Bovine sexed semen production and utilization

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Abstract

It has been 30 years since the first publication describing the birth of live offspring resulting from sexed mammalian sperm and ~ another 15 years have passed since the technology first became commercially available to cattle producers. Significant research investments on flow cytometry engineering and sperm biology ensured continuous improvements of the technology and current processing methods now allow producers access to SexedULTRA™, sexed semen with 90% gender purity and fertility comparable to that obtained with conventional semen. Sexed semen is a product now offered by all major bovine genetic companies around the world and is an important tool in capturing additional genetic gain through its strategic use in breeding programs. Current state of the art in the fundamentals of sperm sex sorting, the biology of the process, industrial production and field results are described in this review.

Keywords: Cattle, artificial insemination, sexed semen, fertility, SexedULTRA™

Introduction

The world's population is expected to grow to ~ 10 billion by 2050. Population growth, combined with increased urbanization and per capita increases in income, is expected to increase food demand by 50% from 2012 levels. Satisfying increased food demands with existing production practices will result in more intense competition for natural resources, increased greenhouse gas emissions, and further deforestation and land degradation.[1,2] The sustainability, even the very existence, of the world's cattle industry relies on strategies and initiatives to meet the protein needs of 10 billion people in a way that is economical, healthy and good for the environment.

Production efficiency (productivity per animal unit and land unit) relates to sustainability through its effects on economics and environmental impacts. Use of artificial insemination enabled large scale genetic selection programs in cattle and those have been the major contributors to increases in animal productivity, efficiency, product quality and environmental and economic advancements in the last half century. As an example, milk production in US increased by 59% with 64% fewer cows in 2007 compared to 1944. Production of the same volume of milk produced in 1944 required only 21% of the cows, 23% of the feedstuff, 10% of the land and 35% of the water in 2007. As a consequence, greenhouse gas production also decreased by 41%.[3]

In the last 15 years, cattle industry was revolutionized by development and adoption of new genetics and breeding technologies, namely the use of genomics for animal selection and commercial use of sexed semen for artificial insemination. Genomic selection reduced generation intervals and accelerated rates of genetic gain in extraordinary fashion in dairy cattle and new programs in beef cattle offer promising results. Although use of sexed semen for artificial insemination is rightfully considered a reproductive biotechnology, it could be argued that it should also be considered genetic selection, since gender is a genetic trait. Most genetic traits can be manipulated through selection, but before sexed semen was available, producers had to accept the probability that births would result in calf ratios of ~ 50:50 female to male. Due to impact of gender on animal production systems, it is described as "the most important genetic trait."[4] As such, sexed semen will continue to be one of the main drivers of cattle production efficiency and sustainability. Sexed semen production greatly improved since the beginning of commercial application, but still continuous to evolve rapidly. Incorporation of the most recent advancements into production of sexed semen resulted in a differentiated product, SexedULTRA 4M™, that now allows producers to obtain > 90% calves of the desired gender with fertility rates comparable to those obtained with conventional semen.

Brief history of sexed semen technology

Sexed semen technology was initially developed at US government research centers. In studies started at the Lawrence Livermore National Laboratory in the 1970s, scientists studying health effects of radiation using mouse sperm as a model to indicate damage to the germline developed flow cytometer techniques that allowed precise measurement of sperm DNA content; this lead to the breakthrough demonstration of the potential use of these techniques to identify X and Y sperm populations, based on DNA content differences.[5] Further development of the technology occurred at the USDA Beltsville Agricultural Research Center in the 1980s and 1990s, when changes to sperm staining methods and further advancements in flow cytometry not only lead to the major breakthrough of live births of rabbits produced with sexed semen,[6] but also supported potential commercial application of the technology.

Following encouraging results using low-dose insemination with fresh semen in cattle, in the mid-1990s USDA granted a license to XY Inc., a company funded by the Colorado State University Research Foundation, Cytomation Inc. and private investors, to commercialize the Beltsville sexed semen technology for nonhuman mammalian sperm.[5] Further developments in rapid speed flow cytometry lead to a leap in production from a few hundred sperm/second to ~ 3000 sperm per second at ~ 90% accuracy.[7] Development of methods for sexed semen cryopreservation[8] and demonstration of acceptable pregnancy rates obtained with frozen-thawed sexed semen [9] further opened doors for commercial application of the technology.

Commercial licenses were granted to bull studs in the early 2000s and commercial tests started being conducted around the world. Development of the technology changed when Sexing Technologies (ST/STgenetics) secured a sorting license in 2004 and started to establish a small number of sorting labs. In 2007, ST acquired XY Inc. and refocused the commercial approach to allow bull studs access to larger and consistently growing amounts of sexed semen of consistent quality at reasonable costs.[10] Today, the world's largest bull studs use ST technology to offer sexed semen from a diverse group of top bulls as an essential and important portion of their product portfolios.

Overview of sexed semen production

Sexed semen production is based on the difference in DNA content between X and Y sperm, resulting from the difference in size between X and Y chromosomes. On average, the difference in DNA content between bull X and Y sperm is ~ 4%, although subtle differences occur among breeds (4.22% in Jerseys, 4.07% in Angus, 4.01% in Holstein and 3.7% in Brahman).[11] Hoechst 33342 (H33342) is a dye that permeates the intact cell membrane and binds selectively to A/T base pairs along the minor groove of dsDNA. Hoechst 33342 exhibits a relatively large Stokes shift (excitation/emission maxima of ~ 350/460 nm), making it very useful in assessing precise amounts of DNA in living cells.[12,13] A flow cytometer is used to quantify sperm DNA content. Briefly, H33342 dye DNA bound molecules are excited by a laser as sperm pass 2 fluorescence detectors that measure the intensity of fluorescence. The strength of the fluorescence signals depends on the number of fluorescing molecules bound to DNA, thus allowing differentiation of X and Y sperm.

There are significant differences in the production processes of conventional and sexed semen. In contrast to conventional semen, which requires minimal intervention and handling, processing sexed semen involves ~ 30 steps (Figure 1). After collection and evaluation, semen must be prepared for sorting. This involves extension with appropriate buffers, removal of seminal plasma, and adjustment of cell concentration to optimal range. The sample is then incubated with optimal concentrations of H33342 for a predetermined interval and an exclusive product, ULTRAsep™ is used to reduce proportion of dead sperm in the sample prior to sorting. Sperm that are not properly oriented, or that contain abnormal DNA amount and therefore fall outside the 'normal' range of fluorescent intensity, are gated out and eliminated from the final population. Use of a stain-quenching compound that is impermeable to intact plasmalemma allows identification, quantification and elimination of all sperm with disrupted membranes (Figure 2). In addition, droplets with sperm that cannot be classified (e.g. signals too close together, > 1 cell in the droplet) also receive no charge and are directed to the discard stream. Therefore, live sperm population is

enriched throughout the process, starting with selection of ejaculates with high motility, removal of a large proportion of dead sperm by ULTRAsep™ during the staining process, gating out non-oriented and dead sperm and eliminating all sperm with undetermined signals during the sort process. In contrast, other commercial sexed semen production technologies actually enrich the population with dead sperm, since no cell is removed from the sample during the process and undesired sperm are actively destroyed using a high-potency laser.

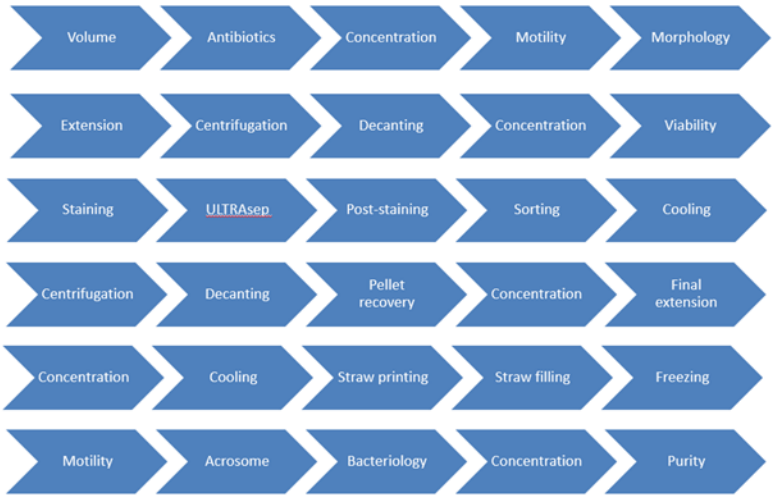

Figure 1. Schematic representation of sexed semen processing.

