sperm. *J. Anim. Sci.* **70**, 8–18. https://doi.org/10.2527/1992.70suppl_28x (1992).
13. Johnson, L. A., Welch, G. R. & Rens, W. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. *J. Anim. Sci.* **77**(Suppl 2), 213–220. https://doi.org/10.2527/1999.77suppl_2213x (1999).
14. Garner, D. L. & Seidel, G. E. Jr. History of commercializing sexed semen for cattle. *Theriogenology* **69**, 886–895. https://doi.org/10.1016/j.theriogenology.2008.01.006 (2008).
15. Suh, T. K., Schenk, J. L. & Seidel, G. E. Jr. High pressure flow cytometric sorting damages sperm. *Theriogenology* **64**, 1035–1048. https://doi.org/10.1016/j.theriogenology.2005.02.002 (2005).
16. Sharpe, J. C. & Evans, K. M. Advances in flow cytometry for sperm sexing. *Theriogenology* **71**, 4–10. https://doi.org/10.1016/j.theriogenology.2008.09.021 (2009).
17. González-Marín, C. *et al.* In vitro sperm quality and DNA integrity of SexedULTRATM sex-sorted sperm compared to non-sorted bovine sperm. *Theriogenology* **114**, 40–45. https://doi.org/10.1016/j.theriogenology.2018.09.039 (2018).
18. Thomas, J. M. *et al.* Evaluation of SexedULTRA 4M (TM) sex-sorted semen in timed artificial insemination programs for mature beef cows. *Theriogenology* **123**, 100–107. https://doi.org/10.1016/j.theriogenology.2018.09.039 (2019).
19. Maicas, C. *et al.* Fertility of frozen sex-sorted sperm at 4×10^6 sperm per dose in lactating dairy cows in seasonal-calving pasture-based herds. *J. Dairy Sci.* **103**, 929–939. https://doi.org/10.3168/jds.2019-17131 (2019).
20. Pirez, M. C., Steele, H., Reese, S. & Kolle, S. Bovine sperm-oviduct interactions are characterized by specific sperm behaviour, ultrastructure and tubal reactions which are impacted by sex sorting. *Sci. Rep.* https://doi.org/10.1038/s41598-020-73592-1 (2020).

21. Carvalho, J. O. *et al.* Flow cytometry sex sorting affects bull sperm longevity and comprises their capacity to bind to oviductal cells. *Livest. Sci.* **207**, 30–37. https://doi.org/10.1016/j.livsci.2017.11.005 (2018).
22. Carvalho, J. O., Sartori, R., Machado, G. M., Mourao, G. B. & Dode, M. A. N. Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in in vitro embryo production. *Theriogenology* **74**, 1521–1530. https://doi.org/10.1016/j.theriogenology.2010.06.030 (2010).
23. Holden, S. A. *et al.* In vitro characterisation of fresh and frozen sex-sorted bull spermatozoa. *Reprod. Fertil. Dev.* **29**, 1415–1425. https://doi.org/10.1071/RD16086 (2017).
24. Pontes, J. H. F. *et al.* Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. *Theriogenology* **74**, 1349–1355. https://doi.org/10.1016/j.theriogenology.2010.06.004 (2010).
25. Pellegrino, C. A. G. *et al.* Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of in vitro-produced embryos in cattle. *Theriogenology* **86**, 888–893. https://doi.org/10.1016/j.theriogenology.2016.03.010 (2016).
26. Merton, J. S., Haring, R. M., Stap, J., Hoebe, R. A. & Aten, J. A. Effect of flow cytometrically sorted frozen/thawed semen on success rate of in vitro bovine embryo production. *Theriogenology* **47**, 295. https://doi.org/10.1016/S0093-691X(97)82422-3 (1997).
27. Lu, K. H., Cran, D. G. & Seidel, G. E. Jr. In vitro fertilization with flow-cytometrically-sorted bovine sperm. *Theriogenology* **52**, 1393–1405. https://doi.org/10.1016/s0093-691x(99)00225-3 (1999).
28. Palma, G. A., Olivier, N. S., Neumuller, C. & Sinowatz, F. Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts. *Anat. Histol. Embryol.* **37**, 67–73. https://doi.org/10.1111/j.1439-0264.2007.00795.x (2008).
