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# The Use Of Sex-Sorted Sperm For Reproductive Programs In cattle

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## 1. Introduction

The biotechnology of sex selection in animals is one of the most studied and also misunderstood in the history. According Garner and Seidel [1], Democratis (470 – 402 AC) had suggested that the right testicle produces male sperm and the left female. For sure that is not true, once there are two sperm populations on the mammals ejaculate, being a portion of it carrier the X sexual chromosome and other Y. During years, researchers have been trying to manipulate the sex of the offspring before conception [1]. The selection of desired sex delivered can be one of the determining factors to increase the genetic progress and farmer profitability in either beef or dairy cattle. For example, in dairy farms, the male calf has little or any zootechnical or economic value. However, in beef farms, the male calf is the product of interest due to its increased potential to produce meat. Considering these particularities, many researches have been developed to predict and/or manipulate the calf sex proportion. The separation of the Y sperm from the X is possible due to the differences on the DNA content of these spermatic cells (X sperm has about 4% more genetic material than Y) by flow cytometry. Nowadays, this is the most efficient method to separate X from Y-spermatozoa in large scale. Some available biotechnologies in commercial scale are the use of the sex-sorted sperm by artificial insemination (AI) with estrus detection, timed artificial insemination (TAI), embryo transfer with superovulation (ET) and timed embryo transfer (TET).

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Reproductive programs based on TAI, have been continuously incorporated in routine of the reproductive management on farms. These programs represent a systematic approach to enhance the use of AI in dairy or beef farms, increasing the benefits of this reproductive biotechnology.

The ET technique has been used widely around the world once it increases the number of offspring that can be obtained from females with great genetic value [2, 3]. The use of sex-sorted sperm could increase the production of a specific calf gender, which would benefit beef and dairy industries worldwide [4]. Likewise, the TET synchronizes the ovulation similar to TAI; however, instead to inseminate before ovulation, the recipient cow receive an embryo fertilized *in vitro* with sex-sorted sperm seven days after ovulation.

Advances in sex sorting of sperm using flow cytometry have enabled its incorporation into commercial reproductive management. Despite the increased use of sex-sorted sperm, pregnancy per AI (P/AI) is still less than when using non sex-sorted sperm [5]. Regardless of these reduced results, suitable spermatozoa concentration at AI time; longer intervals from the induction of ovulation to the AI (i.e., closer to the expected moment of ovulation); AI into the uterine horn ipsilateral to the expected ovulation; the size of the follicle from which ovulation occurs; occurrence of estrus from progesterone (P4) source removal to the TAI and the identification and use of bulls with proven fertility producing spermatozoa resistant to the sexing process have increased the likelihood of pregnancy in females inseminated with sex-sorted sperm [6-8], thereby optimizing the use of sex-sorted sperm in TAI and ET programs.

There is a huge interest in sex-sorted sperm around the world. There are many opportunities and challenges associated to the use of this semen in farms. The aim of this review is to bring into focus a summary of our current understanding of the use of sex-sorted sperm in TAI and ET programs and some strategies to optimize the use of sex-sorted sperm. Before describing the researches results and opportunities for its use, it is important to understand how sperm are sorted and the critical points associated to the process.

## 2. Basic principles of sexing

The biotechnology of sex sorting is based on the information that X-sperm has about 4% more genetic material than Y-sperm. In this manner, the flow cytometry associates the laser, differential coloration of the viable and non-viable spermatozoa and hydrodynamic force which direct the sperm at the moment of the reading during the process of X and Y sperm separation. Moreover, there are differences among bovine breeds according to the amount of DNA present in the Y chromosome. According Garner [9], the X-Y sperm nuclei DNA content difference (%) is: 4.22 for Jersey, 4.07 for Angus, 4.01 for Holstein, 3.98 for Hereford and 3.7 for Brahman. Such differences, do not determine the fertility after sexing process. These differences mainly determine the speed and efficiency of the sexed semen production and have to be considered when using flow cytometry.

Recent advances in the form of the tip of the flow cytometry, the positioning of the sperm cells at the moment of the passage through the laser, as well as changes in pressure and the type of staining cells have significantly improved separation process gametes X and Y [9]. The X-Y sperm separation speed is relatively slow with approximately 300,000 to 400,000 cells per minute. In this way, for a higher process efficacy, the semen dose in the straw normally used is set to be $2.1 \times 10^6$ cells in a 0.25cc straw.

The process of sorting X and Y-bearing sperm likely results in some damage to the sperm that compromise fertilization [4, 10]. According to Gonsálvez et al. [11], the sorting process produce an interaction of the DNA with fluorophores, laser exposure, spermatozoa separation in micro-droplets, acceleration of spermatozoa through geometrically-pressured fluid channels and centrifugation. All of these para-biological spermatozoa-media or mechanical interactions would theoretically have the potential to produce changes in cell structures, including the DNA molecule. When considering cell structures, spermatozoa appear to be partially capacitated during the flow cytometry process used for sex pre-determination [12]. This total or partial capacitation is induced by the conditions that sperm are subjected during preparation for flow cytometric-sorting and during sorting [13]. Lu and Seidel [12] emphasize that it could be due to the condition that the sperm are pre-incubated with Hoechst 33342 at 34.5 8C for 45 min before sorting. During sorting, sperm are subjected to laser light and various physical forces, such as exiting the sorter at nearly 90 km/h before entering the collecting medium. The process of sorting results in an extremely diluted sample with 800,000 sperm/ml, and subsequently sperm are gently centrifuged to provide a concentrated sample suitable for packaging and cryopreservation.

Thus, this process could also led to a shorter functional sperm life compared to non-sorted sperm [12, 14, 15], which could include pre-capacitation and a reduced number of viable spermatozoa for the insemination [6, 16]. The thermo-resistance test showed that the motility decline in sex-sorted sperm was faster compared to non-sorted sperm. Also, there is an effect associated with samples from a specific bull and sex-sorted sperm insemination dose [5] and some samples from certain bulls can tolerate the stress of sorting in a more desirable manner [17].