Stained sperm are pumped in a stream in front of a laser beam, causing illuminated sperm to emit a very bright blue fluorescence. This fluorescence is measured as sperm flow single file in front of a photomultiplier tube (PMT). Specialized software part of the Cytome Genesis™ system is used to analyze relative fluorescence of the X and Y sperm populations and select population(s) to be captured (Figure 2). A crystal vibrator is used to break the fluid stream into individual droplets containing a single sperm. Sperm are then sorted by placing opposite electrical charges on droplets containing X sperm from those containing Y sperm. Droplets fall past positive and negative electrical fields that separate droplets into 2 streams for collection, whereas a third stream of uncharged droplets is discarded (Figure 3).

Sorted sperm are collected into tubes containing appropriate buffers to protect cells during sorting and cooling. After sorting, tubes are slowly cooled to 5°C, additional extenders containing cryoprotectants are added and tubes are centrifuged to obtain concentrated sperm pellets. The number of recovered cells is determined and extenders added to obtain the desired concentration. After a period of equilibration, semen is loaded into straws and frozen in a programmable freezer using the optimal freezing curve. [12,13]

Quality control

Quality control procedures are performed according to guidelines established by the National Association of Animal Breeders/Certified Semen Services,[14] in addition to analyses used exclusively for sexed semen.

Quality control includes evaluation of raw ejaculates for obvious signs of contamination, including debris, urine, water, blood or white blood cells. Any sign of contamination results in ejaculate discard. Additionally, sperm motility and morphology are evaluated in each ejaculate using phase contrast and DIC microscopy, respectively. Sperm morphology defects are classified as primary (sperm acrosome and head defects) and secondary (sperm tail defects). Only ejaculates that meet minimum requirements are processed further.

After production, thawed straws are incubated for 3 hours at 36°C prior to evaluation of sperm motility and presence of sperm agglutination using phase-contract microscopy, and acrosome integrity

using DIC microscopy. Motility videos are recorded and archived with the batch records. All videos are also remotely reviewed by an independent reviewer before any batch is released. Post-thaw motility and acrosome integrity obtained after a recent QC personnel training exercise and implementation of computer-assisted sperm analysis (CASA) equipment at Northern American laboratories are described in Figure 4. Enrichment of live sperm population throughout the process results in high post-thaw sperm motility and acrosome integrity even after 3 hours of post-thaw incubation.

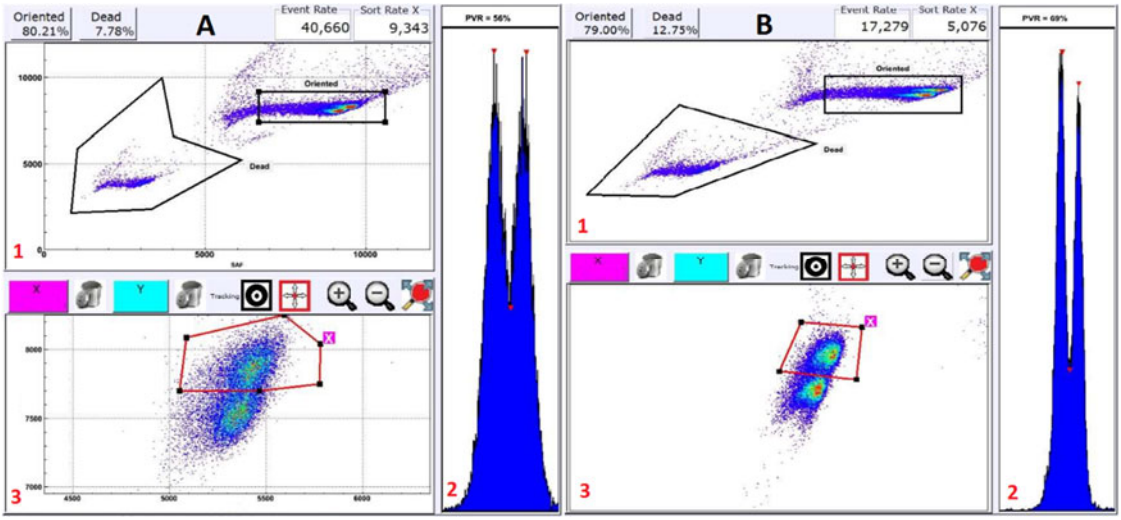

Figure 2. Flow cytometry histograms used to analyze the relative fluorescence of the X and Y sperm populations and select the sorted population. In histograms (1), the dead and properly oriented sperm populations can be differentiated and gated. The degree of difference (peak-to-valley ratio or PVR) in fluorescence intensity between the X and Y sperm in the oriented population can be visualized in histograms (2), whereas the population of interest (desired gender) is gated in histograms (3). High-Productivity sorting (A) results in the maximum number of straws produced per allotted time and requires a high event rate to obtain a high sorting rate; in this example, > 40,000 sperm/second going through the sorter and >9,300 sperm/second sorted. High-Efficiency (B) sorting results in the maximum number of straws produced from the allotted amount of ejaculate and requires adjusting the event rate to maximize the proportion of sperm sorted from the overall sperm population; in this example, over 29% of available sperm are sorted for the desired gender (> 5,000 sperm/second sorted for ~ 17,000 sperm/second going through the sorter). Different sorting modes allow bull studs to strategically plan production according to bull age, availability of semen, and demand to adequately fulfill their customers' needs for sexed semen.[10]

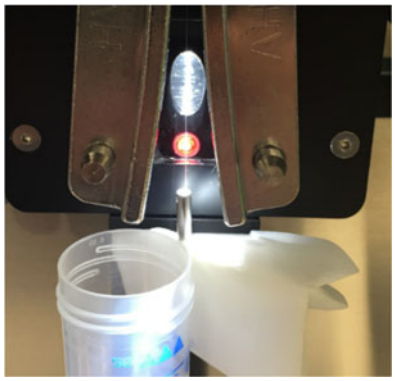

Figure 3. High-voltage plates used to deflect electrically charged droplets containing the desired sperm. A thin stream of fluid can be observed as it is deflected by the plate on the left and into the collecting tube. Droplets containing non-desired sperm, dead sperm, non-oriented sperm, and multiple sperm are not charged and are simply collected into the waste stream (metal tube in the middle). A common misconception is that X and Y sperm are always simultaneously sorted, and that sperm of the non-preferred gender is commercialized as conventional semen. In fact, the vast majority of production involves production of only one type of sperm and the other type is completely discarded.

