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Application and Commercialization of Flow Cytometrically Sex-Sorted Semen

D Rath[1] and LA Johnson[2]

[1]*Institute of Farm Animal Genetics, Mariensee (FLI), Neustadt, Germany;* [2]*Mount Airy, MD, USA*

Contents

The current technology to sort X and Y chromosome bearing sperm population requires individual identification and selection of spermatozoa in a modified high-speed flow cytometer. For farm animal species, the technology is capable of producing sexed sperm at greater than 90% purity. However, only in the bovine, the technology has reached a developmental level that allows its commercial application. Meanwhile, the demand for female calves has grown rapidly, which encourages the demand for sex-sorted semen from high genetic value bulls. The success of the technology will depend mainly on the fertilizing capacity of the sorted spermatozoa, as this is the most affecting and economically relevant factor. To date, fertility is still variable and is quite dependant on post-sort processing. New processing techniques are under investigation and will likely be able to improve the fertility rates after AI with sex-sorted semen. It is of great importance to select the right bulls and to test the sorted samples on a routine basis. In addition to the demand for sex-sorted semen by the cattle industry, there is also a significant demand expressed by pig farmers. However, it is still unknown if the use of sex-sorted semen through commercial pig AI will be economically feasible. For the pig, the combination of *in vitro* fertilization with sexed semen and non-surgical embryo transfer is an alternative that merits further scientific attention. Recent developments in ovine AI and ET will make it very likely that commercial sheep industry will adopt the sexing technology in their breeding concepts.

Introduction

Controlling the sex of offspring prior to conception permits the livestock industry to produce the optimal proportion of males and females to take advantage of sex-limited and sex-influenced traits, thus providing economically flexible management practices for the producer. Controlling sex ratio permits faster genetic progress, higher productivity, improves animal welfare by decreasing obstetric difficulties in cattle, avoiding castration in pigs, and producing less environmental impact due to the elimination of the unwanted sex before they grow to adulthood. Sex pre-selection in animals must be effective and efficient, must result in fertility equal to or better than with unsexed semen, and must be reasonably inexpensive and convenient to be widely applied (Foote and Miller 1971). Currently, the only known means of effectively producing separate populations of X and Y sperm in mammals is through the use of DNA differentiation by high-speed flow cytometry (Beltsville Sperm Sexing Technology; Johnson et al. 1989). Applicable to virtually all mammals, this method can produce populations of X or Y sperm with greater than 90% purity and subsequent offspring whose phenotypic sex is consistent with initial purity of the sorted sperm population. Limitations of the technology are entwined with the physiology and anatomy of the specific species in terms of the number of sperm required for fertilization and subsequent production of offspring. The current technology requires that each sperm be separately interrogated for DNA content, thus limiting the number of sorted X or Y sperm in cattle, sheep, swine and horses to approximately 12–20 million sperm per hour (Johnson and Welch 1999; de Graaf et al. 2007c; Heer 2007).

As flow cytometers and computers were developed and improved, it soon became apparent that flow cytometry held the key to separation of viable X and Y chromosome bearing sperm based on their DNA content, which could then be used for fertilization. Successful development of a viable sperm sexing procedure encompassed four steps to achieve a verifiable method of controlling sex ratio in mammals. These steps were:

(1) modification of a commercially available flow cytometer/cell sorter into a sperm cell sorter by adding a forward fluorescence detector and a bevelled sample injection needle to accommodate sperm orientation and minimize DNA variability (Johnson and Pinkel 1986),
(2) development of a method to stain living sperm with a vital fluorescent dye (Hoechst 33342, Sigma-Aldrich, München, Germany) in order to maintain viability through the sorting process and to the time of fertilization (Johnson et al. 1987a),
(3) merging the analytical and sorting capability of the sperm sorter to produce separate populations of living X and Y sperm based on differential DNA content (Johnson et al. 1989) and
(4) development of a method to re-analyse separate sorted populations of X and Y living sperm for their DNA content to verify the purity of X or Y sperm in the laboratory (Johnson et al. 1987b; Welch and Johnson 1999) to be a predictor of ultimate phenotypic sex of the live offspring (Johnson et al. 1989; Johnson 1991) and prove the efficacy of using DNA to differentiate X and Y sperm that would be used to achieve fertilization.

The limitations of single-cell measurement technology have been followed throughout the development of the technology and continued even to this day in commercial application. Consistent with these limitations, the method is applied most easily in the bovine (2 million sperm per AI; Seidel et al. 1997), ovine (1–5 million sperm per AI; de Graaf et al. 2007c) but hardly at all in swine (50–100 million sperm per AI; Rath et al. 2003; Vazquez et al. 2003; Grossfeld et al. 2005). Even with the obvious limitations, the technology has begun to flourish in commercial bovine practice. It is estimated

that more than 2 million sexed calves have been produced since the technology was first applied to commercial practice in 2000. Although the license to begin commercialization using AI was granted by the US Department of Agriculture in 1996 (Patent #5,135,759) to XY-Inc. Fort Collins, CO, USA, commercial production of bovine sexed sperm did not begin until 2000 (Cogent Breeding Ltd., Chester, UK). However, insufficient development of the technology by the parent company hampered faster and wider commercial application. Within the past year, XY-Inc. was sold to a sub-licensee (Sexing Technologies, Navasota, TX, USA). Prior to this sale, Sexing Technologies had some 20 sorters operating in North America, which has now been expanded to more than 50 sorters in numerous locations in North and South America, Europe and other parts of the world. These recent developments suggest that sexed bovine sperm will soon be available to most cattle producers around the world. Intensive research is still required to further improve the existing technology or find alternatives and/or combinations with other biotechniques. Increased technology development is critical in order to develop the large commercial opportunity that exists for sexed semen cattle and in other livestock species, especially sheep, swine and horses. In this review, the current status of the sex-sorting technology associated with commercial applications and the consequences for farm animal agriculture are summarized.