Although the above information stated, it is important to highlight that AI with sex-sorted sperm does not alter pattern of return to estrus and does not affect the likelihood of heifers to conceive from subsequent AI [18]. Also, Holstein heifers inseminated with sexed semen had similar pregnancy loss from 29 ± 1 to 50 ± 1 d after AI compared with heifers inseminated with conventional semen [17], and there is no difference on abortion rate from 2 mo of gestation to parturition. Farmers in general are interested to know if calves produced by sexed semen are different that those from conventional semen. To address this question Tubman et al. [19] analyzed data from 1,169 calves produced from sexed semen and 793 calves from conventional semen. They did not observed difference in gestation length, birth weight, calving ease, calf vigor, weaning weight, abortion rate, and death rates (neonatal and through weaning) among calves produced by sexed or conventional semen. When *in vitro* models are used to verify the efficiency of sex-sorted sperm to produce embryos, there are inconsistent results concerning the embryo development which mainly depend on the

sire used [20-22]. In general, P/AI of females inseminated with sex-sorted is not resultant from increased late embryonic and fetal losses. Therefore, calves produced from sexed semen grew and developed normally both pre- and postnatally.

## 3. Sperm concentration for sex-sorted sperm in reproductive programs

Commercially, the established spermatozoa number by dose of sexed semen is $2.1 \times 10^6$ cells/dose. This amount is much lower than that from conventional semen ($\sim 20 \times 10^6$ cells/dose). To achieve this proportion many studies have been done to specify the best straw concentration considering commercial aspects. Holstein heifers and cows have same conception rate when inseminated with sex-sorted sperm in a dose of 2.1 or $3.5 \times 10^6$ sperm/dose; however, not only in heifers but also in cows, there is an increasing on conception when AI is performed with conventionally processed sperm ($15 \times 10^6$ sperm/dose) as presented on Table 1.

| | Sex-sorted sperm (dose) | | Non sex-sorted sperm (dose) |
|---|---|---|---|
| | $2.1 \times 10^6$ | $3.5 \times 10^6$ | $15 \times 10^6$ |
| Heifers (%) | 43.9[a] (2,752/6,268) | 45.7[a] (2,864/6,268) | 60.7[b] (3,805/6,268) |
| Cows (%) | 23.0[a] (1,257/5,466) | 25,4[a] (1,388/5,466) | 31,5[b] (1,722/5,466) |

**Table 1.** Different superscript letters ([a, b]) in row indicates statistical difference (P < 0.01).Adapted from Dejarnette et al. [23].Conception rates Holstein heifers and cows after artificial insemination with 2.1 or $3.5 \times 10^6$ sex-sorted sperm or $15 \times 10^6$ non sex-sorted sperm.

Dejarnette et al. [24] compared the effects of sperm dosage (2.1 vs. $10 \times 10^6$ sperm/dose) and sex-sorting (conventional vs. sexed) on conception rates of Holstein heifers (n = 9,172) and observed difference among groups as follows on Table 2. In a field trial study with three different breeds (Holstein, Jersey and Danish Red) Borchersen and Peacock [25] observed a reduction of 12% for Holstein, 7% for Jersey, and 5% for Danish Red in conception rate using sexed semen.

| | Sperm dosage | |
|---|---|---|
| | $2.1 \times 10^6$ | $10 \times 10^6$ |
| Sex-sorted sperm (%) | 38[a] (881/2,319) | 44[c] (1,003/2,279) |
| Non sex-sorted sperm (%) | 55[b] (1,255/2,282) | 60[d] (1,375/2,292) |

**Table 2.** Different superscript letters ([a, b, c] and [d]) indicates statistical difference (P < 0.01).Adapted from Dejarnette et al. [24].Conception rates of Holstein heifers according semen type (sexed vs. conventional) and sperm dosage combinations (2.1 vs. $10 \times 10^6$ sperm/dose).

In a combination of some experiments, Seidel et al. [26] observed that the conception rate of Holstein heifers inseminated with sex-sorted sperm vary from 40% to 68%, and with non-sex-sorted sperm vary from 67% to 82%. Also, Seidel and Schenk [17] observed a lower pregnancy rate when using sex-sorted sperm (31% to 42%) than non sex-sorted sperm (43% to 62%). Although the greater variability on the pregnancy outcomes of cattle inseminated of with sex-sorted sperm by literature, most part of the researches with heifers indicates that conception rate after AI upon estrous detection with sex-sorted sperm is about 70% to 90% (according to the farms handling) from the conception obtained following the use of conventional semen [27]. In accordance, Sá Filho et al. [28] showed that overall P/AI rates were reduced with sex-sorted sperm compared with non sex-sorted sperm (i.e., 83.8% pregnancy was obtained with the non-sex-sorted sperm). This reduced P/AI could be attributable to several factors including a shorter lifespan in the female reproductive tract, reduced number of sperm per straw, and sperm damage from the staining, identification, and separation processes [5, 6, 14]. The Table 3 summarizes the main studies with sex-sorted sperm, stating the pregnancy rate and the proportion of pregnancy sexed/conventional semen.

In a first report to evaluate the fertility of lactating dairy cows under field conditions, females where inseminated with same low concentration ($2 \times 10^6$) of sex-sorted or non sex-sorted frozen-thawed sperm [29], it was observed the same pregnancy per AI among females inseminated with sex-sorted (27.6%, n = 105) or non sex-sorted (28.1%, n = 64) sperm. Although the inconsistent results with cows presented by literature, most part of the researches with heifers indicates that conception rate after estrus detection by observation and AI with sex-sorted sperm is about 70% to 90% (it depends on to the farms handling) form the conception rate obtained by conventional semen [27].