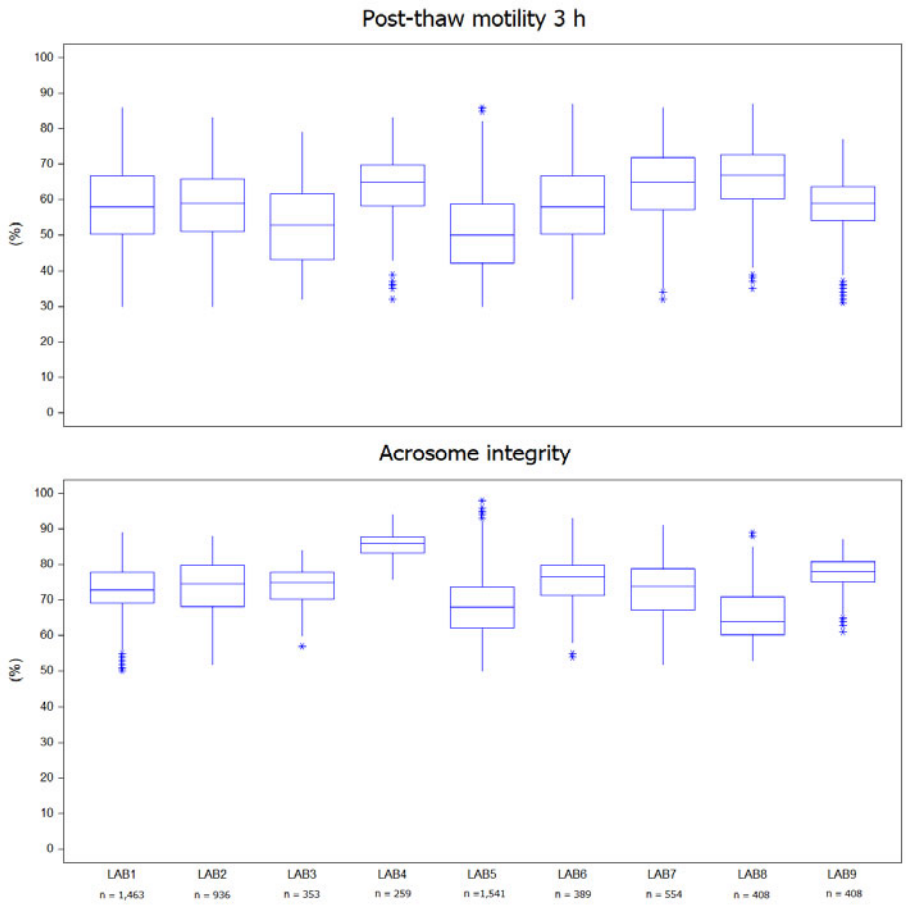

Figure 4. SexedULTRA™ post-thaw sperm motility (top) and acrosome integrity (bottom) after 3-hours incubation at 36ºC. Results obtained using Hamilton-Thorne IVOS II CASA and IDENT method (motility) and DIC microscopy (acrosome integrity). Data from North American laboratories for Holstein and Jersey bulls between February 1 and May 10, 2019. Overall (mean ± SEM) post-thaw motility = 57.2 ± 0.2% and acrosome integrity = 70.1 ± 0.1%.

Total sperm number in the straw is determined using the NucleoCounter (Chemometec®, Denmark), using automatic quantification of propidium iodide fluorescently labeled sperm DNA within a known volume and calculation of sperm concentration. Because sperm identification is specific, there is no interference from seminal plasma composition and gel, lubricants, extenders, or debris, yielding very accurate and precise evaluations. Aerobic bacterial count is determined on all batches by streaking processed semen on Trypticase Soy Agar with 5% Sheep Blood (TSA) plates. Samples are incubated at 37ºC for 24 - 36 hours and number of colony forming units (CFU) determined.

Gender purity is evaluated using Genesis™ high-resolution flow cytometry, which allows precise targeting of purity. Samples from sorting systems (i.e. production equipment) are collected and verified multiple times throughout each batch production to ensure equipment settings are properly setup and accurate so that the targeted gender purity is obtained, while maintaining optimal levels of productivity. Gender purity is also evaluated on each produced batch as part of the quality control process (Figure 5).

Before sample analysis, the Genesis™ high resolution flow cytometer is calibrated using standard, certified sperm nuclei to ensure pre-determined specifications are met, including percentage of oriented cells for the side fluorescence detector path and quality of resolution on DNA content on the forward fluorescence detector path. After the instrument is properly calibrated, analyses are conducted at a low event rate, ensuring that percentage of oriented sperm is > 80%, and evaluating a large number of cells (3,000 to 5,000 sperm). Images generated for purity analysis are recorded and archived with batch

records (Figure 6). All images are also remotely reviewed by a qualified technical expert based in the US before any batch is released.

Figure 5. Use of Genesis™ high-resolution flow cytometry allows precise targeting of Sexed ULTRA™ gender purity. High-resolution flow cytometry is used throughout the production process to ensure sorting equipment are properly calibrated and setup (A: note Genesis 1 analyzer on the left being used in a production laboratory). High-resolution flow cytometry is also used as part of the quality control process to ensure minimum gender purity on all approved semen batches (B).

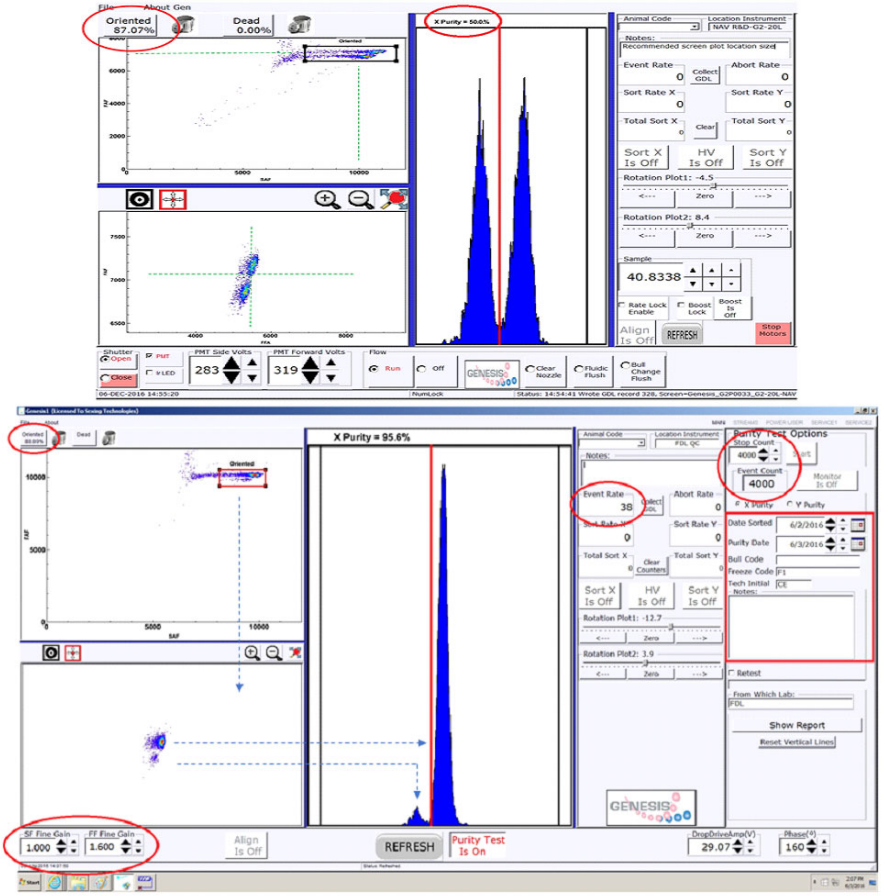

Figure 6. Genesis™ high-resolution flow cytometry for analysis of semen gender purity. Before analysis, the instrument is calibrated using certified sperm nuclei as standard to ensure predetermined specifications are met, including percentage of oriented cells for the side fluorescence detector path and quality of resolution on DNA content on the forward fluorescence detector path; note the clear distinction of between the X and Y sperm in the histogram in the middle of the figure (top). After the instrument is properly calibrated, analyses are conducted at low event rate, ensuring that gains are properly set, percentage of oriented sperm is greater than 80%, sperm plots are properly aligned, centered, rotated and gated. Two distinct peaks are observed in the histogram in the middle of the figure and purity is determined by evaluating 3,000 to 5,000 sperm (bottom).

Flow cytometry analysis is the only well-validated method to evaluate semen gender purity. Validation refers to the observation of gender skew of live offspring closely resembling the skew in X or Y chromosome bearing sperm in the semen sample, as demonstrated in the first study describing the birth of live animals obtained with sexed semen.[6] Several studies describing results involving use of commercial sexed bovine semen have provided support for validity of flow cytometry analysis to evaluate batch purity, as gender skew rates after birth of hundreds of thousands of live calves have ranged from 89 to 93% (Table 1). When calving data from individual bulls were cross-referenced with batch production records, proportion of female calves closely resembled the average batch gender purity determined during quality control. Batches with average gender purity of 91% resulted in 8,306 female calves from 9,148 births or 91% female ratio (Table 2).

Table 1. Field reports of female calf ratio after artificial insemination using sexed semen.