The Limitations of Single-Cell Measurement Technology

The current technology requires single sperm identification, individual recognition of the orienting position in front of the laser beam and single droplet charging. Although there has been significant progress in the number of sperms sorted per unit time in the last 12 years (1–2 million sperm per hour to the current level of approximately 20 million sperm per hour), the process remains inefficient in the production of sperm for AI. Standard AI dose numbers of fresh or frozen unsexed sperm are out of reach for this technology. Efficient utilization of sex-sorted semen requires a significant reduction in spermatozoa per AI dose or for use in *in vitro* fertilization (IVF) and other biotechniques. For example, 2 million *live* spermatozoa from many bulls or for some bulls even below seem to be sufficient for AI. The usability of these bulls for sex-sorting depends only partly on the strength of their spermatozoa to survive the sorting process. It is well known that the fertilizing ability with unsorted spermatozoa varies among individual bulls when low sperm concentrations are used for AI (Den Daas et al. 1998).

In addition to the effects of reduced sperm number, high dilution effects diminish the fertilizing potential of spermatozoa as the protecting and regulating substances of seminal plasma are also diluted or eliminated (Maxwell and Johnson 1999; Centurion et al. 2003).

The differential sperm DNA content is identified with the Bis-benzimide Hoechst 33342 that emits a blue fluorescence when excited with UV light. Hoechst 33342 was selected based on the fact that it was a vital stain and one that effectively was able to penetrate the living sperm membrane and bind to the DNA of the highly condensed chromatin of the sperm nucleus (Johnson et al. 1987a). In several experiments, the influence of the Hoechst 33342 on DNA integrity was tested. Johnson et al. (1989) postulated that fluorochrome dyes reduce embryonic viability by mid-gestation. This coincides with the findings of Spinaci et al. (2005) with boar spermatozoa. Co-incubation with Hoechst dye as well as the sorting process itself diminished the percentage of live spermatozoa. The damaged ability to fertilize and carry the embryo to term may be the result of the combined effects of the dye and UV laser or of either individually. Higher laser intensity is more damaging than the lower laser intensity as shown for rabbit spermatozoa by Johnson et al. (1996). Recently, Schenk and Seidel (2007) reported a similar finding for bovine semen. However, less laser intensity diminishes the resolution and indirectly the sort rates. Guthrie et al. (2002) saw no differences on embryo development when pig spermatozoa were illuminated with 125 or 25 mW laser power, but optimum resolution during sorting between X and Y intact sperm required at least 125 mW laser power.

Catt et al. (1997) labelled human spermatozoa on microscope slides with the Hoechst dye and exposed them to UV laser light. No changes were found in the frequency of endogenous DNA nicks. This is in agreement with a recent study from Parrilla et al. (2004), indicating no genotoxic effects of Hoechst 33342 in porcine spermatozoa. Boe-Hansen et al. (2005) used the neutral Comet assay and the sperm condensation structure assay (SCSA) to evaluate sperm chromosome integrity. Both tests showed that sperm integrity is improved in the sorted population when compared with unsorted semen. This finding is no doubt due to the presence of FD#40 red food dye, which is added to the pre-sort sample to eliminate membrane damaged sperm from the sorting process (Johnson et al. 1999). Similar results were obtained by De Ambrogi et al. (2006), who found no effect on the defragmentation index after sorting, and de Graaf et al. (2007a,b) getting even better fertilization and ET results with sorted semen when compared with high diluted controls.

A significant factor during sorting is also the repeated electrical charging and electrostatic deviation. Membranes of the mid-piece of the sperm tail are sensitive to the electric field and may undergo depolarization. Furthermore, we believe that mitochondrial activity is reduced due to the presence of reactive oxygen species (ROS) produced by electric forces (Klinc and Rath 2007; Klinc et al. 2007). Similar positive results were reported for sex-sorted boar spermatozoa when media were supplemented with PSP I/II heterodimers (García et al. 2007), which increased motility and mitochondrial activity (Centurion et al. 2003). However, in a study reported by de Graaf et al. (2007b), they were unable to see similar benefits to ram semen antioxidants or seminal plasma.

Commercial Application of Sex-Sorted Spermatozoa in Livestock Production

Excellent reviews on the commercialization, especially of bovine sexed semen, have been published by Amann (1999), Seidel (2003a,b), Garner (2006) and Garner and Seidel (2008). Although their calculation models are mainly directed to the American farm situation, they are helpful guidelines for other countries too. The situation in other species had been summarized by Maxwell et al. (2004) and for pigs by Johnson et al. (2005). Sperm sorting based on the Beltsville Sperm Sexing Technology has reached a certain technical standard that makes it robust enough for use under practical conditions, even if it has clear limitations as described above. Its utilization differs significantly among species mainly because of the biological differences related to site of semen deposition, length of cycle, demand on sperm numbers, sortability and freezability of spermatozoa as well as commercial demands and management conditions.