29. Wilson, R. D. *et al.* In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows. *J. Dairy Sci.* **88**, 776–782. https://doi.org/10.3168/jds.S0022-0302(05)72741-7 (2005).
30. Blondin, P. *et al.* Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* **71**, 30–38. https://doi.org/10.1016/j.theriogenology.2008.09.017 (2009).
31. Steele, H., Makri, D., Maalouf, W. E., Reese, S. & Kolle, S. Bovine sperm sexing alters sperm morphokinetics and subsequent early embryonic development. *Sci. Rep.* **10**, 6255. https://doi.org/10.1038/s41598-020-63077-6 (2020).
32. Mikkola, M., Andersson, M. & Taponen, J. Transfer of cattle embryos produced with sex-sorted semen results in impaired pregnancy rate and increased male calf mortality. *Theriogenology* **84**, 1118–1122. https://doi.org/10.1016/j.theriogenology.2015.06.012 (2015).
33. Siqueira, L. G. B., Dikmen, S., Ortega, M. S. & Hansen, P. J. Postnatal phenotype of dairy cows is altered by in vitro embryo production using reverse X-sorted semen. *J. Dairy Sci.* **100**, 5899–5908. https://doi.org/10.3168/jds.2016-12539 (2017).
34. Lenz, R. W. *et al.* SexedULTRA^{TM}, A new method of processing sex-sorted bovine sperm improves conception rates. *Reprod. Fertil. Dev.* **29**, 203–204. https://doi.org/10.1071/RDv29n1Ab190 (2016).
35. Crites, B. R. *et al.* Conception risk of beef cattle after fixed-time artificial insemination using either SexedUltra (TM) 4M sex-sorted semen or conventional semen. *Theriogenology* **118**, 126–129. https://doi.org/10.1016/j.theriogenology.2018.05.003 (2018).
36. Dawod, A. & Elbaz, H. T. Effect of sexed semen, puberty and breeding ages on fertility of Holstein dairy heifers treated with double Ovsynch protocol. *Trop. Anim. Health Prod.* **52**, 2925–2930. https://doi.org/10.1007/s11250-020-02306-6 (2020).
37. Maicas, C. *et al.* Fertility of fresh and frozen sex-sorted semen in dairy cows and heifers in seasonal-calving pasture-based herds. *J. Dairy Sci.* **102**, 10530–10542. https://doi.org/10.3168/jds.2019-16740 (2019).
38. Oikawa, K. *et al.* Effects of use of conventional and sexed semen on the conception rate in heifers: A comparison study. *Theriogenology* **135**, 33–37. https://doi.org/10.1016/j.theriogenology.2019.06.012 (2019).
39. Drake, E. *et al.* Evaluation of delayed timing of artificial insemination with sex-sorted sperm on pregnancy per artificial insemination in seasonal-calving, pasture-based lactating dairy cows. *J. Dairy Sci.* **103**, 12059–12068. https://doi.org/10.3168/jds.2020-18847 (2020).
40. Karakaya, E. *et al.* Fertility in dairy cows after artificial insemination using sex-sorted sperm or conventional semen. *Reprod. Domest. Anim.* **49**, 333–337. https://doi.org/10.1111/rda.12280 (2014).
41. Andersson, M., Taponen, J., Kommeri, M. & Dahlbom, M. Pregnancy rates in lactating Holstein-Friesian cows after artificial insemination with sexed sperm. *Reprod. Domest. Anim.* **41**, 95–97. https://doi.org/10.1111/j.1439-0531.2006.00625.x (2006).
42. Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & Grp, P. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *J. Clin. Epidemiol.* **62**, 1006–1012. https://doi.org/10.1016/j.jclinepi.2009.06.005 (2009).
43. Sterne, J. A. & Egger, M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. *J. Clin. Epidemiol.* **54**, 1046–1055. https://doi.org/10.1016/s0895-4356(01)00377-8 (2001).
44. Hooijmans, C. R., IntHout, J., Ritskes-Hoitinga, M. & Rovers, M. M. Meta-analyses of animal studies: An introduction of a valuable instrument to further improve healthcare. *Ilar J.* **55**, 418–426. https://doi.org/10.1093/ilar/ilu042 (2014).
