Introduction

Gender selection of offspring has considerable benefit for livestock management systems. Planning the sex of the offspring prior to conception is the most cost-effective means of achieving the desired outcome. Utilization of gender-selected semen allows production of male and female offspring to take advantage of sex-limited and sex-influenced traits.  

To be widely used, gender preselection should be effective and efficient, result in acceptable fertility, and be reasonably inexpensive and convenient. In mammals, sex is determined by gonosomes: an XX chromosome combination determines a female, whereas an XY results in a male. Since X and Y chromosomes differ significantly in length, discrimination of X and Y chromosome-bearing sperm populations is possible due to the difference in their DNA content. Several methods to separate X and Y chromosome-bearing sperm populations have been investigated. The only accurate and potentially cost-effective approach for achieving gender selection currently involves separating X from Y chromosome by flow cytometry, followed by its use for artificial insemination (AI) for breeding or multiple embryo production systems, or for in vitro fertilization (IVF) with subsequent embryo transfer programs. This method can produce populations of X or Y sperm with up to 90% accuracy and subsequent offspring whose phenotypic sex is consistent with the initial accuracy of the sex-sorted sperm population. Limitations of the technology are associated with the number of sperm required for fertilization, timing of insemination, individual bull variation in sexed-sperm sorting, and subsequent production of offspring. This technology examines each sperm separately for its DNA content, thus limiting the number of sorted X or Y sperm to approximately 5–6 million sperm per hour.

Sorting sperm by DNA content for gender selection

Flow cytometric examination of the DNA content of X and Y chromosome-bearing sperm of cattle (Bos taurus and Bos indicus) indicated a difference in their DNA content. The difference in total DNA content between those from bulls and cows is approximately 4.2%. This average DNA content difference is sufficient to detect by flow cytometric systems (Table 72.1) and subsequent sorting of X and Y chromosome-bearing sperm. 

The sperm sex-sorting procedure involves several steps. Briefly, sperm DNA is quantitatively stained with Hoechst 33342, and the sperm are then forced in a stream in front of a laser beam at specific wavelengths. The illuminated stained sperm emit a very bright blue fluorescence. This fluorescence is rapidly measured using a photomultiplier tube as the sperm flow in single file in front of the tube. A computer is used to rapidly analyze the relative fluorescence of the X and Y sperm populations as they flow through the instrument in a fluidic stream. A crystal vibrator is used to break the stream into individual droplets, many of which contain a sperm. The fluorescently stained sperm are sorted by DNA content by placing opposite charges on droplets containing X sperm from those containing Y sperm (Figures 72.1 and 72.2). The droplets fall past positive and negative electrical fields, and since opposite charges attract, the droplets separate into two streams for collection. A third stream of uncharged droplets is discarded, as these droplets have sperm that could not accurately be sexed (over half), no sperm, rarely two sperm, and dead sperm (gating out dead sperm is a valuable additional benefit). This sperm-sorting technique is known as the Beltsville sperm sexing technology.
Commercialization of bovine gender-selected semen

Advances in sex sorting sperm have enabled its incorporation into commercial reproductive management. Currently, gender-selected semen is more applicable on a large-scale basis to the dairy industry than to the beef industry. Sexed semen will contribute to increased profitability of dairy and beef cattle production in several ways. It could be used to produce offspring of the desired sex from a particular mating to take advantage of differences in value of males and females for specific marketing purposes. Dairy herds are more interested in producing replacement heifers, as dairy bull calves are inherently less valuable. Advantages are production of more replacement heifers, thereby enabling greater selection pressure, contributing to more rapid genetic gains. Furthermore, there are opportunities for herd size increase, sale of more females, and simultaneous increase in milk production and decrease in milk price (the latter is an advantage for consumers). Furthermore, once replacement heifer needs are met, dairy producers could also use sexed semen to produce beef crossbred bulls from the remainder of their cow population. Seed-stock dairy producers gain their income from selling genetically superior animals in addition to selling milk. Their goal is to maximize genetic merit of breeding stock for economically important commercial production traits in a cost-effective manner. Sexed semen will be useful to seed-stock breeders if it allows them to increase rate of response to selection, or if it reduces the cost of achieving genetic change.