Considering that maybe straw concentration would still be low, our research group performed an experiment inseminating Jersey heifers once or twice [7]. Aimed at, 576 virgin Jersey heifers were synchronized with two injections of PGF2$\alpha$ apart and had their estrus observed twice daily (based upon removal of tail-head chalk). The AI was performed with a single insemination dose ($2.1 \times 10^6$ sperm) 12 h after estrus detection (n=193), a double dose at 12 h (n=193), or a double dose involving insemination 12 and 24 h after estrus detection (n=190). It was not observed any effect of treatments on P/AI (87/193 = 45.1%, 85/193 = 44.0%, and 94/190 = 49.5%, respectively; P = 0.51). However, P/AI was influenced by the number of AI service (First, 115/208 = 55.3%[a]; Second, 94/204 = 46.1%[a]; and Third, 57/165 = 34.8%[b]; P = 0.004). Similar results have also been described by Dejarnette et al. [30] where, pregnancy rate has been reduced in heifers when the number of AI service has been increased (First service = 47%; Second = 39% ; Third = 32%). Accordingly, in Jersey heifers, the increasing on the spermatozoa number, 2.1 to 4.2 million, to be used in insemination after estrus detection and to perform a double insemination (12 hour interval) have not changed the conception rate.

The use of sex-sorted sperm in suckled beef cows in the post-partum have not been much explored scientifically. Most part of the papers use few cows per treatment, making the results inconclusive. A study in suckled Angus cows (n = 212), Doyle et al. [34] compared the following treatments:

1.  Frozen conventional semen (40 x $10^6$ sperm/dose) deposited at uterus corpus;

2.  Frozen conventional semen at low concentration (1 x $10^6$ sperm/dose);

3.  Frozen sexed semen (1 x $10^6$ sperm/dose);

4.  Cooled sexed semen (5 x $10^6$ sperm/dose).

Semen for the Treatment 1 was deposited into the uterus corpus. Semen for the other three treatments was shared where each half was deposited into each uterine horn. Pregnancy was lower for the sex-sorted sperm treatments (frozen = 23% and cooled = 25%) than for the non sex-sorted sperm (conventional = 67% and low concentration = 49%)

| Breed | Category | Pregnancy rate based on type of semen used in AI | | | |
| --- | --- | --- | --- | --- | --- |
| | | Conventional (%) (n/n) | Sexed (%) (n/n) | Proportion (%) | Reference |
| *Timed Artificial insemination* | | | | | |
| Beef | Cows | 54.2 (232/428) | 45.4 (193/425) | 83.7 | Sá Filho et al. [31] (Exp 1) |
| Beef | Cows | 54.7 (134/245) | 45.9 (113/246) | 83.9 | Sá Filho et al. [31] (Exp 2) |
| Beef | Cows | 51.8 (100/193) | 41.8 (82/196) | 80.7 | Sales et al. [8] |
| Beef | Cows | 55.3 (105/190) | 40.9 (79/193) | 74.0 | Sales et al. [8] |
| Dairy | Cows | 27.1 (44/162) | 13.0 (21/161) | 48.0 | Souza et al. 2006 unpublished data |
| *Artificial insemination with estrus detection* | | | | | |
| Beef | Heifers | 67.6 (96/142) | 53.7 (130/242) | 79.4 | Seidel and Schenk [17] (Exp.1) |
| Beef | Heifers | 67.0 (85/126) | 52.6 (129/245) | 78.5 | Seidel and Schenk [17] (Exp.2) |
| Dairy | Heifers | 60.0 (1375/2292) | 38.0 (881/2319) | 63.3 | DeJarnette et al. [24] |
| Dairy | Cows and Heifers | 37.7 (160/426) | 22.9 (51/223) | 60.7 | Mellado et al. [32] |
| Dairy | Heifers | 56.0 (30082/53718) | 45.0 (17893/39763) | 80.3 | DeJarnette et al. [30] |
| Dairy | Cows and Heifers | 37.4 (34/91) | 28.8 (38/132) | 77.0 | Bodmer et al. [29] |
| Dairy | Cows | 46.0 (69/149) | 21.0 (33/157) | 45.6 | Andersson et al. [33] |

| | | Pregnancy rate based on type of semen used in AI | | | |
|---|---|---|---|---|---|
| Dairy | Heifers | 60.0 (74/124) | 46.7 (114/244) | 77.8 | Seidel and Schenk [17] (Exp.4) |
| Dairy | Heifers | 62.0 (163/263) | 42.1 (225/534) | 67.9 | Seidel and Schenk [17] (Exp.5) |
| | Overall | 55.9% (32753/58549) | 44.3% (19982/45080) | 79.2 | |

**Table 3.** Pregnancy rate of females inseminated with conventional or sexed semen and the pregnancy proportion obtained by sexed semen based on conventional. (sexed/conventional).

In brief, the fertility of cows and heifers is influenced when the dose of the sex-sorted sperm is considerably increased [for example to 10 x 10$^6$ sperm/AI; [24]]. However, the high cost of increasing the insemination dose would make this commercially unviable. Certainly duo to the low sorting rate per hour provided by flow cytometry method. Nowadays, sexing companies just offer sexed semen in a dose of 2.1 x 10$^6$ sperm/straw, and studies have been done using this dose as pattern'. Also, data suggest a better use of sex-sorted sperm in the first/second service.

## 4. Timing for AI using non sex-sorted or sex-sorted sperm

### 4.1. AI following estrus detection

The optimal time at which insemination should take place relative to ovulation (IOI) depends primarily on the lifespan of spermatozoa and on the viability of the oocyte in the female genital tract [35]. Several experiments [36-38] have demonstrated that 6 h is the minimum time needed for a viable sperm population capable of fertilization to pass through the oviduct. Furthermore, the number of progressive motile sperm peaked from 8 to 18 h after insemination. In terms of the oocyte, the most desirable period for fertilization appears to be between 6 and 10 h after ovulation [39]. Also, Dransfield et al. [40] and Roelofs et al. [41] demonstrated that the probability that conception will occur decreases when AI is performed near the time of ovulation (less than 12 or 6 h before ovulation, respectively). According to Roelofs et al. [42], fertilization rate drastically decreases when AI occurs after ovulation. Artificial insemination should occur near the time of ovulation to maximize sperm access to the ovum, but not so late that an aging ovum awaits sperm arrival [43]. The ovulation occurs 28-30 h after the estrus beginning. The optimal AI time was between 24 and 12 h before ovulation for the most desirable rate of fertilization and 16–12 h for greatest percentage of greater quality embryos [89% of recovered embryos; [42]. More precisely, Maatje et al. [44] obtained an optimal pregnancy rate when AI was performed 16.2 h before ovulation.