45. Deeks, J. J., Higgins, J. P. T., & Altman, D. G. Identifying and measuring heterogenity. in *Cochrane Handbook for Systematic Reviews of Interventions: Version 5* (ed. Higgins, J. P. T. & Green, S.) 243–296 https://training.cochrane.org/handbook/archive/v5.0.0 (2008).
46. Deeks, J. J. & Higgins, J.P.T. Statistical Algorithms in Review Manager 5. https://training.cochrane.org/handbook/statistical-methods-revman5 (2010).
47. Riley, R. D., Higgins, J. P. T. & Deeks, J. J. Interpretation of random effects meta-analyses. *Br Med J* **342**, 549. https://doi.org/10.1136/bmj.d549 (2011).
48. Deeks, J. J. H., J.P.T., Altman, D.G. in *Cochrane Handbook for Systematic Reviews of Interventions* (ed J. P. T.; Thomas Higgins, J.; Chandler, J.; Cumpston, M.L.T.; Page, M.L.; Welch, V.A.) Ch. 10, (2019).
49. Abdalla, H., Ali, M. A. & El-Tarabany, M. S. Fertility of commercial sexed semen and the economic analyses of its application in Holstein heifers. *Adv. Anim. Vet. Sci.* **2**, 535–542. https://doi.org/10.14737/journal.aavs/2014/2.9.535.542 (2014).
50. An, L. *et al.* Fertility in single-ovulating and superovulated dairy heifers after insemination with lose dose sex-sorted sperm. *Reprod. Domest. Anim.* **45**, 344–350. https://doi.org/10.1111/j.1439-0531.2009.01574.x (2010).
51. Bodmer, M. *et al.* Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions. *Theriogenology* **64**, 1647–1655. https://doi.org/10.1016/j.theriogenology.2005.04.011 (2005).
52. Borchersen, S. & Peacock, M. D. Danish A.I. field data with sexed semen. *Theriogenology* **71**, 59–63. https://doi.org/10.1016/j.theriogenology.2008.09.026 (2009).
53. Chebel, R. C., Guagnini, F. S., Santos, J. E., Fetrow, J. P. & Lima, J. R. Sex-sorted semen for dairy heifers: Effects on reproductive and lactational performances. *J. Dairy Sci.* **93**, 2496–2507. https://doi.org/10.3168/jds.2009-2858 (2010).
54. Chebel, R. C. & Cunha, T. Optimization of timing of insemination of dairy heifers inseminated with sex-sorted semen. *J. Dairy Sci.* **103**, 5591–5603. https://doi.org/10.3168/jds.2019-17870 (2020).
55. Colazo, M. G. & Mapletoft, R. J. Pregnancy per AI in Holstein heifers inseminated with sex-selected or conventional semen after estrus detection or timed-AI. *Can. Vet. J.* **58**, 365–370 (2017).
56. Cooke, R., Bohnert, D., Cappellozza, B., Mueller, C. & DelCurto, T. Incorporation of sexed semen into reproductive management of range cow-calf operations. *Reprod. Domest. Anim.* **47**, 420–420 (2012).
57. DeJarnette, J. M., Nebel, R. L. & Marshall, C. E. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology* **71**, 49–58. https://doi.org/10.1016/j.theriogenology.2008.09.042 (2009).

58. DeJarnette, J. M. *et al.* Effects of 2.1 and 3.5x10(6) sex-sorted sperm dosages on conception rates of Holstein cows and heifers. *J. Dairy Sci.* **93**, 4079–4085. https://doi.org/10.3168/jds.2010-3181 (2010).
59. DeJarnette, J. M. *et al.* Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen plausible?. *J. Dairy Sci.* **94**, 3477–3483. https://doi.org/10.3168/jds.2011-4214 (2011).
60. Djedovic, R. *et al.* Involuntary reduction in vigour of calves born from sexed semen. *Acta Vet. Hung.* **64**, 229–238. https://doi.org/10.1556/004.2016.023 (2016).