The Commercial Use of Bovine Sex-Sorted Semen

Stage of applicability

Due to the high sorting index (131), an approximation of the ability to flow cytometrically sort sperm consisting of the head profile area (μm^2) × X-Y sperm DNA difference (%; Garner 2006), bovine spermatozoa are more suitable for high-speed sorting than other species. The method has reached a standard that is likely to make it profitable in dairy herds soon. However, impairments to the fertilizing capacity of sorted bull sperm are obvious and impairment of fertilizing capacity because of the impact of sorting and high dilution on capacitation and seminal plasma components continue to be limitations to be dealt with. Further improvement of the pre- and post-sort handling of the semen is critical and requires intensive research to minimize negative effects on the post-thaw lifespan of sexed spermatozoa as the fertile phase has direct implications on AI regimens. In a recent study employing a modified sperm handling protocol during sorting and freezing and in combination with a new extender for both sorting and freezing (Sexcess®, Masterrind GmbH, Verden, Germany), we were able to extend the post-thaw viability and progressive motility for several hours as shown by thermo tolerance test and calving rates (Klinc 2005; Klinc et al. 2007). In this AI study, sorted semen (2 million live sperm/straw) was thawed and inseminated in accordance with the existing protocols for conventional semen (thawing at 37°C for 20 s; AI 12–24 h after onset of heat; semen deposition in the uterine body or if possible without force into the ipsilateral horn). Pregnancy and calving rates were equal to controls. Similar data were also obtained for sorted liquid semen stored for up to 72 h (Klinc and Rath 2007). Significant technical improvements in sperm quality after sorting were developed using the latest laser technology and replacing the water-cooled Argon gas laser with a pulsed solid-state laser. Shorter exposure to the laser light seems to further reduce sperm stress, and observations with ram spermatozoa indicate increased fertility rates (de Graaf et al. 2007c). Another stress factor is the hydrodynamic pressure. The percentage of live bull and stallion spermatozoa increased significantly when the fluid pressure was lowered from 2.07 to 2.59 mm Hg and increased developmental rates of bovine IVF embryos (Campos-Chillon and de la Torre 2003; Suh et al. 2005).

For many reasons, it may be helpful to sort conventionally frozen semen after thawing and refreeze the sorted samples. Such methods are under investigation. Hollinshead et al. (2004) showed for the first time that bull semen survives an adapted protocol from ram (Hollinshead et al. 2003) employing a gradient centrifugation to separate egg yolk from the thawed samples and to enrich intact sperm prior to sorting. Maxwell et al. (2007) tested PureSperm® (Labotect, Göttingen, Germany) as effective gradient for high diluted, unsorted bull sperm, and in a recent study, Parrilla et al. (unpublished) showed that PureSperm and Bovipure® (Labotect, Göttingen, Germany) are suitable for refreezing sexed bull sperm and to produce embryos to the same extent as with unsorted sperm from the same ejaculates.

Altogether, the technical improvements made in the recent years allow for the maintenance of an acceptable standard of bovine semen quality. Nevertheless, it is self-evident to control the sorted sperm quality on a daily basis. Some suggested prerequisites for sex-sorted bull semen of optimal quality are as follows:

(1) Bulls must be pre-tested and those failing with unsorted low-dose insemination should be excluded from their use to produce sex-sorted sperm.
(2) A complete spermiogram should be made from each ejaculate before sorting. The test should include: macroscopical evaluation, mass movement categorization, single sperm motility estimation, evaluation of sperm concentration and a complete morphological evaluation test, especially as a faster acrosome reaction is commonplace in sex-sorted sperm (Mocé et al. 2006).
(3) After sorting, aliquots of each batch should be tested for single sperm motility, motility pattern in a thermal tolerance test preferably for 6 h at 38°C, precise morphological evaluation for acrosome integrity and total morphology, FACS Analysis (FITC-PNA/Syto17/PI; SCSA), and a general test of microbiological contamination. These tests may be performed with sorted sperm of the unwanted sex. Additionally, one straw from each batch of the wanted sex needs to be reanalysed for sorting purity and the total number of spermatozoa per straw. Ballester et al. (2007) confirmed that it is not sufficient to test semen quality directly after thawing. Incubation of the samples at 38°C revealed a reduction of linearity, sperm viability (Seminaphtharhodafluor) and acrosome integrity.
It is recommended to put at least 2 million live spermatozoa in a straw as the absolute minimum. Andersson et al. (2004, 2006) clearly showed that more than 2 million spermatozoa are necessary to obtain satisfactory pregnancy and calving rates. Caution must also be exercised to account for high individual bull effects. In order to avoid losses of the highly diluted semen, the liquid column in the straw should be divided into a first segment containing

extender only, which will close the cotton plug, and a second segment containing the sexed spermatozoa. Both segments need to be divided by an air bubble. Automatic systems to fill the straws in this manner are commercially available.
(4) It is recommended that only heifers are inseminated with sex-sorted semen at the present time. From several studies on our research farm, we have no indication that neither timing nor variability of ovulation nor site of insemination is responsible for the large differences in non-return rates between heifers and cows.

On the female side, it is a prerequisite to provide optimal herd management to attain high female fertility (Seidel 2003a,b). Virtually, all fertility results to date from the AI of sex-sorted semen in cattle have been from heifers. Using AI with sorted semen in cows frequently produces unacceptable non-return rates, possibly because of the inability to consistently pinpoint ovulation for optimum AI.

Several groups (Cran et al. 1994; Merton et al. 1997; Lu et al. 1999) reported similar cleavage rates but reduced blastocyst development following fertilization *in vitro* with sorted bovine sperm cells. In a recent study, we demonstrated for the first time that bovine IVF-embryos derived from sex-sorted spermatozoa display a reduction in the relative abundance of developmentally important genes like Gluc-3 and G6PD, compared with their counterparts derived from unsorted semen (Morton et al. 2007). It requires further in-depth study as epigenetic changes already occurring in the early embryo are thought to be involved in postnatal abnormalities. These are deleterious effects of sperm sexing on spermatozoa beyond those previously recognized and reflected in the developmental competence of embryos. Similarly, cleavage rates after IVF with sex-sorted spermatozoa were 30% below those of unsorted spermatozoa of the same ejaculate, blastocyst formation on day 8 was 30–40% lower than for the controls (Bermejo-Alvarez et al. 2007) and cell cycles were reduced (Beyhan et al. 1999) or disturbed in timing (Cran et al. 1993; Lu et al. 1999; Morton et al. 2005). On the contrary, Seidel et al. (1999) found no excess embryonic loss between 1 and 2 months of gestation in heifers inseminated with sorted sperm.