Reference	Country	Inseminations	Female ratio
Borchersen & Peacock[15]	Denmark	1,588	91%
DeJarnette et al.[16]	USA	19,546	89%
Norman et al.[17]	USA	128,702 heifers	91%
		25,910 (cows)	89%
Joezy-Shekalgorabi et al.[18]	Iran	1,154	91%

Table 2. Association of Sexed ULTRA™ gender purity determined using Genesis™ high-resolution flow cytometry with field reports of female calf ratio. Calving data from herd management programs of STgenetics collaborating dairies in the US.

Bull	Semen batches	Semen purity*	Calves	Female calves	Female ratio
Jersey A	52	92%	2,895	2,663	92%
Jersey B	49	91%	1,966	1,809	92%
Jersey C	110	91%	1,513	1,362	90%
Jersey D	87	91%	1,434	1,262	88%
Jersey E	18	92%	773	711	92%
Jersey F	42	90%	567	499	88%
Total	358	91%	9,148	8,306	91%

*Average across batches

Industrialization of sexed semen production

Progress in adoption of technologies requires several intersecting elements to converge, e.g. price, efficiency, performance, ease of use and acceptance. In the case of sexed semen, there were 2 critical elements to successful industrialization of the technology. The first was improvements in understanding and manipulating sperm biology, which were directly related to fertility performance (more on this below). The second element was development of flow cytometry engineering and technology. Original equipment used for sexed semen production was adapted from medical research. These instruments were expensive, bulky, difficult to operate and with low throughput, which represented significant barriers for scaling-up production. Modern equipment has been developed and manufactured by Cytonme Inc., a company of the ST group (www.cytonome.com). Cytonome is a biotechnology engineering and manufacturing company that designs and develops cutting-edge cell sorting platforms, offering powerful application-specific technology for industrial markets.

Equipment development included reduction of fluidic instability and pressure, laser noise, electronic and photodetector noise and acoustic vibration, while improving sperm orientation, light collection efficiency, resolution and signal processing. Current equipment uses a solid-state laser for UV excitation, dual orthogonal detectors (at 0º and 90º to the laser), an orienting nozzle, and digital electronics to provide sorted subpopulations of X or Y sperm at rates of ~ 8,000 sperm/second when operating at an input event rate of 40,000 sperm per second.[19,20]

Cytome Genesis™ is the system developed on the Hydris™ platform used for production of sexed semen (Figure 7). The system is highly automated and designed around user needs, providing simplified operation, reduced instrument size and lower cost in a streamlined workstation layout. Cytome Genesis™ utilizes a compact proprietary multisorter design. Each of the 3 sort units can be operated

independently and dedicated sort electronics ensure stable, accurate, highspeed sorting across multiple sorting units. The system is operated via a touchscreen and can be remotely accessed and controlled over the internet. Control settings (i.e. alignment, droplet setup and droplet delay) are highly automated and require minimal user intervention. The operator can easily control each independent sorting unit using the built-in sort monitoring functions and real time displays. Since each sorting unit is independent, interruption of any individual sorting unit will not affect remaining sorting units. These capabilities provide ease of use in busy, high-throughput sorting facilities, critical in a 24/7 environment.

Figure 7. Genesis™ is the latest generation of sexed semen flow cytometry sorters manufactured by Cytonome/ST, Inc. The system has a multi-sorter design and the fastest overall sort rate of any conventional high-speed cell sorter of its size. The Cytonome Genesis™ is highly automated and designed around user needs, providing simplified operation, reduced instrument size and lower cost in a streamlined workstation layout.

Cytome Genesis™ is a technology marvel that ushered a new era of large-scale, industrial sexed semen production. In May 2019, SexedULTRA™ technology is being utilized for production of sexed semen in 27 laboratories from all major bovine genetic companies across 15 countries. These include 5 STgenetics bull studs, 18 ST managed service laboratories and 4 licensee laboratories that combined operate > 500 flow cytometry sorters and produce > 13 million sexed semen straws per year.

SexedULTRA™ and SexedULTRA 4M™

Continuous research and development investment in sexed semen production technology resulted in significant improvements in semen quality and fertility, so much so that a new product label was created. Although the SexedULTRA™ label was officially launched in 2013, it is important to understand that the product is a culmination of a series of innovations that combined to create a product significantly different from that produced using XY Inc. legacy technology. In addition to improvements to flow cytometry technology described above, other innovations included optimization of flow cytometry media (sheath fluid) and extenders, large scale media and extender production for global distribution, optimization of staining conditions and worldwide adoption of modern, standard equipment (Figure 8).

Initial laboratory evaluations indicated that results from in vitro semen quality tests, including sperm motility and acrosome integrity, were superior when semen was processed using SexedULTRA™ technology when compared to XY legacy technology (Figure 9). In addition, use of SexedULTRA™ semen for in vitro fertilization resulted in greater production of blastocysts and greater proportion of freezable embryos (Table 3).[21] Further evaluations of SexedULTRA™ post-thaw sperm motility, viability and acrosome integrity revealed those to be equal or better than conventional semen. Also, the decline in semen quality after 3 hours of in vitro incubation was lower in SexedULTRA™ compared to conventional semen (Figure 10).[22] Interestingly, DNA fragmentation in conventional semen was not high at the baseline (~ 2%), but dropped to nearly zero in sexed semen, indicating that sperm with damaged DNA are removed during the sorting process. In addition, sperm DNA was more stable in SexedULTRA™ semen and fragmentation did not increase after several periods of in vitro incubation for both frozen-thawed and fresh semen, whereas fragmentation increased significantly in conventional semen (Figure 11).[22]

Figure 8. SexedULTRA™ technology was the culmination of a series of innovations that combined to create a superior product. Substantial investments in Research & Development ensure the continuous cycle of innovations to improve product quality.

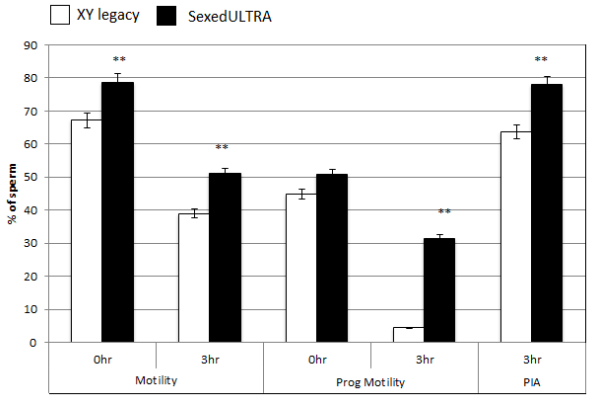

Figure 9. Effect of SexedULTRA™ technology on in vitro semen quality tests. Sperm motility and progressive motility were determined using computer-assisted semen analysis (CASA) and percentage intact acrosome (PIA) was determined using DIC microscopy (n = 12 bulls). **Bars with superscripts differ ($p < 0.001$). Adapted from Gonzalez-Marin et al.[21]

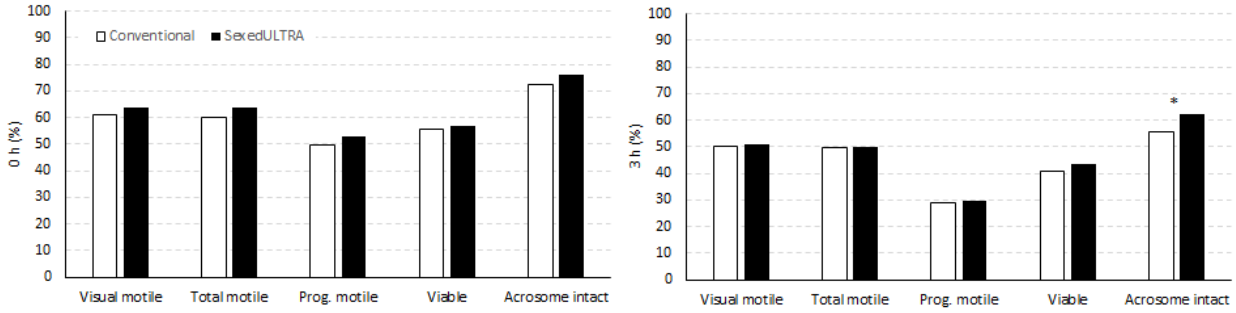

Figure 10. Comparison of post-thaw SexedULTRA™ and conventional semen quality using contemporaneous ejaculates of the same bull (n = 10). After thawing, semen was incubated at 37°C for 3 h and at 18°C for 8 h and 24 h. Sperm motility was evaluated visually and using Hamilton-Thorne IVOS II CASA. Sperm viability and acrosome integrity were determined using flow cytometry with Hoechst 33342, propidium iodide, FITC-PNA fluorescent stains. *Bars with superscripts differ ($p < 0.005$). Adapted from Gonzalez-Marin et al.[22]