In order to produce genetically superior replacement heifers using sexed semen, commercial dairy farmers should first rank their cows according to estimated genetic and economic merit for dairy production traits and then use X chromosome-enriched semen from genetically superior bulls to inseminate a sufficient number of the highest-ranking cows to produce the needed number of replacements. By selecting only the best cows to produce replacement heifers, farmers would increase selection pressure for dairy production traits. Methods to assess the economic impact, generated through increased response to selection among dams of commercial cows, of a unit of semen with an increased proportion of X chromosome-bearing sperm have been discussed. It was reported that the predicted impact on annual genetic progress for sexed semen technology is a 9% or 15% increase compared with a mating system using AI with conventional semen.

The commercial beef cattle farmer produces calves for eventual sale as slaughter animals and for female herd...
replacements. Depending on the planned role for a calf, males and females are of quite different value, so sexed semen potentially is a powerful technology to affect genetic and economic efficiency. With beef cattle herds, the value of shifting the calf crop gender ratio to favor bulls or heifers depends on herd genetics and marketing plans. For beef cattle seed-stock producers, bull sales to commercial cattle producers are often the most significant source of revenue. Replacement heifers are also important for genetic improvement. For beef cattle producers who depend on bull sales and require fewer replacement heifers, utilization of male (Y-bearing) gender-selected semen could be practical. Steers are usually heavier at weaning and more valuable than heifers. Thus, for commercial cattle producers who market feeder calves, utilization of Y-bearing semen in a commercial setting would lead to more steer calves that could offset increased semen cost. Furthermore, if heifers are retained as replacements, individual hand-selected matings could be done to produce heifers from more maternal dams and bull calves from terminal crosses. It should also be noted that in some beef cattle breeding programs, heifers are more valuable than their male counterparts.

**Artificial insemination**

Sexed semen technology became practical with the live birth of offspring of the predicted sex. Continued research recognized the ability of flow sorting to consistently produce approximately 90% gender-selected offspring and facilitated eventual commercial adaptation. The technology was introduced into commercial application in the United Kingdom in 2000. Currently, several American AI centers have this technology and market sexed semen from their bulls worldwide.

Conception rates to AI in cattle are related to sire, semen processing, sperm dosage, timing of insemination, and service number. Despite successes, the invasive nature of the sex-sorting procedure has detrimental effects on sperm viability and quality. In addition, for economic reasons, the number of sperm in sex-sorted samples is reduced to approximately 2 million sperm per dose. In breeding programs that use conventional semen, some factors that cause differences in fertility are amenable to increased sperm numbers in an insemination dose (compensable), whereas others are not (uncompensable). An extensive insemination dosage trial concluded that the compensable component is mostly satisfied at total sperm dosages of approximately 4.0 million; however, considering differences among sires, the range is 1–11 million sperm per dose. Seidel and Schenk predicted that commercial application of doses of 2.1 million sex-sorted sperm in Holstein heifers would yield conception rates approximately 75–80% of those obtained with conventional semen, consistent with field results in the United States. Increasing sperm number in the insemination dose seems to be a plausible solution to increase fertility. A recent study compared conception rates for 2.1 or 10 million sperm dosages of sex-sorted or conventional sperm, respectively. Across herds and sires, the conception rates of sperm dosage by semen type combinations were 38 and 44% for 2.1 and 10 million sex-sorted semen, and 55 and 60% for 2.1 and 10 million conventional semen dosages, respectively. A marginal improvement in conception rates was observed with insemination dose 10 million for some sires, suggesting that a portion of the decrease in conception rates with sex-sorted sperm may be compensable. However, that conception rates achieved with 10 million gender-selected sperm are not comparable to either dose of conventional semen dose in this study implies that a portion of the decrease in conception rates of sex-sorted semen is induced by the staining and/or sorting procedure. Further, although the sorting method is standardized, bull-related dilution effects may contribute to the decreased fertility independent of sorting stress, but this remains unproven as insemination of high numbers of sex-sorted sperm (10 million) led to reduced pregnancy rates compared with unsorted sperm inseminated at the reduced dose (2.1 million). Further, reducing the available insemination sex-sorted semen dose to 20% to achieve a 6% increase in pregnancy rate is a poor use of genetic potential.