61. Dominguez, J. H. E., Costa, D. S., Centurion, V. J. & Brumatti, R. C. Análise marginal dos custos da utilização de sêmen sexado macho para produção de touros nelore. *Archivos de Zootecnia* **61**, 111–118 (2012).
62. Duarte, A. M., Diniz, E. G., Mattos Nascimento, M. R. B., Tavares, M. & Resende Franco, V. R. Pregnancy rates in heifers inseminated with semen of nelore bulls sexed by flow cytometry. *Ciência Animal Brasileira* **8**, 566–573 (2007).
63. Frijters, A. C. J. *et al.* What affects fertility of sexed bull semen more, low sperm dosage or the sorting process?. *Theriogenology* **71**, 64–67. https://doi.org/10.1016/j.theriogenology.2008.09.025 (2009).
64. Healy, A. A., House, J. K. & Thomson, P. C. Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. *J. Dairy Sci.* **96**, 1905–1914. https://doi.org/10.3168/jds.2012-5465 (2013).
65. Holden, S. A. *et al.* Relationship between in vitro sperm functional assessments, seminal plasma composition, and field fertility after AI with either non-sorted or sex-sorted bull semen. *Theriogenology* **87**, 221–228. https://doi.org/10.1016/j.theriogenology.2016.08.024 (2017).
66. Joezy-Shekalgorabi, S., Maghsoudi, A. & Mansourian, M. R. Reproductive performance of sexed versus conventional semen in Holstein heifers in various semiarid regions of Iran. *Ital J Anim Sci* **16**, 666–672. https://doi.org/10.1080/1828051x.2017.1321473 (2017).
67. Ketchum, J. N. *et al.* Evaluation of later timepoints for split-time artificial insemination when using sex-sorted semen among beef heifers following the 14-d CIDR (R)-PG protocol. *Anim. Reprod. Sci.* https://doi.org/10.1016/j.anireprosci.2020.106649 (2021).
68. Klinc, P., Frese, D., Osmers, H. & Rath, D. Insemination with sex sorted fresh bovine spermatozoa processed in the presence of antioxidative substances. *Reprod. Domest. Anim.* **42**, 58–62. https://doi.org/10.1111/j.1439-0531.2006.00729.x (2007).
69. Kurykin, J. *et al.* Effect of insemination-related factors on pregnancy rate using sexed semen in Holstein heifers. *Czech J. Anim. Sci.* **61**, 568–577. https://doi.org/10.17221/12/2016-Cjas (2016).
70. Mallory, D. A., Lock, S. L., Woods, D. C., Poock, S. E. & Patterson, D. J. Hot topic: Comparison of sex-sorted and conventional semen within a fixed-time artificial insemination protocol designed for dairy heifers. *J. Dairy Sci.* **96**, 854–856. https://doi.org/10.3168/jds.2012-5850 (2013).
71. Mellado, M., Coronel, F., Estrada, A. & Rios, F. G. Fertility in Holstein x Gyr cows in a subtropical environment after insemination with Gyr sex-sorted semen. *Trop. Anim. Health Prod.* **42**, 1493–1496. https://doi.org/10.1007/s11250-010-9585-3 (2010).
72. Mellado, M. *et al.* Effects of month of breeding on reproductive efficiency of Holstein cows and heifers inseminated with sex-sorted or conventional semen in a hot environment. *Trop Anim Health Pro* **46**, 265–269. https://doi.org/10.1007/s11250-013-0470-8 (2014).
73. Naniwa, Y., Ueda, M. & Uchiyama, K. Production technology and application of bovine sexed semen in Japan. *Jpn. J. Embryo Transf.* **39**, 169–175 (2017).
74. Noonan, E. J., Kelly, J. C. & Beggs, D. S. Factors associated with fertility of nulliparous dairy heifers following a 10-day fixed-time artificial insemination program with sex-sorted and conventional semen. *Aust. Vet. J.* **94**, 145–148. https://doi.org/10.1111/avj.12430 (2016).
75. Norman, H. D., Hutchison, J. L. & Miller, R. H. Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. *J. Dairy Sci.* **93**, 3880–3890. https://doi.org/10.3168/jds.2009-2781 (2010).