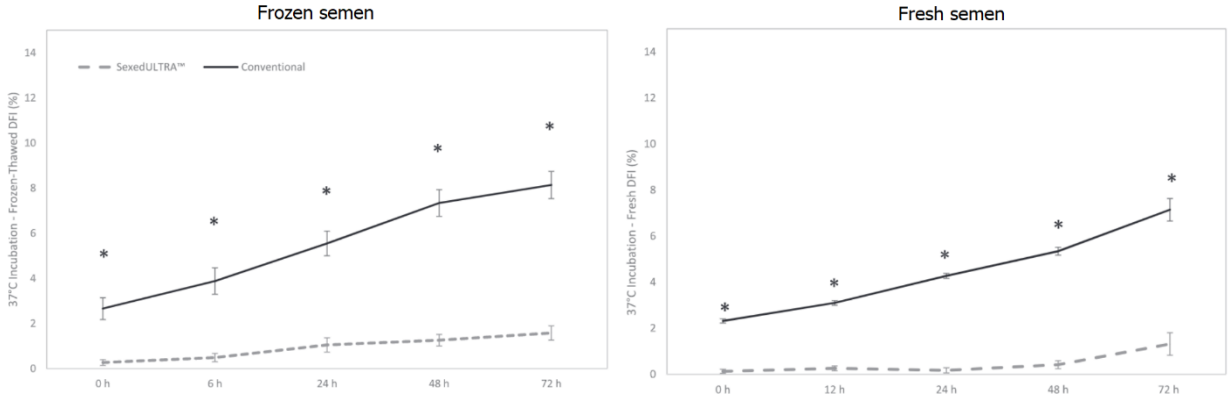

Figure 11. Comparison of SexedULTRA™ and conventional semen DNA fragmentation index (DFI) using contemporaneous ejaculates of the same bull in frozen (n = 10) and fresh (n = 7) semen. After thawing, semen was incubated at 37ºC for 3 hours and at 18ºC for 8 and 24 hours. Fresh semen was incubated at 37ºC throughout the experimental period. DFI was determined using the Bull sperm Halomax® commercial kit (Halotech DNA, Madrid, Spain). *Values with superscripts differ ($p < 0.05$). Adapted from Gonzalez-Marin et al.[22]

Table 3. Effect of SexedULTRA™ technology on in vitro embryo production. Adapted from Gonzalez-Marin et al.[21]

	No. of oocytes	Cleavage rate	Blastocyst rate	Freezable embryos*
XY legacy	5,082	32.7%	18.4%[a]	9.2%[a]
SexedULTRA™	5,081	34.8%	22.3%[b]	13.2%[b]

*Grades 1 and 2.
[a,b]Rows with different superscripts differ ($p < 0.05$).

In an initial field trial with a small number of inseminations involving industry partners, Holstein and Jersey heifer conception rates were 7.4% greater when SexedULTRA™ was compared to XY legacy technology (Table 4). A larger field trial in collaboration with Select Sires in 41 Holstein commercial herds in the US indicated that heifer conception rates were 4.5% greater when SexedULTRA™ technology was used (Table 4).[23] Data compiled by researchers from the USDA on sexed semen usage for Holstein females in the United States demonstrated the positive effects of SexedULTRA™ technology on conception rates. Data on sexed semen inseminations in heifers and cows between 2007 and 2015 showed a consistent reduction in conceptional rate differences between sex sorted and conventional semen, coinciding with global introduction of SexedULTRA™ in 2013 (Figure 12).[24]

Table 4. Effect of SexedULTRA™ technology on heifer conception rates. Select Sires trial results from Vishwanath.[23]

	No. of inseminations	Conception rate
Sexing Technologies trial		
XY legacy	1,166	47.3%[a]
SexedULTRA™	957	54.7%[b]
CR improvement		7.4%
Select Sires trial		
XY legacy	3,384	41.6%[a]
SexedULTRA™	3,546	46.1%[b]
CR improvement		4.5%

[a,b]Rows with different superscripts differ ($p < 0.01$) within trial.

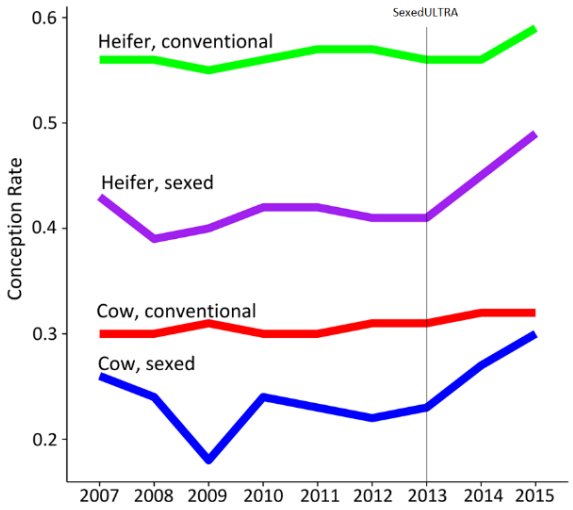

Figure 12. Conception rates in Holstein females in the United States. Only inseminations from 2007 through 2015 with confirmed outcomes were included: 5,963,876 heifer inseminations (1,323,721 to sexed semen) and 42,232,502 cow inseminations (253,586 to sexed semen). Mean conception rates for heifer sexed semen inseminations increased due to improved technology (42% in 2007 compared to 49% in 2015). Comparable conception rates for heifer conventional inseminations were 56, and 59% for 2007, and 2015, respectively. Conception rates for sexed-semen inseminations to cows were 26% in 2007, and 30% in 2015 compared to 30, and 32% for conventional inseminations during the same years. Adapted from Hutchison and Bickhart.[24]

Successful sexed semen production must address susceptibilities of sperm to staining, laser exposure, high dilution, elevated pressure and resistance to several changes in media composition that occur during the process. Historically, compounding deleterious effects of these factors resulted in what could be described as uncompensable changes to sperm, as increasing insemination dosage from the 2.1 million sperm used as the industry standard resulted in little to no significant gain in conception rates. Although some sire by dosage interactions were observed, across sires, sexed semen dosages of 2.1, 3.5 or 5 million sperm had no effect on conception rates in Holstein heifers and cows.[25,26] In another study comparing sexed and conventional semen dosages of 2.1 and 10 million sperm, sexed semen resulted in a decrease in conception rates by an almost identical magnitude within both sperm dosages. Although sexed semen conception rates were improved by the 10 million sperm dosage, conception rates were not comparable to either dosage of conventional semen.[27]

One of the most interesting observations since implementation of SexedULTRA™ technology is that not only have deleterious effects of semen processing been minimized, but also that resulting adverse biological changes to sperm became compensable. In a study conducted in collaboration with German Genetics International, ejaculates from 5 bulls were split 4 ways and processed using XY legacy technology with 2.1 million sperm dosage or using SexedULTRA™ technology with 2.1, 3 and 4 million sperm dosages; contemporaneously produced conventional semen with 15 million sperm dosage served as control. Nonreturn rates (56 days) were evaluated after insemination of 7,855 heifers with sexed semen and 62398 heifers with conventional semen. As expected, XY 2.1 million resulted in lower conception rates when compared to both SexedULTRA™ and conventional treatments. Although SexedULTRA™ 2.1 and 3 million sperm dosages produced results lower than conventional semen, increasing the dosage to 4 million sperm resulted in conception rates comparable to conventional semen (Figure 13).[28] These results were the first to demonstrate: (i) consistently improved conception rates with increased sexed semen dosage; and (ii) conception rates equivalent to conventional semen with SexedULTRA 4M™ sexed semen. STgenetics adopted SexedULTRA 4M™ as its standard product in 2015 and officially launched the product in 2017. Some bull studs also conducted internal trails and have recently announced the release of similar products (see SELECTed™ SexedULTRA™ 4M from Select Sires and GenChoice™ 4M from Genex).