Current recommendations for use of sex-sorted semen in a commercial operation are as follows.

1. Use only in herds with AI pregnancy rates of 60% or more with conventional semen.
2. Select healthy cycling females with good body condition, preferably heifers.
3. Inseminate only animals observed in standing estrus. If using fixed-time AI, make sure a high percentage of the animals were in estrus before fixed-time AI.
4. Inseminate heifers with sex-sorted semen once and subsequently with conventional semen if they do not conceive.
5. Be extremely careful with semen thawing and handling.
6. The inseminator should have experience and be known to achieve good pregnancy rates.

At present, the recommendation for use of sex-sorted semen is restricted to females exhibiting estrus. However, the use of sex-sorted semen in conjunction with estrus synchronization for a timed AI has huge commercial appeal. Besides, estrus synchronization schemes are of particular value for insemination with sex-sorted sperm, as highly accurate insemination time is extremely important. The current focus is to develop an optimal protocol for fixed-timed AI of sex-sorted sperm. Insemination with sex-sorted sperm closer to ovulation and insemination of females with large follicles at the time of AI increased the likelihood of pregnancy. Additionally, uterine horn insemination could improve the conception rate when using sex-sorted sperm. However, placement of sex-sorted sperm deeper into the uterus did not increase pregnancy rates to timed AI.

**Multiple ovulation and embryo transfer**

Several studies have reported in vivo embryo production from superfertilized cows and heifers following insemination with sex-sorted sperm. In many of those studies, there was a reduced fertilization rate and increased percentage of degenerate embryos from sexed sperm, which resulted in fewer recovered transferable embryos. Suboptimal sperm quality and improper timing of AI were regarded as major factors contributing to fewer embryos per flush from
sorted sperm.\textsuperscript{28,29} In contrast to single-ovulating cattle, multiple inseminations are believed necessary to cover the interval required for multiple ovulations.\textsuperscript{30} Because the number of sperm per insemination dose is limited by the high cost of sexed semen, multiple inseminations with low dose (2 million) sorted sperm have been adopted.\textsuperscript{29,30} When donors were inseminated with sexed semen 12 and 24 hours after detected estrus, the number of transferable embryos was increased compared with donors receiving only one dose of sexed semen,\textsuperscript{30} indicating that two inseminations of sexed semen were more effective than one insemination at attaining embryo production characteristics similar to those for conventional semen. However, another study claimed that superstimulated donor cows inseminated four times had fewer Grade 1 embryos and more unfertilized ova with sexed versus conventional semen.\textsuperscript{30} Additionally, when sexed semen was inseminated at various times after a detected estrus,\textsuperscript{30} the number of transferable embryos was decreased compared with donors receiving conventional semen.

Higher numbers of sperm are normally required for superovulated cows. The successful use of sexed sperm in bovine multiple ovulation and embryo transfer (MOET) schemes critically depends on the AI dose. The dose of sex-sorted semen that is used for regular AI, 2 million sperm, is insufficient.\textsuperscript{29} The success with higher doses (10–20 million sperm) seems to depend on the sire or MOET conditions. Further, increasing the dosage to 20 million sex-sorted sperm lowered fertilization rates compared with controls.\textsuperscript{29} Superstimulated heifers inseminated with 5 million sexed (X-sorted; $n=5$) or unsexed ($n=5$) frozen-thawed sperm from one bull at 12 and 24 hours after detection of estrus showed no difference in rates of transferable embryos (53.4 vs. 68.1%).\textsuperscript{31} Furthermore, rates of degenerate embryos and unfertilized ova between sexed and unsexed sperm were 24.8 versus 26.6% and 21.8 versus 5.3%, respectively.\textsuperscript{31} Producing higher numbers of quality transferable embryos and better pregnancy rates after transfer has great potential for a significant impact on the efficiency of utilization of sex-sorted sperm.