Commercial demands

The demand for sex-sorted semen may vary between countries. Based on the American market situation, advantages are foreseeable for the decoupling of production of replacement heifers and cows from the number of culled cows. More heifers are available for herd replacement and by this a stronger selection will be possible. A higher degree of female selection will have an impact on the genetic development of the population as females will contribute with up to 15% on genetic selection, which was based so far on sires (Weigel and Barlass 2003). As the number of replacement animals is satisfied, herd size may grow and more females are sold. In parallel, the milk production can rise and the market price may decrease as benefit for consumers. In addition, the costs for progeny testing and embryo transfer will decrease and eventually the economic benefit for genetic markers will be enhanced as more siblings of the same sex can be produced of the best bulls and cows. The more the technology develops and more reasonable pricing is able to be applied the more the prices for replacement and export heifers will decrease. Therefore, only the early users will benefit most by selling surplus heifers at substantially higher prices than the cost to raise or replace (De Vries et al. 2008). It is essential that as commercialization increases, more data on fertility, embryonic losses, foetal deformations, abortions and heifer health can be collected. Such information will help to evaluate the technology correctly for its practical use.

Status of commercial implementation

The technology is the most highly developed for bovine semen and was introduced into commercial application in the United Kingdom in 2000. Recently, several American AI centres have contracted for the technology and offer sexed semen from their bulls worldwide. As this manuscript is written, there are about 50 sorters in the USA and three new centres in Europe starting production of sexed bull semen. At the current situation, the high price for sexed semen may be adequate as long as the demand is high for heifers but its value will decrease with decreasing milk, heifer and cull cow prices, with increasing feed costs and lower pricing of conventional semen. If the sex-sorted semen is highly fertile and female prices are $200 (approximately 130€) above males, Seidel (2003a) considered an additional price of $21.56 per AI dose as affordable for marketing.

In a recent study, Ettema et al. (2007) calculated the situation in Denmark as an example for the European market. They developed a spreadsheet model including price for springing heifers, replacement costs, price per beef calf, price of sexed semen, conception rates with sexed semen, replacement rates, sex ratio of sexed semen, incidence of dystocia and stillbirth. The main factors affecting price are lowered fertility, the high cost of equipment, necessity for skilled personnel and the cost for intellectual property. From this data, it is obvious that the net return to assets (NRA) will be negative for the first year and it will take 3–4 years before herds have reached a steady state with respect to raising and selling replacements.

In summary, sex-sorted bull semen is in high demand. The success of a widespread application depends on its price. It has to be moderate enough to allow a reasonable profit for farmers, and it has to be high enough to make its production lucrative (Seidel et al. 2003). A prerequisite for integration in genetic programmes is the quality of the sorted semen. Under optimal conditions, fertility is beginning to reach satisfactory levels though semen variability and management factors continue to be critically important to success in commercial practice.

The Commercial Use of Ovine Sex-Sorted Semen

Stage of applicability

In sheep, insemination with less than 1 million motile unsexed sperm has been demonstrated to result in acceptable fertility (Walker et al. 1984; 5 million motile, Eppleston et al. 1986) but the results vary considerably (Salamon and Maxwell 2000). Lambs from sex-sorted semen were produced via laparoscopy by Cran et al. (1997). Subsequent experiments with 1–16 million sex-sorted frozen-thawed spermatozoa produced disappointing levels of fertility (Hollinshead et al. 2002, 2003). Even inseminations with up to 40 million total sorted, cryopreserved spermatozoa were inconclusive (Hollinshead et al. 2003). However, in a very recent study using an adapted protocol, de Graaf et al. (2007c) demonstrated that dosages of 1 or 5 million sorted motile ram sperm gave superior fertility to non-sorted sperm when laparoscopically inseminated. These results have significance for the future commercialization of sex pre-selection technology in sheep as a reduction in the minimum effective sperm number will allow a corresponding decrease in the associated cost per dose and set aside a larger percentage of the sheep industry to utilize sex pre-selection. In another trial, the same group showed for the first time for any species that frozen-thawed spermatozoa, after sex-sorting and a second cryopreservation step are capable of producing offspring of the predicted sex following AI (de Graaf et al. 2006). Sorting of frozen semen and subsequent refreezing with the birth of lambs had only been described so far after IVF and ET (Hollinshead et al. 2004). Sex-sorted ram sperm can be used effectively with intra-cytoplasmic single sperm injection (ICSI; Catt et al. 1996; Hollinshead et al. 2002) and IVF (Rhodes et al. 1994; Maxwell et al. 2004; Morton et al. 2004). de Graaf et al. (2007a) also showed that the potential of sex-sorted frozen-thawed ram semen is equal to unsorted semen when used for laparoscopic insemination, or intrauterine insemination in superovulated ewes and subsequent embryo transfer of morula and blastocysts.

Status of commercial implementation

Commercialization of sex-sorted ram spermatozoa for laparoscopical insemination may play a role in the near future, though it is questionable, whether its use will be sufficiently profitable to make it routinely available. The latest results of the University of Sydney group (de Graaf et al. 2006, 2007c) are very promising and the implementation of sex-sorted ram sperm in the commercial practice may have a significant impact on the Australian and New Zealand markets.

The Commercial Use of Porcine SexSorted Semen

Stage of applicability

In principle, porcine sperm are as easy to sort as other livestock species. Its sorting index (115) ranges between bull and ram sperm (Garner 2006). The primary limitations are the large amount of sperm necessary for AI and the sensitivity of sorted sperm against high dilution and cryopreservation. Factors associated with processing boar sperm for sexing lead to capacitation like membrane changes (Ashworth et al. 1994; Maxwell et al. 1997, 1998; Barrios et al. 2000). Seminal plasma as a constituent of the sample medium and of the catch medium has been shown to be beneficial to decapacitate sorted spermatozoa (Caballero et al. 2004).