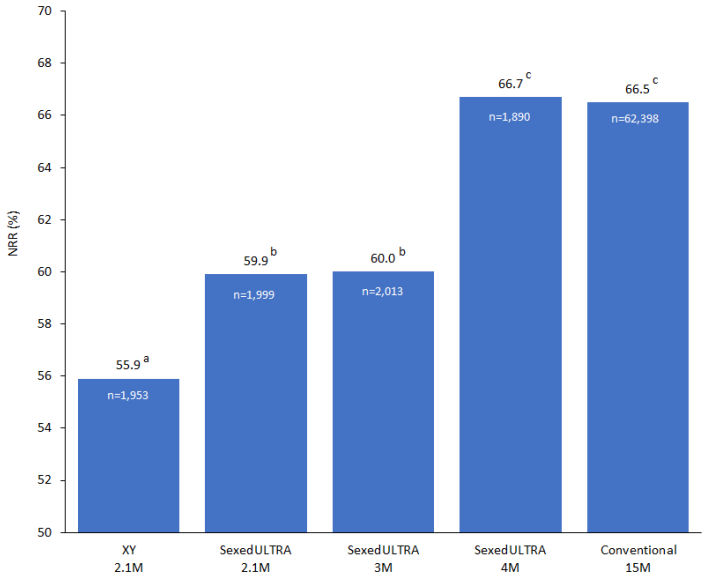

Figure 13. Effect of SexedULTRA 4M™ on 56 days non-return rates (NRR) recorded at German Genetics International (n = 5 bulls). [a,b]Bars with different superscripts differ ($P < 0.001$). Adapted from Lenz et al.[28]

Sexed semen field fertility

Field fertility data from STgenetics collaborating dairies across the US between 2012 and 2017 have been analyzed. Records were obtained directly from dairy management programs (e.g. DairyComp, PCDART) and included 2,123,153 conventional semen inseminations and 1,105,969 sexed semen inseminations from 9,085 Holstein and 1,682 Jersey sires. A linear mixed model was fitted to the data that included an interaction term between year-month of insemination and semen type, the insemination number (1-3) and the age of the service sire at the time of insemination. Random effects included the service sire and a herd-year-season of insemination effect. The model was fitted separately for heifers and cows, whereas the cow model included the lactation number (1 - 2) as an additional fixed effect. Least square means (LSM) on the interaction between year-month and semen type were used to describe the changes in conception rates over time while averaging over the remaining fixed effects:

$$y = X\beta + Zs + Wh + \epsilon$$

where,

y = vector of insemination results [0,1]
X = fixed effect design matrix
β = solution vector for fixed effects
Z = service sire design matrix
s = solution vector random sire effect that follows $MVN(0, I\sigma_s^2)$
W = Herd − Year − Season (HYS) design matrix
h = solution vector for random HYS effect that follows $MVN(0, I\sigma_h^2)$
ϵ = vector of i. i. d. residuals $\sim MVN(0, I\sigma_\epsilon^2)$

Models were fitted using Restricted Maximum Likelihood as implemented in the lme4 package for R.[29,30] Least square means and multiple contrasts were estimated using the emmeans package.[31]

Conventional and sexed conception rates in Holstein and Jersey are shown in Figures 14 and 15. Conception rates in cows had strong seasonality, consistent over years with any semen type. However, seasonality was not observed in heifers. Conception rate for conventional semen in cows in January 2012 was 38%, whereas sexed semen reached 25%. In heifers the difference in conception rates was more

pronounced, i.e. 58% for conventional and 38% for sexed semen. Conception rates started to improve in 2013 with introduction of SexedULTRA™. In June 2014, conventional and sexed semen conception rates in cows were almost at the same level for the first time (34 and 33%, respectively). In 2015, conception rates of sexed semen were stable 88 and 86% of conventional semen conception rates in cows and heifers, respectively. Since mid-2016, relative conception rates of sexed semen were consistently > 90% in cows.

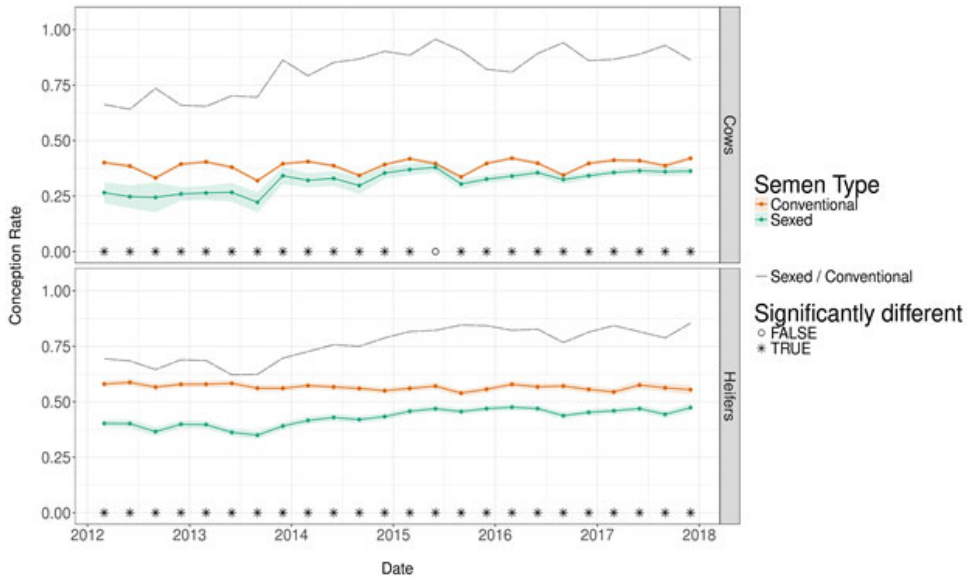

Figure 14. Least square means of conception rates for conventional (n = 1,880,094 inseminations) and sexed semen (n = 558,007 inseminations) from Holstein bulls (n = 9,085) in cows (n = 1,496,740) and heifers (n = 941,361). Data from herd management programs of STgenetics collaborating dairies across the US (n = 181). Bands indicate standard errors.

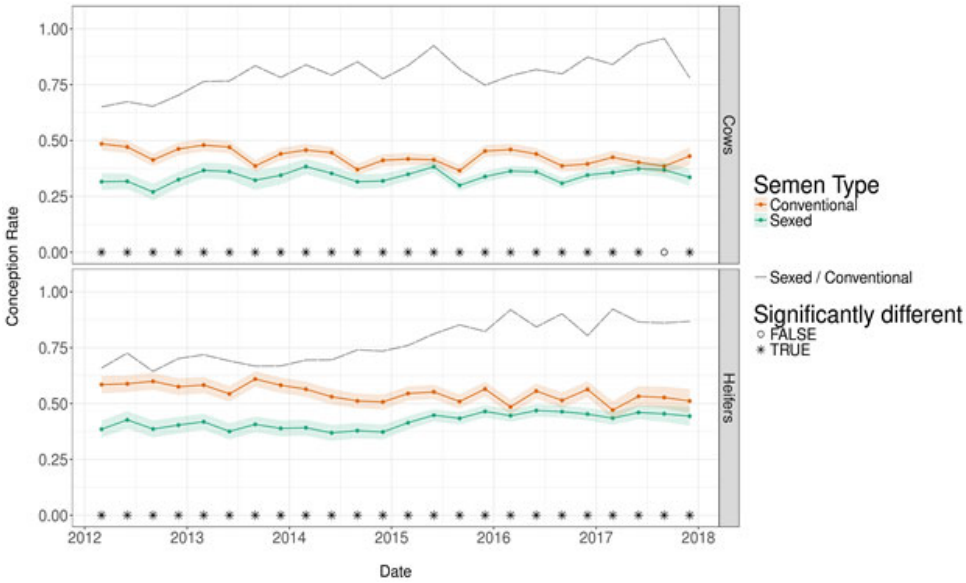

Figure 15. Least square means of conception rates for conventional (n = 243,059) and sexed semen (n = 547,962 inseminations) from Jersey bulls (n = 1,682) in cows (n = 547,962) and heifers (n = 367,779). Data from herd management programs of STgenetics collaborating dairies across the US (n = 115). Bands indicate standard errors.