In a review by Seidel et al. [26], crossbred beef heifers inseminated with sex-sorted sperm, present conception rate about 40%, lower than AI with non sex-sorted sperm (75%).

Sá Filho et al. [7] have been evaluated the different times to perform the AI. Thereby, 638 Jersey heifers have been inseminated after estrus detection according those times (12 a 16h; 16 a 20h; 20 a 24h e 24 a 30h) and the estrus has been detected using radio telemetry (Heat Watch®). The P/AI of heifers inseminated from 12 to 16 h after the onset of estrus ($40/10^6$ = 37.7%) was less (P = 0.03) than those inseminated from 16.1 to 20 h (85/164 = 51.8%), and 20.1 to 24 h (130/234 = 55.6%). However, the P/AI for heifers inseminated from 24.1 to 30 h (61/134 = 45.5%) did not differ from that of any other interval.

Pharmacological manipulation is expected to increase the reproductive performance even in management with estrus detection. Exogenous GnRH given at the onset of estrus [45, 46] or concurrent with AI [47, 48] have improved fertility, but the effects have not been consistent [45-48]. Therefore, reproductive strategies to enhance fertility with exogenous hormones, optimizing estrus detection, or improving the timing of AI relative to estrus detection, could enhance the use of sexed semen in dairy cattle breeding. Following this idea, our group aimed to develop strategies to improve P/AI (35 to 42 d after AI) in virgin Jersey heifers bred by AI of sex-sorted sperm after being detected in estrus [7]. Nevertheless, giving 100 µg of GnRH at first detection of estrus, with AI 12 h later, did not affect P/AI in females with estrus detected by tail-head chalk [GnRH=47.2% (100/212) vs. No GnRH=51.7% (104/201); P = 0.38] or by radio telemetry [HeatWatch® ; GnRH=53.1% (137/258) vs. No GnRH=48.6% (122/251); P = 0.43]. In the referred study, GnRH treatments were done 7.4 h after the onset of estrus, identified by HeatWatch® system, due to the management schedule (i.e. twice daily 07:00 or 19:00). Previous studies demonstrated that the onset of estrus, the peak of the 17β-estradiol in plasma, and the release of the ovulatory LH surge occurred at approximately the same moment [49, 50]. However, treatment with GnRH following a spontaneous LH surge resulted in a surge of LH of shorter duration and decreased magnitude compared to an ovulatory LH surge [51]. Additionally, treatment with GnRH at AI tended to decrease subsequent progesterone concentrations in synchronized beef heifers [48]. Therefore, the positive effect of GnRH treatment at estrus appeared to be most beneficial in females with decreased fertility, or when the treatment was performed close to the onset of estrus (i.e., close to the spontaneous LH surge).

### 4.2. AI following synchronization of ovulation

The use of a P4/progestin plus E2 based TAI protocols has been the most commercially used type of fixed time synchronization protocol in South America [52-55]. In females, a common aspect among the estrus synchronization protocols for TAI is the insertion of an intravaginal device containing P4 or an ear implant containing norgestomet plus administration of estradiol benzoate (EB; 2mg i.m.) on Day 0; an injection of prostaglandin (PG) F2α on Day 8 or 9 at the moment of device withdrawal plus 300 to 400 IU of equine chorionic gonadotropin (eCG). Different ovulation inducers with similar efficiency could be used such as estradiol cipionate (EC; 0.5 mg i.m.) at moment or EB (1mg i.m.) 24 h after the P4/progestin implant

removal. Timed artificial insemination use to occur 48 to 60 hours after P4/progestin source withdrawal [54-58].

A possibility to improve the use of sex-sorted sperm is controlling the ovulation time variation through the use of synchronization techniques; thus, increasing the efficiency of AI programs using sexed semen. For instance, in beef and dairy cows, P4 and E2 based synchronization induce ovulation around 70-72h after the P4 device removal [59-61].

The P4-based synchronization protocol is a well-established protocol to synchronize the ovulation. Despite the satisfactory predictability of the moment of ovulation provided by the P4 plus estradiol-based estrus synchronization protocol (averaging 66 to 72 h after P4 device removal), the timing of ovulation is influenced by the diameter of the follicle at the time of the ovulatory stimulus treatment [62]. Neves [62] evaluated the time of ovulation in a large number of suckled *Bos indicus* cows (n = 312) and observed a significant effect of the diameter of the ovulatory follicle at the moment of synchronized ovulation (average of 71.8 ± 7.7 h after P4 device removal). The author reported that cows experiencing premature ovulation (i.e., ovulation occurring from 48 to 59 h after P4 device removal) presented a larger ovulatory follicle (14.0 ± 2.2 mm) than cows with delayed ovulation (11.4 ± 2.2 mm; 73 to 96 h after P4 device removal) and that cows that ovulated at the expected time of ovulation (60 to 72 h after P4 device removal) showed ovulatory follicles of intermediate diameter (13.6 ± 2.1 mm).