Embryos from IVF with sexed boar semen were first produced 15 years ago (Rath et al. 1993). Subsequently, offspring were produced after sex-sorting and IVF of non-frozen spermatozoa (Rath and Niemann 1996, 1997; Abeydeera et al. 1998). In addition, ICSI has been used successfully to produce male offspring (Probst and Rath 2003).

A major step forward was made with the idea of low-dose insemination (for review see Rath 2002) and with the development of a flexible catheter (Firflex®, Minitüb, Tiefenbach, Germany) for deep intrauterine insemination (DIU) in Spain (Martinez et al. 2001, 2002; Cuello et al. 2005). Progeny have been produced after DIU of non-frozen bulk-sorted (Vazquez et al. 2003) and sex-sorted non-frozen sperm (Rath et al. 2003; Grossfeld et al. 2005), using as few as 50×10^6 spermatozoa in a volume of 2 ml (Rath et al. 2003). However, the method is only available for sows and not for primiparous gilts. Placing the semen directly in front of the uterotubal junction may avoid major sperm losses during uterine transportation. Unilateral insemination is sufficient to find adequate number of embryos in both horns (Tummaruk et al. 2007).

Boar semen has been sex-sorted, frozen, thawed and surgically inseminated into the oviduct to produce live offspring (Johnson et al. 2000). However, a repeatable method for freezing sex-sorted boar sperm remains to be developed. Although attempts to freeze sex-sorted boar semen resulted in sufficient post-thaw sperm quality, no pregnancies were able to go to term after non-surgical DUI (Bathgate et al. 2008). Similarly, embryos derived from IVF with sex-sorted frozen sperm and transferred non-surgically did not develop to term (Bathgate et al. 2007).

Commercial demands

In swine, the greatest general demand for sex-sorted semen is to produce female piglets for meat production. Several European countries will soon begin to forbid castration of male piglets; therefore a shift in sex ratios will be a necessity for pig producers under such regulations.

Status of commercial implementation

The Beltsville Sperm Sexing Technology, as it is currently used in research and commercial practice, is unable to produce sufficient numbers of sperm to make swine AI feasible at the present time. In its current stage of development, sex-sorted pig sperm could be used in specialized commercial practice, e.g. nuclear herds, if combined with IVF/ET and/or laparoscopic insemination (Rath et al. 1999; Vazquez et al. 2006; García et al. 2007). Optimum utilization of sex-sorted boar sperm

would suggest that it would be advantageous and essential to freeze sex-sorted boar spermatozoa so that the greatest flexibility for insemination and ovulation can be exercised.

The Commercial Use of Equine Sex-Sorted Semen

Semen from horses can be sorted even though the efficiency is lower than in other livestock species. However, the value of a single dose may cover all production costs. Buchanan et al. (2000) reported a 40% pregnancy rate in mares after AI of 25×10^6 sex-sorted non-frozen spermatozoa and pregnancies have even been obtained with as few as 5×10^6 liquid or frozen-thawed spermatozoa using hysteroscopic insemination (Lindsey et al. 2002a, b). Recently, a filly was born after hysteroscopic insemination with low numbers of frozen-thawed sexed spermatozoa into the uterotubal junction. Highly diluted unsorted semen from the same ejaculates did no better. Undiluted semen inseminated at a dose of 5×10^8 spermatozoa yielded satisfactory conception rates (Clulow et al. 2007). Therefore, the poor fertility after hysteroscopic insemination with low doses of sex-sorted or non-sorted spermatozoa may be directly attributable to the low-dose insemination conditions with frozen-thawed rather than sex-sorted spermatozoa. In order to improve the post-thaw quality of sex-sorted stallion semen, different approaches were focused on cushion centrifugation before and after sorting as well as pre-selection of a stable sperm population using a PureSperm gradient (Knop et al. 2005; Heer 2007) and different automated freezing protocols (Buss 2006; Clulow et al. 2007). The quality of the sorted frozen semen was improved stepwise and insemination trials have yet to be performed. The major difficulty with equine semen is its variability among stallions and ejaculates. The commercialization of sexed stallion semen will depend on the consistency of the fertility after sorting and freezing, a broader number of stallions with sortable ejaculates and improvements of the current hysteroscopic insemination technique.

References

Abeydeera LR, Johnson LA, Welch GR, Wang WH, Boquest AC, Cantley TC, Rieke A, Day BN, 1998: Birth of piglets preselected for gender following in vitro fertilization of in vitro matured pig oocytes by X and Y chromosome bearing spermatozoa sorted by high speed flow cytometry. Theriogenology **50,** 981–988.

Amann RP, 1999: Issues affecting commercialization of sexed sperm. Theriogenology **52,** 1441–1457.

Andersson M, Taponen J, Koskinen E, Dahlbom M, 2004: Effect of insemination with doses of 2 or 15 million frozen-thawed spermatozoa and semen deposition site on pregnancy rate in dairy cows. Theriogenology **61,** 1583–1588.

Andersson M, Taponen J, Kommeri M, Dahlbom M, 2006: Pregnancy rates in lactating Holstein-Friesian cows after artificial insemination with sexed sperm. Reprod Domest Anim **41,** 95–97.

Ashworth PJC, Harrison RAP, Miller NGA, Plummer JM, Watson PF, 1994: Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. Reprod Fertil Dev **6,** 173–180.

Ballester J, Johannisson A, Saravia F, Håård M, Gustafsson H, Bajramovic D, Rodriguez-Martinez H, 2007: Post-thaw viability of bull AI-doses with low-sperm numbers. Theriogenology **68,** 934–943.

Barrios B, Pérez-Pé R, Gallego M, Tato A, Osada J, Muiño-Blanco T, Cebrián-Pérez JA, 2000: Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod **63,** 1531–1537.