Field fertility data with the use of SexedULTRA™ in beef cattle is somewhat limited. However, when used in conjunction with a variety of fixed time artificial insemination (FTAI) strategies, relative conception rates of ~ 80 - 85% have been observed in heifers and lactating cows (Table 5).

Table 5. Conception rates in beef cattle using conventional and SexedULTRA™ semen and various fixed-time artificial insemination strategies.

Reference	Female category	Conventional	SexedULTRA™	Relative
Thomas et al.[32]	Heifers	59.9% (257/429)	51.7% (218/422)	86%
Baruselli et al.[33]	Lactating zebu cows	50.3% (75/149)	45.0% (127/282)	89%
Crites et al. [34]	Heifers and lactating cows	56.7% (114/201)	49.2% (95/193)	87%
Colazo et al.[35]	Heifers	59.1% (394/667)	47.6% (316/664)	81%
Bo et al.[36]	Heifers	58.3% (247/424)	49.3% (210/426)	85%
Thomas et al.[37]	Lactating cows	64.7% (525/812)	47.9% (387/808)	74%

Insemination timing - closing the fertility gap

In cattle, it has been estimated that a minimum of 6 hours is required for inseminated sperm to reach the oviducts and undergo the necessary physiological changes to acquire fertilization potential (i.e. capacitation), with the number of capacitated sperm increasing progressively 8 - 18 hours after insemination.[38,39] Conversely, although the oocyte may retain fertilization potential for up to 20 hours, the optimum period is much shorter and is estimated to be only 6 - 10 hours after ovulation.[40] These physiological phenomena result in a quadratic association between insemination to ovulation interval and conception rate. Conception rates increase as the interval decreases, until a point when conception starts to decrease in proximity to and after ovulation. Early inseminations result in high levels of unfertilized ova due to inadequate sperm lifespan, whereas late inseminations result in poor embryo quality, most likely due to aging oocytes.[41] In practice, optimum insemination timing is a compromise aimed to increase the probability of achieving the highest number of capacitated sperm in the oviduct from the time of ovulation to 6 - 10 hours later.

Functional in vitro studies demonstrated that sex sorting changes sperm protein tyrosine phosphorylation and CTC staining patterns that resemble capacitation.[42] Sorted ram sperm bind in fewer numbers to oviduct epithelial cell monolayers in vitro and detach more rapidly than unsorted sperm.[43,44] In a more recent study, binding of sexed porcine sperm to oviduct cells was reduced by more than half compared to conventional controls, but the percentage of sperm that bound to purified soluble glycans and the location of binding was similar between control and sexed sperm.[45] This changed ability of sexed sperm to bind to oviduct cells suggests that these sperm might be at more advanced stages of capacitation and require less time to complete capacitation in the oviduct than non-sexed sperm.

Another important aspect to consider in relation to differences in fertility between sexed and conventional semen is physiological semen heterogeneity. Any given semen sample contains distinct sub populations of sperm that become ready for fertilization at different intervals post-insemination. This heterogeneity is directly related to the window of time that a given semen sample retains fertilizing potential. In fact, variation in fertility of an individual semen sample or amongst multiple semen samples from the same individual has been attributed to the heterogeneity of the sperm population within said sample.[46] Therefore, if semen heterogeneity is altered, it may lead to changes in fertility, depending on insemination timing. Given that the entire sperm population is exposed to the same conditions (i.e. temperature, pH, lipoproteins, antioxidants, etc.) during the sorting process and that sex sorting is a highly selective process, it is likely that the sexed semen is more homogeneous than conventional semen, with most sperm within similar stages of capacitation.

Observations from studies using FTAI and estrus detection systems have provided tacit evidence that sexed semen might contain a more homogeneous sperm population that require less time to complete capacitation. When using conventional semen, conception rates are optimal when females are inseminated from mid-estrus to the end of standing estrus. Considering the interval between onset of estrus to ovulation as ~ 26 - 30 hours and duration of estrus as ~ 12 - 18 hours, optimum insemination timing is then ~ 12 - 22 hours prior to ovulation. Although the same quadratic association between insemination to ovulation interval and conception rate is observed with sexed semen, conception rates seem optimal when inseminations are performed closer to ovulation (~ 6 hours prior to until 10 hours after ovulation).

Jersey heifers inseminated with sexed semen 6.5 hours before presumptive ovulation when using a CIDR/estradiol benzoate/$PGF_{2\alpha}$ FTAI protocol had a conception rate 1.9 fold greater than heifers

inseminated 12.5 hours before presumptive ovulation (i.e. insemination either 54 or 60 hours after CIDR removal). Timing of insemination (6.5 or 12.5 hours prior to ovulation) did not affect conception rate when heifers where inseminated with conventional semen.[47] In lactating Jersey cows fitted with heat-rumination long-distance collars, conception rates with sexed semen were greatest when insemination was performed within 22 - 36 hours after reaching activity threshold. Since ovulation was determined to have occurred ~ 26 hours after activity threshold, optimum insemination was therefore between 4 hours before to 10 hours after ovulation. Conception rates obtained with optimum insemination timing were close to 50%, but dropped to < 30% when insemination was performed > 14 hours before ovulation.[48] In beef heifers and cows, the approach of delaying insemination by 24 hours and administering GnRH at the time of insemination for females that had not shown signs of estrus 66 hours after $PGF_{2\alpha}$ treatment in a 14 day CIDR PG FTAI protocol (split time insemination) seemed to improve overall conception rates.[33,37]

In beef cattle, there is an increasing body of evidence demonstrating the positive association of estrus expression with sexed semen fertility, using a variety of FTAI strategies. Estrus expression is associated with high circulating estradiol concentrations, known to regulate several physiological processes critical for establishment and maintenance of pregnancy, including effects on follicular cells, oocytes, gamete transport and preparation of the uterine environment. Estrus expression is also a proxy for effectiveness of hormonal treatments in inducing synchronous ovulations. Conception rates in females that expressed estrus inseminated with SexedULTRA™ were similar to that observed using conventional semen, but were much lower in females that did not express estrus (Table 6). Based on these observations, recommendations have been made for incorporating estrus detection aids (e.g. chalk, scratch pads) into FTAI strategies and selectively use sexed and conventional semen based on estrus expression by the time of insemination. With this approach, no decline is conception rate is expected and the overall resulting gender skew is directly related to efficacy of the program in result in estrus expression. For example, if the FTAI strategy produces estrus in 75% of the females and the conception rates are 55% for both females with signs of estrus inseminated with SexedULTRA™ and females not in estrus inseminated with conventional semen, than an overall conception rate of 55% and gender skew of 80% (semen gender purity 90%) would be expected.

Table 6. Conception rates in beef cattle using conventional and SexedULTRA™ semen and various fixed-time artificial insemination strategies according to expression of estrus by the time of insemination.

	Conventional	SexedULTRA™ with estrus	SexedULTRA™ without estrus
Thomas et al.[32]	59.9%	54.7%	28.6%
Baruselli et al.[33]	50.3%	53.1%	26.7%
Crites et al. [34]	56.7%	59.8%	27.3%
Colazo et al.[35]	59.1%	56.7%	27.8%
Bo et al.[36]	58.3%	53.2%	38.8%
Thomas et al.[37]	64.7%	52.3%	35.0%

These observations clearly indicate that breeding management practices optimized for conventional semen might not necessarily be optimal for sexed semen. Minor adjustments to management, especially insemination timing, significantly improve conception rates obtained with sexed semen and help close the fertility gap.