76. Sá Filho, M. F. *et al.* Optimizing the use of sex-sorted sperm in timed artificial insemination programs for suckled beef cows. *J. Anim. Sci.* **90**, 1816–1823. https://doi.org/10.2527/jas.2011-4523 (2012).
77. Sales, J. N. S. *et al.* Timing of insemination and fertility in dairy and beef cattle receiving timed artificial insemination using sex-sorted sperm. *Theriogenology* **76**, 427–435. https://doi.org/10.1016/j.theriogenology.2011.02.019 (2011).
78. Schenk, J. L., Cran, D. G., Everett, R. W. & Seidel, G. E. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. *Theriogenology* **71**, 717–728. https://doi.org/10.1016/j.theriogenology.2008.08.016 (2009).
79. Seidel, G. E. *et al.* Insemination of heifers with sexed sperm. *Theriogenology* **52**, 1407–1420. https://doi.org/10.1016/S0093-691x(99)00226-5 (1999).
80. Seidel, G. E. Jr. & Schenk, J. L. Pregnancy rates in cattle with cryopreserved sexed sperm: effects of sperm numbers per inseminate and site of sperm deposition. *Anim. Reprod. Sci.* **105**, 129–138. https://doi.org/10.1016/j.anireprosci.2007.11.015 (2008).
81. Thomas, J. M. *et al.* Delayed insemination of nonestrous cows improves pregnancy rates when using sex-sorted semen in timed artificial insemination of suckled beef cows. *J. Anim. Sci.* **92**, 1747–1752. https://doi.org/10.2527/jas.2013-7131 (2014).
82. Thomas, J. M. *et al.* Effective use of SexedULTRA (TM) sex-sorted semen for timed artificial insemination of beef heifers. *Theriogenology* **98**, 88–93. https://doi.org/10.1016/j.theriogenology.2017.03.018 (2017).
83. Tubman, L. M., Brink, Z., Suh, T. K. & Seidel, G. E. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. *J. Anim. Sci.* **82**, 1029–1036 (2004).
84. Xu, Z. Z. Application of liquid semen technology improves conception rate of sex-sorted semen in lactating dairy cows. *J. Dairy Sci.* **97**, 7298–7304. https://doi.org/10.3168/jds.2014-8507 (2014).
85. Murphy, C., Shalloo, L., Hutchinson, I. A. & Butler, S. T. Expanding the dairy herd in pasture-based systems: The role of sexed semen within alternative breeding strategies. *J. Dairy Sci.* **99**, 6680–6692. https://doi.org/10.3168/jds.2015-10378 (2016).
86. Sorensen, M. K., Voergaard, J., Pedersen, L. D., Berg, P. & Sorensen, A. C. Genetic gain in dairy cattle populations is increased using sexed semen in commercial herds. *J. Anim. Breed. Genet.* **128**, 267–275. https://doi.org/10.1111/j.1439-0388.2011.00924.x (2011).
87. Seidel, G. E. Jr. & Garner, D. L. Current status of sexing mammalian spermatozoa. *Reproduction* **124**, 733–743. https://doi.org/10.1530/rep.0.1240733 (2002).
88. Vishwanath, R. & Moreno, J. F. Review: Semen sexing—current state of the art with emphasis on bovine species. *Animal* **12**, s85–s96. https://doi.org/10.1017/S1751731118000496 (2018).
89. Boe-Hansen, G. B., Morris, I. D., Ersboll, A. K., Greve, T. & Christensen, P. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology* **63**, 1789–1802. https://doi.org/10.1016/j.theriogenology.2004.08.004 (2005).
90. Balao da Silva, C. M., Ortega-Ferrusola, C., Morrell, J. M., Rodriguez Martinez, H. & Pena, F. J. Flow cytometric chromosomal sex sorting of stallion spermatozoa induces oxidative stress on mitochondria and genomic DNA. *Reprod. Domest. Anim.* **51**, 18–25. https://doi.org/10.1111/rda.12640 (2016).
91. Leahy, T. *et al.* Flow-sorted ram spermatozoa are highly susceptible to hydrogen peroxide damage but are protected by seminal plasma and catalase. *Reprod. Fertil. Dev.* **22**, 1131–1140. https://doi.org/10.1071/RD09286 (2010).