Bathgate R, Morton KM, Eriksson BM, Rath D, Sieg B, Maxwell WM, Evans G, 2007: Non-surgical deep intrauterine transfer of in vitro produced porcine embryos derived from sex-sorted frozen-thawed boar sperm. Anim Reprod Sci **99,** 82–92.

Bathgate R, Grossfeld R, Susetio D, Ruckholdt M, Heasman K, Rath D, Evans G, Maxwell WM, 2008: Early pregnancy loss in sows after low dose, deep uterine artificial insemination with sex-sorted, frozen-thawed sperm. Anim Reprod Sci **104,** 440–444.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A, 2007: Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiol Genomics **17,** 264–272.

Beyhan Z, Johnson LA, First NL, 1999: Sexual dimorphism in IVM-IVF bovine embryos produced from X and Y chromosome-bearing spermatozoa sorted by high speed flow cytometry. Theriogenology **52,** 35–48.

Boe-Hansen GB, Morris ID, Annette B, Ersbølla K, Greve T, Christensen P, 2005: DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. Theriogenology **63,** 1789–1802.

Buchanan BR, Seidel GE, McCue PM, Schenk JL, Herickhoff LA, Squires EL, 2000: Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenology **53,** 1309–1321.

Buss H, 2006: Improvement of the freezability of sex-sorted stallion spermatozoa. Thesis, University of Veterinary Medicine Hannover, Hannover, Germany. (in German).

Caballero I, Vazquez JM, Centurión F, Rodríguez-Martinez H, Parrilla I, Roca J, Cuello C, Martinez EA, 2004: Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. Reprod Domest Anim **39,** 370–375.

Campos-Chillon LF, de la Torre JF, 2003: Effect on concentration of sexed bovine sperm sorted at 40 and 50 psi on developmental capacity of in vitro produced embryos. Theriogenology **59,** 506. (Abstract).

Catt SL, Catt JW, Gomez MC, Maxwell WMC, Evans G, 1996: Birth of a male lamb derived from in vitro matured oocyte fertilized by intracytoplasmic injection of a single 'male' sperm. Vet Rec **139,** 494–495.

Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WMC, Evans G, 1997: Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human sperm. Mol Hum Reprod **3,** 821–825.

Centurion F, Vazquez JM, Calvete JJ, Roca J, Sanz L, Parrilla I, Garcia EM, Martinez EA, 2003: Influence of porcine spermadhesins on the susceptibility of boar spermatozoa to high dilution. Biol Reprod **69,** 640–646.

Clulow JR, Buss H, Sieme H, Rodger JA, Cawdell-Smith AJ, Evans G, Rath D, Morris LH, Maxwell WM2007: Field fertility of sex-sorted and non-sorted frozen-thawed stallion spermatozoa. Anim Reprod Sci. doi:10.1016/j.anireprosci.2007.08.015. (Epub ahead of print)

Cran DG, Johnson LA, Miller NGA, Cochrane D, Polge CE, 1993: Production of bovine calves following separation of