Sexed semen utilization

Data compiled by researchers from the USDA on Holstein breeding involving 5,963,876 heifer inseminations (1,323,721 to sexed semen) and 42,232,502 cow inseminations (253,586 to sexed semen) in the United States indicated that sexed semen utilization rate in heifers increased from 22.5% of total inseminations in 2013 to 30.7% in 2015. Although sexed semen utilization was still low in cows, rates increased from 0.5% in 2013 to 1% in 2015.[24]

Data obtained from STgenetics collaborating dairies across the US show that relative use of sexed semen increased from 7.6% in 2012 to 24% in 2017 in Holstein and from 31 to 84% in Jersey (Table 7). There has been a steady increase of sexed semen usage across all lactations, most prominently in heifers,

for which sexed semen has been the dominant semen type since 2015, but also in first- and second-lactation cows for which use of sexed semen is becoming more common. It is also evident the trend of almost exclusive use of sexed semen in Jersey; relative sexed semen usage has been > 80% in heifers and first and second lactation cows since 2016. The increase in sexed semen utilization is likely associated with the desire to increase selection pressure in heifers (see below) and improvements in fertility in both heifers and cows.

Table 7. Absolute number of inseminations using sexed semen and relative proportion of overall inseminations across lactations and years in Holstein and Jersey. Data from herd management programs of STgenetics collaborating dairies in the US (n = 296) including 1,105,696 sexed semen inseminations and 2,123,153 conventional semen inseminations.

	Holstein			Jersey		
Year	Heifers	Lactation 1	Lactation 2	Heifers	Lactation 1	Lactation 2
2012	47,424	2,508	416	17,620	5,954	4,236
	(30%)	(2%)	(0%)	(59%)	(26%)	(24%)
2013	66,301	3,245	486	19,648	6,121	3,740
	(40%)	(2%	(0%)	(54%)	(24%)	(21%)
2014	71,358	5,206	548	23,259	12,043	7,519
	(43%)	(4%)	(0%)	(49%)	(39%)	(35%)
2015	90,063	11,627	3,289	64,410	40,026	18,560
	(55%)	(7%)	(3%)	(81%)	(70%)	(64%)
2016	106,162	19,992	5,972	90,885	64,686	34,518
	(66%)	(12%)	(5%)	(90%)	(85%)	(82%)
2017	90,401	23,361	9,648	68,696	46,841	19,200
	(71%)	(19%)	(11%)	(94%)	(86%)	(70%)

Sire availability

Genomic selection has had a tremendous impact on the age of dairy sires with available conventional and sexed semen (Figure 16A). Whereas sire age for conventional semen was ~ 50 - 60 months in 2012, it decreased to < 40 months by 2017. Sire age for sexed semen 2012 was ~ 70 months, indicating that sexed semen from mostly old and proven sires were available at that time. Since 2015, there has been no difference between the age of sires for conventional or sexed semen, as most bovine genetic companies offer sexed semen on a diversified lineup of proven and young genomic bulls.

The genetic merit of sires with available sexed semen was relatively low when commercial application of the technology started in the 2000's, a reality that is very different from today. Evaluation of a core genetic index, 'Net Merit $', reveals that average genetic value of sires with sexed semen is now similar to sires with conventional semen. This trend is more pronounced in Holsteins than in Jerseys (Figure 16B).

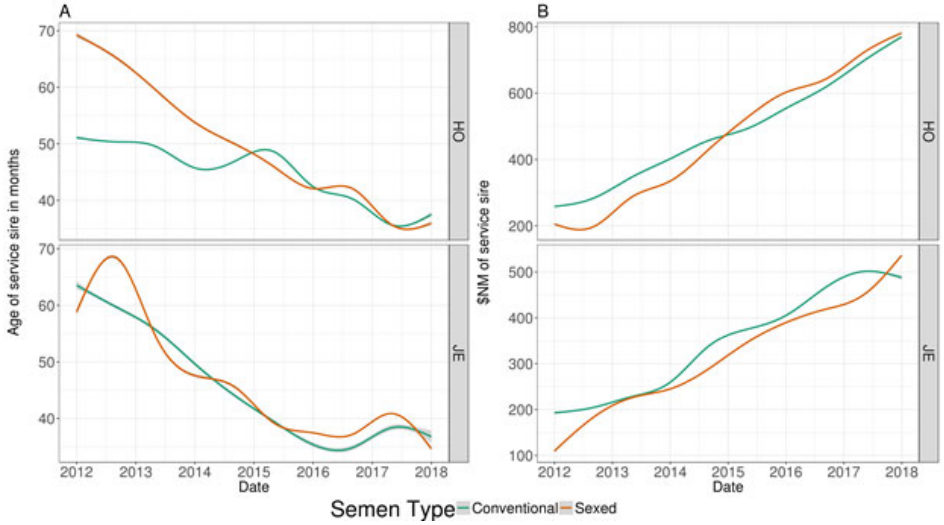

Figure 16. (A) Smoothed sire age and (B) net merit $ according to breed (HO: Holstein, JE: Jersey), semen type, and year.

With increasing interest in beef sexed semen, including for use in beef on dairy breeding schemes, there has also been an increase in availability of beef sires. In May 2019, sexed female and male semen are available in the US for > 75 beef sires of several breeds, including Angus, Red Angus, Simmental, Hereford, Wagyu and Brahman through STgenetics alone.

Genomic selection and sexed semen

Genetic progress for a given population can predicted using the Breeder's Equation:[49]

$\Delta G = (I \times R \times \sigma)/GI,$

where ΔG is the progress in genetic standard deviations per year, I is the selection intensity, R is the accuracy of selection, σ is the genetic standard deviation in the population under selection, and GI is the generation interval.

Genomics and sexed semen allow producers to affect the breeder's equation and genetic progress in multiple ways. Genomic prediction increases prediction accuracy of selection (R) of candidates without progeny information from ~ 0.5 to 0.8 for most traits; however, ability to make accurate selection decisions very early in life decreases the generation interval (GI) for sires of bulls from 6.5 to 1.75 years.[50] Accordingly, genomic selection alone might double genetic progress per year across all pathways of selection.

The biggest potential of sexed semen is evident in commercial dairies. Historically, there was little to no selection on the female side on commercial dairies. With replacement rates of 40% and accounting for calf losses and a sex ratio of 50% females, dairy producers needed to keep every female simply to maintain herd size. However, by increasing the proportion of females to ~ 90%, sexed semen offers a way of increasing selection intensity (I) on replacement females.

Table 8 shows an example of how genomics and sexed semen can affect genetic progress on the female side on commercial dairies. Given that everything else is equal, the use of sexed semen can increase ΔG for the example trait, milk yield, from 38.5 kg per year to 105.6 kg per year. Sexed semen in conjunction with genomic selection can leverage that progress even further to 184.8 kg per year. Making use of the available technologies can therefore enhance genetic progress in dams by a factor of 3 - 5.

Table 8. Predicted genetic progress for milk yield in dams of dams using different breeding strategies, including traditional (parent average and conventional semen), incorporation of sexed semen, and incorporation of both sexed semen and genomic selection.

Strategy	σ	P	I	R	GI	ΔG
Traditional	1100	0.8	0.35	0.4	4	38.5
Sexed Semen	1100	0.4	0.96	0.4	4	105.6
Sexed semen + genomic selection	1100	0.4	0.96	0.7	4	184.8

σ: genetic standard deviation in the population under selection; P: proportion of animals selected as replacements; R: accuracy of selection; I: selection intensity; GI: generation interval; ΔG is the progress in genetic standard deviations (milk kg per year).

Conclusion

SexedULTRA™ represents the 'coming of age' of sexed semen production technology. With 90% gender purity and fertility comparable to that obtained with conventional semen, this proven product now allows producers worldwide different opportunities to strategically manage breeding and genetic improvement programs to increase productivity and profitability.

Conflict of interest

There are no conflicts of interest to declare.

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