Once the sex-sorted sperm present lower viability on the reproductive tract than conventional semen [6, 14], our research group has evaluated the delay on AI using of sex-sorted sperm in heifers. A study [8] breeding 420 cyclic Jersey heifers at either 54 or 60 h after P4-device removal, using either sex-sorted ($2.1 \times 10^6$ sperm/straw) or non-sorted sperm ($20 \times 10^6$ sperm/straw) from three sires (2 x 2 factorial design). There was an interaction (P = 0.06) between time of AI and type of semen on pregnancy per AI (P/AI, at 30 to 42 d after TAI); it was greater when sex-sorted sperm (P < 0.01) was used at 60 h (31.4%; 32/102) than at 54 h (16.2%; 17/105). In contrast, altering the timing of AI did not affect conception results with non-sorted sperm (54 h = 50.5%; 51/101 versus 60 h = 51.8%; 58/112; P = 0.95). There was an effect of sire (P < 0.01) on P/AI, but no interaction between sire and time of AI (P = 0.88).

Based on previous results, Sales et al. [8] evaluated the ideal period to perform the TAI with sex-sorted sperm in a P4-based protocol of synchronization of ovulation. Suckled *Bos indicus* cows (n = 339) were randomly assigned to receive TAI with sex-sorted sperm at 36, 48, or 60 h after P4 device removal. Ultrasonographic examinations were performed twice daily in all cows to confirm ovulation. On average, ovulation occurred 71.8 ± 7.8 h after P4 removal, and greater P/AI was achieved when insemination was performed closer to ovulation. The P/AI was greatest (37.9%, 36/95) for TAI performed between 0 and 12 h before ovulation, whereas P/AI was significantly less for TAI performed between 12.1 and 24 h (19.4%, 21/108) or > 24 h (5.8%, 5/87) before ovulation (P = 0.001) as shown on Table 4. In the Table 5, it is presented a summary of studies when AI with sex-sorted sperm is performed at different times after protocol of synchronization of ovulation.

| Interval from TAI to ovulation (h) | No. cows | Pregnant (%) No./No. | Adjusted OR[x] (95% CI) | P |
|---|---|---|---|---|
| "/ 24 | 87 | 5.8 (5/87)[c] | 0.24 (0.08-0.70) | 0.01 |
| "/ 12 to 24 | 108 | 19.4 (21/108)[b] | Reference group | |
| "/ 0 to 12 | 95 | 37.9 (36/95)[a] | 2.34 (1.22-4.51) | 0.01 |
| After ovulation[z] | 22 | 36.4 (8/22)[ab] | 1.80 (0.64-5.03) | 0.27 |

**Table 4.** a,b Within a column, proportions without a common superscript differed (P _ 0.05).x OR, odds ratio; CI, confidence interval.y Reference, reference group for adjusted risk ratio, which is the industry standard for the optimal timing of AI with non-sorted sperm.z Inseminations were performed within 0–12 h after ovulation.Adapted from [8]Risk of pregnancy based on the interval between TAI and ovulation in suckled B. indicus cows inseminated with sex-sorted sperm.

| | | Pregnancy per AI % (n/n) | | |
|---|---|---|---|---|
| Reference | Animal category | Early AI time | Late AI time | P value |
| Sales et al. (2011) [8] | Suckled Nelore cows | 42.8 (100/193) | 50.8 (99/195) | 0.11 |
| Schenk et al. (2009) [6] | Angus heifers | 34 (11/32) | 49 (17/35) | "/0.10 |
| Sales et al. (2010) [63] | Jersey heifers | 16.2 (17/105) | 31.4 (32/102) | <0.05 |
| Neves et al. (2010) [62] | Nelore cows | 20.8 (27/130) | 30.9 (38/123) | <0.05 |
| Overall | | 25.1 (92/366) | 37.0 (123/357) | <0.01 |

**Table 5.** Influence of the AI moment in synchronization of ovulation protocols on the pregnancy rate.

Importantly to note, the use of in vitro fertilized (IVF) embryos with sexed semen is expected to have its use increased throughout the years associated to TET. The overall percentages of oocytes fertilized with sorted and unsorted frozen bovine sperm appear to be similar using current IVF methods [20]. While TAI uses one dose of sexed semen by cow, the TET allows the optimization of the semen use. Just one sexed semen dose is capable to fertilize about 80 oocytes (equivalent to the aspiration of four females; mean of 20 oocytes per aspirated cow), resulting in the production of approximately 30 viable embryos. Considering that TET provides 40 – 50% of pregnancy rate, transferring the 30 embryos fertilized with just one sexed semen dose, would result theoretically, in 12 – 15 pregnancies. In the case where for TAI is considered the same conception rate of 40 – 50%, each inseminated sexed semen dose would result in just 0.4 – 0.5 pregnancies.

## 5. Bull effect on the efficiency of reproductive programs using sex-sorted sperm

An important factor to consider in the timing of AI with sex-sorted sperm is the variation in the fertility of individual bulls. Whereas sperm sorting has significantly decreased fertility of certain bulls, sperm sorting does not affect fertility of other bulls [25]. Sales et al. [63] have been evaluated the use of the sexed or conventional semen of 3 different sires to inseminate Jersey heifers after the estrus detection by radio telemetry (Heat Watch®). Wherefore, the conventional semen [64.2% (238/371)] had been a higher conception rate than sexed semen [49.5% (189/382); P = 0.001]. Moreover, there was a bull effect on the conception rate [Bull A = 50.0% (108/216)b; Bull B = 63.4% (211/333)a and Bull C = 53.5% (107/200)b; P = 0.008]. Thus, some bulls can present lower conception rate using sexed or conventional semen for insemination (Figure 1). Other studies also have described that conception rates vary in magnitude for individual sires [5, 25, 64].
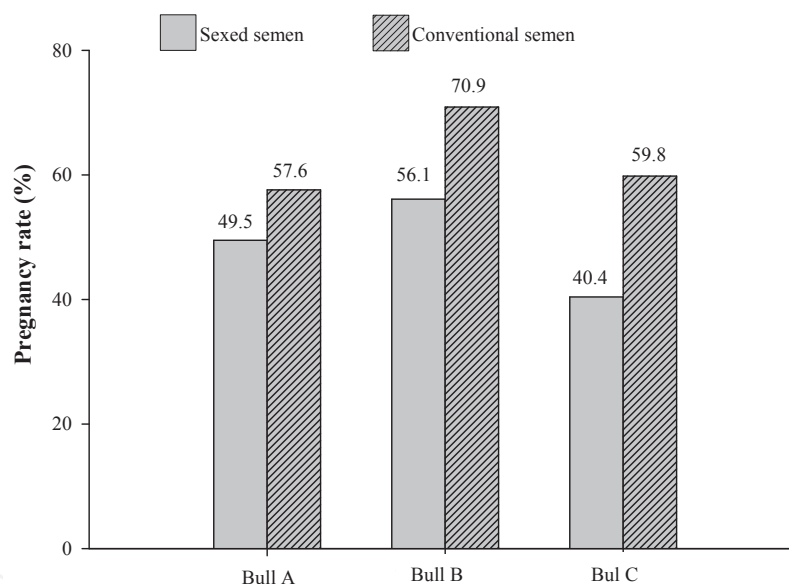