X- and Y-bearing sperm and in vitro fertilization. Vet Rec **132,** 40–41.
Cran DG, Cochrane DJ, Johnson LA, Wei H, Lu KH, Polge C, 1994: Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF. Theriogenology **41,** 183. (Abstract).
Cran DG, McKelvey WAC, King ME, Dolman DF, McEvoy TG, Broadbent PJ, Robinson JJ, 1997: Production of lambs by low dose intrauterine insemination with flow cytometrically sorted and unsorted sperm. Theriogenology **47,** 267. (Abstract).
Cuello C, Berthelot F, Martinat-Botté F, Venturi E, Guillouet P, Vázquez JM, Roca J, Martínez EA, 2005: Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. Anim Reprod Sci **85,** 275–286.
De Ambrogi M, Spinaci M, Galeati G, Tamanini C, 2006: Viability and DNA fragmentation in differently sorted boar spermatozoa. Theriogenology **66,** 1994–2000.
De Vries A, Overton M, Fetrow J, Leslie K, Eicker S, Rogers G, 2008: Exploring the impact of sexed semen on the structure of the dairy industry. J Dairy Sci **91,** 847–856.
Den Daas JH, De Jong G, Lansbergen LM, Van Wagtendonk-De Leeuw AM, 1998: The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J Dairy Sci **81,** 1714–1723.
Eppleston J, Maxwell WMC, Battye KM, Roberts EM, 1986: Effect of thawed motility and intrauterine dose of motile sperm on fertility in ewes. Proc Aust Soc Reprod Biol **18,** 19.
Ettema JF, Hoag DL, Seidel DE Jr, 2007: Economic opportunities for sexed semen on commercial dairies. West Dairy News **7,** 67–68.
Foote RH, Miller P, 1971: What might sex ratio control mean in the animal world? In: Kiddy CA, Hafs HD(eds), Sex Ratio at Birth Prospects for Control. American Society of Animal Science, Champaign, pp. 1–9.
García EM, Vázquez JM, Parrilla I, Calvete JJ, Sanz L, Caballero I, Roca J, Vazquez JL, Martínez EA, 2007: Improving the fertilizing ability of sex sorted boar spermatozoa. Theriogenology **68,** 771–778.
Garner DL, 2006: Flow cytometric sexing of mammalian sperm. Theriogenology **65,** 943–957.
Garner DL, Seidel GE Jr, 2008: History of commercializing sexed semen for cattle. Theriogenology **69,** 886–895.
de Graaf SP, Evans G, Maxwell WM, O'Brien JK, 2006: In vitro characteristics of fresh and frozen-thawed ram spermatozoa after sex-sorting and re-freezing. Reprod Fertil Dev **18,** 867–874.
de Graaf SP, Beilby K, O'Brien JK, Osborn D, Downing JA, Maxwell WM, Evans G, 2007a: Embryo production from superovulated sheep inseminated with sex-sorted ram spermatozoa. Theriogenology **67,** 550–555.
de Graaf SP, Evans G, Gillan L, Guerra MMP, Maxwell WM, O'Brien JK, 2007b: The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of sorted and non-sorted ram spermatozoa. Theriogenology **67,** 217–227.
de Graaf SP, Evans G, Maxwell WM, Downing JA, O'Brien JK, 2007c: Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep. Reprod Domest Anim **42,** 648–653.
Grossfeld R, Klinc P, Sieg B, Rath D, 2005: Production of piglets with sexed semen employing a non-surgical insemination technique. Theriogenology **63,** 2269–2277.
Guthrie HD, Johnson LA, Garrett WM, Welch GR, Dobrinsky JR, 2002: Flow cytometric sperm sorting: effects of varying laser power on embryo development in swine. Mol Reprod Dev **61,** 87–92.
Heer P, 2007: Adjustment of cryopreservation of stallion spermatozoa to the Beltsville Sperm Sexing Technology. Doctorial Thesis, University of Veterinary Medicine Hannover, Hannover, Germany (in German).
Hollinshead FK, O'Brien JK, Maxwell WMC, Evans G, 2002: Production of lambs of predetermined sex after the insemination of ewes with low numbers of frozen-thawed sorted X- or Y-chromosome-bearing spermatozoa. Reprod Fertil Dev **14,** 503–508.
Hollinshead FK, Gillian L, O'Brien JK, Evans G, Maxwell WMC, 2003: In vitro and in vivo assessment of functional capacity of flow cytometrically sorted ram spermatozoa after freezing and thawing. Reprod Fertil Dev **15,** 351–359.
Hollinshead FK, Evans G, Evans KM, Catt SL, Maxwell WMC, O'Brien JK, 2004: Birth of lambs of a predetermined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. Reproduction **127,** 557–568.
Johnson LA, 1991: Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. Reprod Domest Anim **26,** 309–314.
Johnson LA, Pinkel D, 1986: Modification of a laser-based flow cytometer for high-resolution DNA analysis of mammalian spermatozoa. Cytometry **7,** 268–273.
Johnson LA, Welch GR, 1999: Sex preselection: high-speed flow cytometric sorting of X- and Y-sperm for maximum efficiency. Theriogenology **52,** 1134–1323.
Johnson LA, Flook JP, Look MV, 1987a: Flow cytometry of X and Y chromosome-bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. Gamete Res **17,** 203–212.
Johnson LA, Flook JP, Look MV, Pinkel D, 1987b: Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. Gamete Res **16,** 1–9.
Johnson LA, Flook JP, Hawk HW, 1989: Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod **41,** 199–203.
Johnson LA, Cran DG, Welch GR, Polge C, 1996: Gender preselection in mammals. In: Miller RH, Pursel VG, Norman HD(eds), Beltsville Symposium XX: Biotechnology's Role in the Genetic Improvement of Farm Animals. American Society of Animal Science, Savoy, pp. 151–164.
Johnson LA, Welch GR, Rens W, 1999: The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci **77**(Suppl. 2)**,** 213–220.
Johnson LA, Guthrie HD, Fiser P, Maxwell WMC, Welch GR, Garrett WM2000: Cryopreservation of flow cytometrically sorted boar sperm: effects on in vivo embryo development. J Anim Sci **78** (Suppl. 1)**,** 198. (Abstract).
Johnson LA, Rath D, Vazquez JM, Maxwell WMC, Dobrinsky JR, 2005: Preselection of sex of offspring in swine for production: current status of the process and its application. Theriogenology **63,** 615–624.
Klinc P, 2005: Improved fertility of flow cytometrically sex selected bull spermatozoa. Doctoral thesis, University of Veterinary Medicine Hannover, Hannover, Germany.
Klinc P, Rath D, 2007: Reduction of oxidative stress in bovine spermatozoa during flow cytometric sorting. Reprod Domest Anim **42,** 63–67.
Klinc P, Frese D, Osmers H, Rath D, 2007: Insemination with sex sorted fresh bovine spermatozoa processed in the presence of antioxidative substances. Reprod Domest Anim **42,** 58–62.
Knop K, Hoffmann N, Rath D, Sieme H, 2005: Effects of cushioned centrifugation technique on sperm recovery and sperm quality in stallions with good and poor semen freezability. Anim Reprod Sci **89,** 294–297.

Lindsey AC, Morris LHA, Allen WR, Schenk JL, Squires EL, Bruemmer JE, 2002a: Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa. Equine Vet J **34,** 128–132.

Lindsey AC, Schenk JL, Graham JK, Bruemmer JE, Squires EL, 2002b: Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. Equine Vet J **34,** 121–127.

Lu KH, Cran DG, Seidel GE, 1999: In vitro fertilization with flow-cytometrically sorted bovine sperm. Theriogenology **52,** 1393–1405.

Martinez EA, Vazquez JM, Roca J, Lucas X, Gil MA, Parrilla I, 2001: Successful non-surgical deep intrauterine insemination with small numbers of spermatozoa in sows. Reproduction **122,** 289–296.

Martinez EA, Vazquez JM, Roca J, Lucas X, Gil MA, Parrilla I, Vazquez JL, Day BN, 2002: Minimum number of spermatozoa required for normal fertility after deep intrauterine insemination in non-sedated sows. Reproduction **123,** 163–170.

Maxwell WMC, Johnson LA, 1999: Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology **52,** 1353–1362.

Maxwell WMC, Welch GR, Johnson LA, 1997: Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev **8,** 1165–1178.

Maxwell WMC, Long CR, Johnson LA, Dobrinsky JR, Welch GR, 1998: The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev **10,** 433–440.

Maxwell WMC, Evans G, Hollinshead FK, Bathgate R, de Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK, 2004: Integration of sperm sexing technology into the ART toolbox. Anim Reprod Sci **82,** 79–95.

Maxwell WM, Parrilla I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D, 2007: Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm density gradient centrifugation. Reprod Domest Anim **42,** 489–494.