92. Balao da Silva, C. M. *et al.* Sex sorting increases the permeability of the membrane of stallion spermatozoa. *Anim. Reprod. Sci.* **138**, 241–251. https://doi.org/10.1016/j.anireprosci.2013.02.021 (2013).

93. Moce, E., Graham, J. K. & Schenk, J. L. Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. *Theriogenology* **66**, 929–936. https://doi.org/10.1016/j.theriogenology.2006.01.063 (2006).
94. Walsh, S. W., Williams, E. J. & Evans, A. C. A review of the causes of poor fertility in high milk producing dairy cows. *Anim. Reprod. Sci.* **123**, 127–138. https://doi.org/10.1016/j.anireprosci.2010.12.001 (2011).
95. Crowe, M. A. Resumption of ovarian cyclicity in post-partum beef and dairy cows. *Reprod. Domest. Anim.* **43**(Suppl 5), 20–28. https://doi.org/10.1111/j.1439-0531.2008.01210.x (2008).
96. Veerkamp, R. F., Beerda, B. & van der Lende, T. Effects of genetic selection for milk yield on energy balance, levels of hormones, and metabolites in lactating cattle, and possible links to reduced fertility's. *Livest. Prod. Sci.* **83**, 257–275. https://doi.org/10.1016/S0301-6226(03)00108-8 (2003).
97. Berry, D. P., Wall, E. & Pryce, J. E. Genetics and genomics of reproductive performance in dairy and beef cattle. *Animal* **8**(Suppl 1), 105–121. https://doi.org/10.1017/S1751731114000743 (2014).
98. Barkema, H. W. *et al.* Changes in the dairy industry affecting dairy cattle health and welfare. *J. Dairy Sci.* **98**, 7426–7445. https://doi.org/10.3168/jds.2015-9377 (2015).
99. Polsky, L. & von Keyserlingk, M. A. G. Effects of heat stress on dairy cattle welfare. *J. Dairy Sci.* **100**, 8645–8657. https://doi.org/10.3168/jds.2017.12651 (2017).
100. Fricke, P. M. Scanning the future—Ultrasonography as a reproductive management tool for dairy cattle. *J. Dairy Sci.* **85**, 1918–1926. https://doi.org/10.3168/jds.S0022-0302(02)74268-9 (2002).
101. Holman, A. *et al.* Comparison of oestrus detection methods in dairy cattle. *Vet. Rec.* **169**, 47. https://doi.org/10.1136/vr.d2344 (2011).
102. Simoes, R. *et al.* Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome. *Reproduction* **146**, 433–441. https://doi.org/10.1530/REP-13-0123 (2013).
103. Bittner, L. *et al.* Negative effects of oxidative stress in bovine spermatozoa on in vitro development and DNA integrity of embryos. *Reprod. Fertil. Dev.* **30**, 1359–1368. https://doi.org/10.1071/RD17533 (2018).
104. Ghaleno, L. R., Valojerdi, M. R., Hassani, F., Chehrazi, M. & Janzamin, E. High level of intracellular sperm oxidative stress negatively influences embryo pronuclear formation after intracytoplasmic sperm injection treatment. *Andrologia* **46**, 1118–1127. https://doi.org/10.1111/and.12202 (2014).
105. McCullock, K. *et al.* Factors affecting economics of using sexed semen in dairy cattle. *J. Dairy Sci.* **96**, 6366–6377. https://doi.org/10.3168/jds.2013-6672 (2013).
106. Ettema, J. F. *et al.* Economic opportunities for using sexed semen and semen of beef bulls in dairy herds. *J. Dairy Sci.* **100**, 4161–4171. https://doi.org/10.3168/jds.2016-11333 (2017).
107. Butler, S. T., Hutchinson, I. A., Cromie, A. R. & Shalloo, L. Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal* **8**(Suppl 1), 165–172. https://doi.org/10.1017/S1751731114000664 (2014).

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Author contributions
Conception and design of work: S.R. and S.K. Acquisition of data: M.C.P., S.R., S.K. Statistical Analyses: S.R. Interpretation of data: S.R., S.K., M.C.P., H.S. Drafting and revision of the manuscript: S.R., M.C.P., H.S., S.K.

Competing interests
The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to S.K.

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