**Figure 1.** Conception rate of Jersey heifers inseminated artificially with sexed or conventional semen according three different the bull (A, n=216; B, n= 333 e C, n=200). Bull effect (P = 0.008) and semen (P = 0.001).

In another study, Sales et al. [8] synchronized Jersey heifers and used sex-sorted sperm from three different sires to inseminate the females. The conception rate was different among sires used in the experiment, indicating once more the existence of individual discrepancy among bulls producing semen to sex-sorting (Figure 2).

The few number of sorting facilities around the world limits the use of high genetic merit bulls because the distance. The capacity to effectively sex-sort and re-freeze previously frozen-thawed sperm would allow commercial sorting undertaking to offer sex-sorted sperm from any sire currently in the frozen semen market. This capacity is mainly influenced by

variation in the physic-chemical semen properties of the individual bull. A study [65] to verify the fertilizing potential of sex-sorted frozen-thawed bull sperm transported cooled or frozen to the sorting facility, has shown a bull effect on the pregnancy rate after AI [Bull 1: conventional semen (control) 63.0%; previously frozen (FS) 8.6%; previously cooled (CS) 10.0%. Bull 2: control 45.5%, FS 0%, CS 4.8%; P = 0.001].
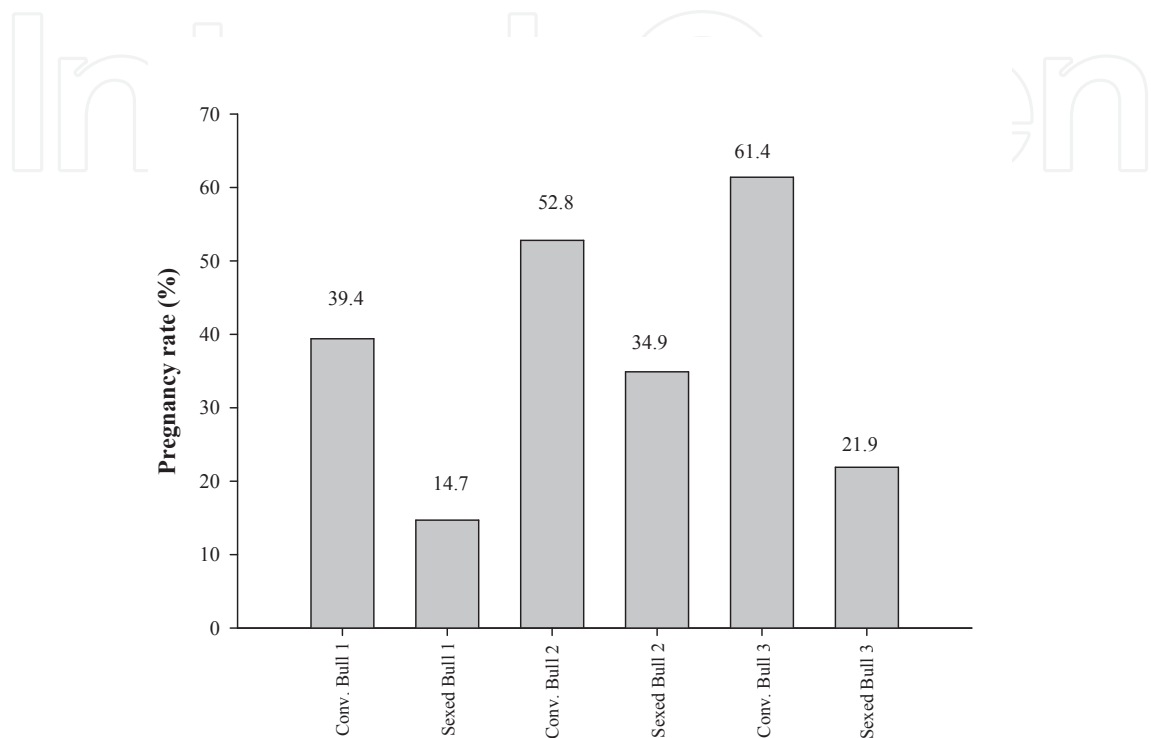


**Figure 2.** Pregnancy rate of Jersey heifers fixed timed artificially inseminated according the bull and type of semen used (conventional or sexed). It was verified a bull effect (P = 0.001) and type of semen (P = 0.001).

Accordingly, the individual difference among bull is an important aspect to consider applying the sex-sorted sperm use at livestock level, allowing the sire selection for higher performance after sexing. Also, it is essential to highlight that this sire effect is one of the most important obstacle to the use of sexed semen in large scale.