Contract matings between elite sires and dams that use MOET greatly increase the probability of producing a desirable offspring, even without sexed semen. Suppose that a MOET procedure resulted in three embryos that produced at least one offspring, even without sexed semen. Suppose that a MOET procedure resulted in three embryos that produced at least one offspring; blastocyst formation on day 8 was 30–40% lower than for the controls and the number of cell cycles was reduced.\textsuperscript{32,33} It was postulated that the impact of sorting on molecular aspects of sperm function, including capacitation, acrosome reaction, zona binding, and embryonic development, are reasons for decreased blastocyst production. Regardless, subsequent reports indicated negative effects of sorting on sperm integrity, but neither cleavage nor blastocyst rates were affected when sperm were centrifuged with a Percoll gradient.\textsuperscript{34} In addition, IVF results are also affected by sperm concentration, which needs to be higher than for IVF with unsexed sperm, and by the hydrodynamic pressure used for sorting.\textsuperscript{35} Additionally, there was a positive correlation between the percentage of progressively moving sorted sperm and blastocyst development.\textsuperscript{36}

Regarding differences in the expression of developmentally important genes, bovine IVF embryos derived from sex-sorted sperm had a reduction in the relative abundances of glucose transporter 3 (Glut3) and glucose-6-phosphate dehydrogenase (G6PD) compared with embryos derived from unsorted sperm, which may be deleterious to the developmental competence of embryos.\textsuperscript{41} Further, it is interesting to note that there are significant sex-related (X- vs. Y-bearing sperm) differences in day 7 blastocysts for several genes, namely glutathione S-transferase M3 (GSTM3), DNA (cytosine-5)-methyltransferase 3A (DNMT3A), and progesterone receptor membrane component 1 (PGRMC1), plausibly contributing to delayed onset of the first cleavage.\textsuperscript{38,44} Additionally, blastocysts produced from sex-sorted sperm are observed with immature mitochondria and rough endoplasmic reticulum, as well as a lower percentage of intact nuclear envelope membranes than the nonsexed counterparts.\textsuperscript{41} Although the sorting process has significant effects on sperm viability, and bull effects are obvious, compromised DNA does not affect reduced fertility, as most nonviable sperm, and those with fragmented DNA, are out-gated during sorting. Regardless, further research is needed to investigate this phenomenon, as there is time-related DNA damage to sex-sorted sperm compared with nonsorted counterparts.

**Reverse sorting**

Reverse sorting is sex-sorting of sperm from previously frozen samples and refreezing those samples after sorting. This technique allows previously frozen sperm to be
separated for sex. This technique is useful for males that are located at a greater distance from a sex-sorting site or which are deceased and thus unable to provide fresh ejaculates for sex-sorting. The “reverse sorting” technique surmounts staining problems by washing frozen sperm through a density gradient, thereby eliminating dead sperm, glycerol, and egg yolk, which may interfere with staining or negatively impact the remaining viable sperm.

The first pre-sexed offspring produced from reverse-sorted sperm were achieved in sheep following AI and embryo transfer, and a few years later using AI. Reverse-sorted bull sperm have poorer motility and viability in vitro compared with frozen-thawed controls. Although pre-sexed calves have been born following AI of reverse-sorted bull sperm, fertility is very low, with increased pregnancy loss. Greater success has been achieved with the use of reverse-sorted bull sperm in IVF, where cleavage and blastocyst production have been comparable to those obtained with sex-sorted frozen sperm and nonsorted frozen controls.

It was apparent that there was a difference among individual sheep with regard to success following reverse sorting. Although the reason for this difference between the species is the source of some disagreement, a better understanding would likely improve fertility of sorted and reverse-sorted bull sperm.