Merton JS, Haring RM, Stap J, Hoebe RA, Aten JA, 1997: Effect of flow cytometrically sorted frozen/thawed semen on success rate of in vitro bovine embryo production. Theriogenology **47,** 295. (Abstract).

Mocé E, Graham JK, Schenk JL, 2006: Effect of sex-sorting on the ability of fresh and cryopreserved bull sperm to undergo an acrosome reaction. Theriogenology **66,** 929–936.

Morton KM, Catt SL, Hollinshead FK, Maxwell WMC, Evans G2004: Lambs born after in vitro embryo production from prepubertal lamb oocytes and frozen-thawed unsorted and sex-sorted spermatozoa. Reprod Fertil Dev **16,** 260. (Abstract).

Morton KM, Catt SL, Maxwell WM, Evans G, 2005: An efficient method of ovarian stimulation and in vitro embryo production from prepubertal lambs. Reprod Fertil Dev **17,** 701–706.

Morton KM, Herrmann D, Sieg B, Struckmann C, Maxwell WM, Rath D, Evans G, Lucas-Hahn A, Niemann H, Wrenzycki C, 2007: Altered mRNA expression patterns in bovine blastocysts after fertilisation in vitro using flow-cytometrically sex-sorted sperm. Mol Reprod Dev **74,** 931–940.

Parrilla I, Vazquez JM, Cuello C, Gil MA, Roca J, Di Berardino D, Martınez EM, 2004: Hoechst 33342 stain and u.v. laser exposure do not induce genotoxic effects in flow-sorted boar spermatozoa. Reproduction **128,** 615–621.

Probst S, Rath D, 2003: Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes. Theriogenology **59,** 961–973.

Rath D, 2002: Low dose insemination in the sow. A review. Reprod Domest Anim **37,** 1291–1304.

Rath D, Niemann H, 1996: In vitro production of swine embryos. Dtsch Tierarztl Wochenschr **103,** 331–336.

Rath D, Niemann H, 1997: *In vitro* fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology **47,** 785–793.

Rath D, Johnson LA, Welch GR, 1993: In vitro culture of porcine embryos: development to blastocysts after in vitro fertilization (IVF) with flow cytometrically sorted and unsorted semen. Theriogenology **39,** 293. (Abstract).

Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL, Johnson LA, 1999: In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci **77,** 3346–3352.

Rath D, Ruiz S, Sieg B, 2003: Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. Vet Rec **152,** 400–401.

Rhodes SL, Maxwell WMC, Evans G1994: DNA analysis and sorting of ram spermatozoa by flow cytometry and subsequent fertilization of in vitro matured sheep oocytes. In: Proceedings of the 7th International Symposium of Spermatology, Cairns, Australia, pp. 9.26. (Abstract).

Salamon S, Maxwell WMC, 2000: Storage of ram semen. Anim Reprod Sci **62,** 77–111.

Schenk JL, Seidel GE Jr, 2007: Pregnancy rates in cattle with cryopreserved sexed spermatozoa: effects of laser intensity, staining conditions and catalase. Soc Reprod Fertil Suppl **64,** 165–177.

Seidel GE Jr, 2003a: Economics of selecting for sex: the most important genetic trait. Theriogenology **59,** 585–598.

Seidel GE Jr, 2003b: Sexing mammalian sperm-intertwining of commerce, technology, and biology. Anim Reprod Sci **79,** 145–156.

Seidel GE Jr, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR, Graham JK, Cattell MB, 1997: Uterine horn insemination of heifers with very low numbers of non-frozen and sexed spermatozoa. Theriogenology **48,** 1255–1264.

Seidel GE Jr, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD, Cran DG, 1999: Insemination of heifers with sexed sperm. Theriogenology **52,** 1407–1420.

Seidel GE Jr, Brink Z, Schenk JL, 2003: Use of heterospermic insemination with fetal sex as the genetic marker to study fertility of sexed sperm. Theriogenology **59,** 515.

Spinaci M, De Ambrogi M, Volpe S, Galeati G, Tamanini C, Seren E, 2005: Effect of staining and sorting on boar sperm membrane integrity, mitochondrial activity and in vitro blastocyst development. Theriogenology **64,** 191–201.

Suh TK, Schenk JL, Seidel GE Jr, 2005: High pressure flow cytometric sorting damages sperm. Theriogenology **64,** 1035–1048.

Tummaruk P, Sumransap P, Techakumphu M, Kunavongkrit A, 2007: Distribution of spermatozoa and embryos in the female reproductive tract after unilateral deep intra uterine insemination in the pig. Reprod Domest Anim **42,** 603–609.

Vazquez JM, Martinez EA, Parrilla I, Roca J, Gil MA, Vazquez JL, 2003: Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa. Theriogenology **59,** 1605–1614.

Vazquez JM, Martinez EA, Parrilla I, Cuello C, Gil MA, Garcia E, 2006: Improving the efficiency of laparoscopic intraoviductal insemination with sex sorted boar spermatozoa. Reprod Fertil Dev **18,** 283. (Abstract).

Walker SK, Smith DH, Little DL, Warnes GM, Quinn P, Seamark RF, 1984: Artificial insemination and transfer of embryos by laparoscopy. In: Lindsay DR, Pearce DT(eds), Reproduction in Sheep. Australian Academy of Science and Australian Wool Corporation, Canberra, pp. 306–309.

Weigel KA, Barlass KA, 2003: Results of a producer survey regarding crossbreeding on US dairy farms. J Dairy Sci **86,** 4148–4154.

Welch GR, Johnson LA, 1999: Sex preselection: laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA. Theriogenology **52,** 1343–1352.

Author's address (for correspondence): D. Rath, Institute of Farm Animal Genetics, Mariensee (FLI), 31535 Neustadt, Germany.
E-mail: detlef.rath@fli.bund.de

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