## 6. Use of sex-sorted sperm in superovulation protocol for embryo production

The use of sex sorted sperm in reproduction program using superovulation to produce female calf in dairy farms or male in beef farms have been used progressively. Our research group has studied this aspect in Nelore (*Bos indicus*) cows superovulated and inseminated with sex-sorted sperm. Animals were synchronized at Day 0 with norgestomet ear implant (Crestar®, MSD) with 2 mg of estradiol benzoate i.m. (Sincrodiol®, Ourofino). The follicle super stimulation was done was induced with 8 decreasing doses of pFSH (12/12 h) beginning

on Day 4). On Day 6, was given PGF2$\alpha$ analog (Sincrocio®, Ourofino). The ear implant was removed 36 h after the PGF2$\alpha$ analog administration, with the application of LH (Luteotropin) 48 h after PGF2$\alpha$ analog. The TAI with sex-sorted (4,2x10$^6$ cell/AI) or non sex-sorted sperm (40x10$^6$ cell/AI) was performed at 12 and 24 h after LH injection. For TAI, it was used semen from same sire. The experimental design used was crossover to avoid individual variation among donors. The Table 6 summarizes the experiment described, demonstrating a decreasing on fresh and frozen embryos, fresh and frozen embryo rate and an increasing on the unfertilized embryos when using sex-sorted sperm. The accuracy on the use of the sexed semen to produce the desired sex was 90% with pregnancy diagnosis 60 days after TAI. The conventional semen produced 52.7% of females.

| | Non sex-sorted sperm (n = 10) | Sex-sorted sperm (n = 10) | Trat | Rep | Trat vs. Rep |
|---|---|---|---|---|---|
| Total of structures (n) | 9.90 ± 0.78 | 8.40 ± 1.40 | 0.28 | 0.81 | 0.71 |
| Transferable embryos (Grade 1, 2 and 3; n) | 6.80 ± 0.66 | 4.20 ± 0.74 | 0.03 | 0.88 | 0.88 |
| Frozen embryos (Grade 1 and 2; n) | 5.9 ± 0.71 | 3.50 ± 0.65 | 0.03 | 0.43 | 0.99 |
| Unfertilized oocyte (n) | 1.50 ± 0.48 | 3.70 ± 0.88 | 0.01 | 0.82 | 0.46 |
| Degenerate (n) | 1.60 ± 0.37 | 0.50 ± 0.16 | 0.04 | 0.54 | 0.78 |
| Transferable embryo rate (%) | 68.70 ± 6.30 | 50.00 ± 5.10 | 0.01 | 0.68 | 0.54 |
| Frozen embryo rate (%) | 59.60 ± 5.10 | 41.70 ± 5.20 | 0.02 | 0.32 | 0.73 |

**Table 6.** Embryo production of superovulated Nelore cows (*Bos indicus*) and inseminated in fixed time with sex-sorted or non sex-sorted sperm.

In a study by our group research [66], we evaluated different intervals for TAI with sex-sorted sperm after pLH treatment in *Bos indicus* and *Bos taurus* donors. The hypothesis was that increased embryo production would occur when TAI with sex-sorted sperm was performed closer to the time synchronized ovulations occurred. In the first experiment, hormonal superstimulation of ovarian follicular development in Nelore donors (n = 71) was performed in randomly allocated animals to one of three treatment groups, and they were inseminated at 12 and 24 h after an ovulatory stimulus with pLH treatment was applied, either with sex-sorted (4.2 x 10$^6$ sperm/insemination; S12/24; n = 17) or non-sorted sperm (20 x 10$^6$ sperm/insemination; NS12/24; n = 18), or they were inseminated at 18 and 30 h using sex-sorted sperm (4.2 x 10$^6$ sperm/insemination; S18/30; n = 19). A greater number of transferable embryos were found when sex-sorted sperm was used to inseminate the animals at 18 and 30 h compared to insemination at 12 and 24 h. However, a greater embryo production was obtained with non-sorted sperm (results are summarized on Table 7).

Additionally, Soares et al. [66] used the same insemination times and semen types in lactating high-production Holstein cows (n = 12). A crossover design was employed in this trial.

A lesser embryo production (0.007) was found in Holstein donors that were inseminated using sex-sorted sperm at 12 and 24 h compared to non-sorted sperm. However, intermediate results were obtained when the inseminations with sex-sorted sperm were performed at 18 and 30 h (Table 8).

| | Treatments | | | P value |
|---|---|---|---|---|
| | Conventional 12/24 | Sexed 12/24 | Sexed 18/30 | |
| Number of cows | 17 | 18 | 19 | ---- |
| Total ova/embryos | 8.0 ± 3.2 | 7.1 ± 3.3 | 9.0 ± 3.8 | 0.14 |
| Transferable embryos (n) | 6.8 ± 2.6[a] | 2.4 ± 1.8[c] | 4.5 ± 3.0[b] | < 0.001 |
| Transferable embryos (%)[d] | 86.1 ± 11.9[a] | 37.3 ± 26.7[c] | 48.2 ± 25.9[b] | < 0.001 |
| Freezable embryos (n) | 6.0 ± 2.4[a] | 2.0 ± 1.4[c] | 3.7 ± 2.8[b] | < 0.001 |
| Freezable embryos (%)[e] | 76.3 ± 19.2[a] | 31.8 ± 24.5[c] | 38.0 ± 26.5[b] | < 0.001 |
| Degenerate embryos | 0.7 ± 0.7 | 0.9 ± 1.6 | 1.6 ± 2.1 | 0.05 |
| Unfertilized oocytes | 0.5 ± 0.7[a] | 3.7 ± 3.6[b] | 2.9 ± 2.6[b] | < 0.001 |

**Table 7.** Rows with different superscripts (a, b, e) indicate P < 0.05. [d] Percentage of transferable embryos based on the number of ova/embryos recovered. [e] Percentage of freezable embryos based on the number of ova/embryos recovered. Adapted from Soares et al. [66]. Embryo production of Nelore cows (*Bos indicus*) superovulated and inseminated in different times with conventional or sexed semen.