Effect of sex-sorting procedure on structural and functional parameters of sperm

During the sorting procedure, sperm are subjected to several steps, including (i) high working pressures during hydrodynamic focusing and passage through the injection nozzle; (ii) DNA staining with the fluorescent dye bis-benzimide (Hoechst 33342); (iii) sperm exposure to wavelengths of the ultraviolet light spectrum; (iv) repeated exposure of sperm DNA to electrical currents and fields; and (v) subsequent passage through the electrostatic deflection field that may cause stress to sex-sorted sperm. Further impact on sperm quality may be related to preservation of stained sperm at low temperature until insemination. In addition, impact on the motility, viability, and functional integrity of sorted sperm may be attributable to a dilution effect following removal of seminal plasma. Perhaps a major reason for a decrease in sorted sperm survival is production of reactive oxygen species (ROS) during several steps of the sorting process. Addition of sodium pyruvate and catalase to the dilution medium and freezing extenders exerted a positive effect on the motility, morphology, acrosomal status, and viability of the sorted-frozen-thawed sperm, confirming production of ROS in several steps during the sorting process.

Hoechst 33342 (bis-benzimide) is suitable for sperm DNA labeling as it preferentially binds to the minor groove of the DNA helix (for review see ref. 58). However, this dye is known to be mutagenic to cells and may induce disturbances in embryo development. Moreover, the fate of this dye transported by the spermatozoon into the oocyte and thereby into the embryo and offspring, is an area that requires further investigation.

Assessment of sperm DNA fragmentation using nonsorted and sex-sorted sperm samples during a 72-hour incubation at 37°C detected reduced DNA longevity in sex-sorted frozen-thawed sperm, with sperm DNA damage appearing between 24 and 48 hours. Although the baseline DNA fragmentation level was higher in conventional frozen-thawed than in sex-sorted frozen-thawed sperm samples initially after approximately 30 hours of incubation there was cross-over in the tendency of DNA fragmentation for both conventional and sex-sorted sperm samples, with higher DNA fragmentation for sex-sorted than conventional semen. Furthermore, it was concluded that sperm chromatin was more resistant to external stressors affecting sperm after sorting.

Cost and revenues

Comparisons of heifers that were inseminated at their first AI breeding with either sexed or conventional semen (in both groups, all services after first AI used conventional semen) were made to evaluate subsequent reproductive performance and health, production, and reproductive performance during their first lactations (refer to ref. 62 for detailed economic outcomes). Briefly, age of heifers at first insemination was 13.1 ± 0.1 and 13.8 ± 0.1 months for sex-sorted and conventional semen, respectively. Rearing cost from first AI to calving was greater for sex-sorted heifers than for conventional heifers ($775.3 ± 6.7 vs. $750.0 ± 5.9). However, calf revenue tended to be greater for sex-sorted heifers ($142.0 ± 7.2 vs. $126.7 ± 6.4) and cost per female calf produced was less for sex-sorted heifers than for conventional heifers ($−809.4 ± 10.8 vs. $−1249.7 ± 10.9). Treatment did not affect calving difficulty, proportion of heifers needing assistance, and incidence of retained fetal membranes or metritis. Among heifers that conceived to first AI, those inseminated with sex-sorted semen were more likely to be culled within 30 days in milk (3.3 vs. 1.6%) and tended to be more likely to be culled within 60 days in milk (5.5 vs. 3.4%) compared with heifers inseminated with conventional semen, but overall replacement cost was not different ($136.8 ± 13.4). Total milk yield (9245.5 ± 84.7 kg) and income over feed cost ($554.7 ± 5.1) were not different. Overall economic return was greater for heifers inseminated with sex-sorted semen than conventional semen ($−83.7 ± 36.7 vs. $−175.3 ± 33.4). Use of sex-sorted semen for first insemination of virgin heifers reduced the cost per female calf produced and increased the economic return during the first lactation. Female to male sex ratios were 0.86 : 0.14 and 0.48 : 0.52 for sex-sorted and conventional semen, respectively. Heifers inseminated with sex-sorted semen were more likely to deliver a dead calf (8.8 vs. 3.4%), and thus the difference in proportion of heifers delivering a live female calf was lower than reported (sex-sorted 79.1%; conventional 47.2%).