| | Treatments | | | P value |
|---|---|---|---|---|
| | Conventional 12/24 | Sexed 12/24 | Sexed 18/30 | |
| Number of cows | 11 | 11 | 11 | ---- |
| Total ova/embryos | 10.4 ± 3.4 | 11.3 ± 4.4 | 12.4 ± 3.8 | 0.40 |
| Transferable embryos (n) | 8.7 ± 2.8[a] | 4.6 ± 3.0[b] | 6.4 ± 3.1[ab] | 0.007 |
| Transferable embryos (%)[d] | 85.9 ± 14.0[a] | 40.7 ± 21.3[c] | 54.2 ± 23.2[b] | < 0.001 |
| Freezable embryos (n) | 6.9 ± 1.8[a] | 3.2 ± 1.8[b] | 5.4 ± 3.4[ab] | 0.007 |
| Freezable embryos (%)[e] | 69.9 ± 16.8[a] | 29.9 ± 15.5[c] | 45.3 ± 26.6[b] | < 0.001 |
| Degenerate embryos | 0.7 ± 0.9 | 1.4 ± 1.8 | 1.3 ± 1.7 | 0.43 |
| Unfertilized oocytes | 0.9 ± 1.4[a] | 5.2 ± 3.1[b] | 4.6 ± 2.6[b] | 0.0003 |

**Table 8.** Rows with different superscripts (a, b, c) indicate P < 0.05. [d] Percentage of transferable embryos based on the number of ova/embryos recovered. [e] Percentage of freezable embryos based on the number of ova/embryos recovered. Adapted from Soares et al. [66]. Embryo production of Holstein cows (*Bos Taurus*) superovulated and inseminated in different times with conventional or sexed semen.

Briefly, it is possible to improve embryo production using sex-sorted sperm in *Bos indicus* and *Bos taurus* superstimulated donors when the inseminations are performed near the same time as time-synchronized ovulations. However, the embryo production for TAI with sex-sorted sperm was still less than the production with non-sorted sperm.

## 7. Strategies to increase the pregnancy in TAI with sex-sorted sperm

After determining the best moment to perform the TAI with sex-sorted sperm, some studies were conducted aiming to verify the effect estrus expression and follicle diameter at the moment of TAI on the conception rate. Earlier studies have demonstrated that females displaying estrus before TAI have better ovarian responses [67] and with bigger follicle size at the moment of TAI [68] have better conception rate when AI is performed with conventional semen. When using this method of the follicle size on TAI with sex-sorted sperm [31], there is an interaction (P = 0.02) between the type of semen and the size of the dominant follicle [conventional ≥ 8mm = 58.9% (126/214); conventional < 8mm = 49.5% (101/204); sexed semen ≥ 8mm = 56.8% (134/236) and sexed semen < 8mm = 31.2% (59/189)]. In this study, it was verified that the difference between the type of semen used (conventional vs. sexed) on the pregnancy probability at 30 days, decrease according the dominant follicle size increase at TAI (P = 0.001).

There is an influence of the number of services with sex sorted sperm and the conception rate in heifers. It was observed by Sá filho et al. [7] working with Jersey heifers (n = 573) showed that P/AI was influenced by the number of AI service (First, 115/208 = 55.3%a; Second, 94/204 = 46.1%a; and Third, 57/165 = 34.8%b; P = 0.004). Similar results were achieved in a study by Dejarnette et al. [30] where pregnancy rate reduced in heifers with more AI service (First = 47%, Second = 39%, and Third = 32%). Thus, heifers which use to fail in the first AI probably will have their pregnancy rate compromised in the later services.

Vazquez et al. [69] in an interesting review, states that the sex-sorted spermatozoa are 'weakened' by the process, giving them a short functional lifespan. Consequently, new AI strategies are necessary in order to achieve high pregnancy rates with a low dose of sex-sorted spermatozoa. The deposition of the spermatozoa higher in the reproductive tract, compared with conventional AI, allows a greater proportion of spermatozoa to survive and colonize the oviduct. Therefore, fewer spermatozoa are necessary to achieve the same probability of fertility than with a dose deposited in a lower part of the reproductive tract, especially when weak spermatozoa are inseminated. However, in a recent study, Sá Filho et al. [31] compared the conception rate of a total of 200 suckled cows presenting LF ≥ 9 mm at TAI were randomly assigned to receive sex-sorted sperm deposited into the uterine body (n = 100) or into the uterine horn ipsilateral to the recorded LF (n = 100). No effect of deeper artificial insemination on P/AI was found (P = 0.57). Several studies have been performed to evaluate the effect of uterine horn insemination [70-77]. The majority of those previous results support the idea that site of semen deposition would play little to no role in P/AI. However, due to the presumably reduced viability of sexed sperm, the insemination closer

to the site of ovulation would potentially provide better results [64, 78]. Also, it is important to mention that in most of those studies, the cornual inseminations were performed by depositing half of the semen straw into the right horn and the other half into the left horn. In the Sá Filho et al. [31] study, the insemination was performed with the full dose in the uterine horn ipsilateral to the expected side of ovulation. Despite these differences, similar P/AI was found when comparing inseminations performed in the horn or in the body of the uterus, which agrees with those previous results with conventional semen.

## 8. Conclusion

The sex sorting process by flow cytometry is aggressive to the spermatozoa, compromising the viability of these cells. Thus, some adjustments in the mode of use the sexed semen in AI have to be done to improve the tangibility in reproductive programs at livestock level. Adjustments in the AI moment in relation to the estrus beginning improve the conception rate. The results of the studies presented, indicates that beef and dairy heifers inseminated with sex-sorted sperm after estrus detection have satisfactory conception rate, and the AI has to be done between 16 to 24 h after the estrus commencement (6 to 14 h before ovulation). Also, when using synchronization of ovulation protocols for TAI in beef and dairy cows, acceptable conception rate is obtained when TAI is done at 60 h after P4 device removal (10 h before ovulation). Additionally, there is a huge individual variation of fertility among semen of sires submitted to the sexing process, and has to be considered at the moment of bull choosing in AI programs.

## Author details

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