Using sex-sorted semen, dairy herds could eliminate 70% of cows in third and greater parity as dams of replacements while maintaining heifer birth rates equal to those of conventional semen. Elimination of older cows as dams of replacements reduces age of dam of replacements and increases genetic merit of dams by about $23. This change doubles the genetic change per year in the dam–daughter path compared with conventional semen, primarily due to shorter generation intervals. Calculated economic values of sexed semen programs for dairy and beef heifers are available.
Incidence of stillbirth

Incidence of stillbirth was similar between heifers inseminated with conventional or sex-sorted semen. Furthermore, there were no breed differences for incidence of stillbirth among Holstein, Jersey, and Danish Red heifers inseminated with sex-sorted or conventional semen from the same bulls. In contrast, a higher incidence of stillbirth following insemination with sex-sorted semen has also been reported. In 3 years of using sex-sorted semen, total incidence of stillbirths was not influenced by the use of sex-sorted semen compared with conventional semen (10.4 vs. 11.8%). Stillbirth for female calves resulting from sex-sorted semen (9.2%) is similar or slightly lower than conventional semen stillbirths. However, the incidence of stillborn births for male calves from sex-sorted semen (19.9%) is much higher than for conventional semen (12.9%). Even though heifers delivering bull calves seemed to have a higher stillbirth rate, only 10% of calves born were male. However, this might be a concern where a higher sex-ratio toward males is preferred. It was suggested that increased incidence of stillbirths may occur when pregnancies result from Y chromosome-bearing sperm that are mistakenly identified as X chromosome-bearing sperm during the sorting process due to the presence of aneuploidy, which would result in greater DNA content and increased risk for malformation. In this case, it should be noted that the Y-bearing sperm sorted for male selection based on sperm having lesser DNA and the Y-bearing sperm that have aneuploidy with greater DNA will not be mixed with this Y-bearing sperm population as they will be selected out. Hence the stillbirth incidence will be similar to conventional semen when selection for male sperm is occurring.

Closing remarks

It is now clear that sexed semen is a commercial reality for cattle, deer, and sheep, and is nearing commercial application in several other species, particularly pigs, horses, and dogs. Reports on sexed semen being applied for conservation purposes have also become common in zoo animals and other wildlife, some of which are of threatened or endangered status. The one aspect that has remained constant until recently and perhaps most concerning is fertility. In cattle and possibly other species it believed that fertility of sexed semen is compromised by about 10 percentage points by an unknown mechanism that appears uncompensable by increasing the number of sperm per inseminate. Sexing Technologies has invested significantly in research and development in modifying processing media as well as biochemically altering the overall sorting process (Vish Vishwanath and Juan Moreno, personal communication). Recently completed trials with Select Sires indicate a significant increase in fertility, which has narrowed the gap between conventional semen and sexed semen down to 3–5 percentage points. It is not unlikely that this gap can be narrowed further, thereby making sexed semen almost as fertile as conventional semen. Some reports do show that in sheep, sexed semen outperforms conventional semen as far as fertility is concerned. Reasons for this are varied but the sorting process does clear out many dead and compromised sperm and if conditions are right and timing of insemination is accurately matched with ovulation, there is a real opportunity to improve the fertilization outcome.

Conclusion

Despite key technological developments for more than three decades, most commercial operations that utilize sorted sperm are dairy herds. Research to improve sperm sexing has been largely focused on improving sample throughput by increasing the rate at which sperm are introduced to the sorter, and on improving measurement resolution, which has increased the proportion of sperm that can be reliably measured and sorted. Although the speed of sorting has greatly increased, sperm still need to be individually assessed and sorted. As a consequence, the use lower sperm dosage for AI has reduced fertility (compared with conventional, sperm) and has contributed greatly to the limited commercial use. Furthermore, more than half of the sperm are discarded. Several improvements have recently made sperm sorting more efficient and less harmful to sperm. Modification of the technology with a harmless sorting method has great promise to improve the longevity and fertilizing capacity of sorted sperm. With further improvements in speed of sorting and precision, the cost of the process will be reduced, particularly for high value samples. In the future, the focus may shift to other aspects of the overall process such as improvement in post-sort sperm quality to reduce bull-to-bull variations and increase the semen supply from high-demand bulls. Further, it is essential that, as commercialization increases, more data regarding fertility, embryonic losses, stillbirth, and heifer health be collected. Such information will help to evaluate the technology correctly for its practical use.

References

Utilization of Sex-sorted Semen


