Sex selection of sperm in farm animals: status report and developmental prospects

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Abstract

Pre-selection of spermatozoa based on the relative DNA difference between X- and Y-chromosome bearing populations by flow cytometry is an established method that has been introduced into commercial cattle production. Although several important improvements have increased the sort efficiency, the fertilising ability of sexed spermatozoa based on offspring per insemination is still behind farmers' expectations. The main stress factors, especially on mitochondria, that reduce the lifespan of spermatozoa are described, and new technical as well as biological solutions to maintain the natural sperm integrity and to increase the sorting efficiency are discussed. Among these methods are the identification of Y-chromosome bearing spermatozoa by bi-functionalised gold nanoparticles and triplex hybridisation *in vivo* as well as new laser-controlled deflection system that replaces the deflection of spermatozoa in the electrostatic field. Additionally, as well as a new nonsurgical transfer system of spermatozoa into the oviduct of cows has been developed and allows a significant reduction of spermatozoa per transfer. Altogether, the improvements made in the recent years will allow a broader use of sex-sorted spermatozoa even in those species that require more cells than cows and sheep.

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Introduction

In 2011, the world's population reached seven billion people and it is predicted that this figure will rise above ten billion by the turn of the century. In order to meet the global food demand associated with this population growth, it will be necessary to utilise modern biotechnologies to promote the sustainable production of animals and agricultural resources. The FAO has recognised that production of pre-sexed livestock by sperm or embryo sexing, when combined with other biotechnologies, genomics, proteomics or phenomics, for example sperm-mediated gene transfer (Kues *et al.* 2008b, De Cecco *et al.* 2010, Niemann *et al.* 2011), offers a promising breeding strategy to help meet the increased demand for food production.

Besides the long-term benefit, farmers can profit directly from the use of sexed spermatozoa by producing optimal proportions of males and females in their animal production systems. The use of sexed spermatozoa increases the rate of genetic progress, especially

in combination with genomic selection of sires. It also has the potential to improve herd management and diminish the incidence of dystocia by avoiding male calves. Sex pre-selection also decouples the quantity of dairy replacement heifers from those required for milk production. In consequence, more meat and milk will appear at least for a certain while on the market and reduce prices for heifers and milk (De Vries *et al.* 2008). Other economic benefits are the production of replacement heifers and heifers for export to developing countries.

In mammals, sex is determined by gonosomes, where an XX chromosome combination determines a female, and an XY chromosome combination a male. As sex chromosomes differ significantly in length, a discrimination of X- and Y-chromosome bearing sperm populations is possible. Several methods to separate X- and Y-chromosome bearing populations of ejaculates have been investigated, but thus far only quantitative, total DNA amount-based differentiation by flow cytometry has proven repeatedly successful and highly accurate.

A possible alternative, interferometry should be mentioned; but the technique has yet to reach practical application ([van Munster 2002](#)). Several reports have described in detail the possibilities and limitations of flow cytometry, which requires the identification of individual spermatozoa. With current high-speed flow cytometry, enrichment of the desired sex >90% can be obtained for most species. Although each spermatozoon has to be individually examined for DNA content, limiting the number of spermatozoa that can be sorted to <20 million/h, several million calves have been born after fertilisation with spermatozoa processed by this technique. In other species, the number of offspring born from artificial insemination (AI) or IVF with sexed spermatozoa is negligibly small.

Recent advances in sex-sorting strategies include innovations in fluid techniques, droplet handling, biological improvements and application of latest nanotechnology methods using gold bioconjugates, which together offer substantial future prospects for application in sperm sexing.

Sex sorting of spermatozoa by flow cytometry

After it was demonstrated that it is not possible to differentiate sex-specific markers by physical methods, [Gledhill et al. \(1982\)](#) published the first experiments on flow cytometry on spermatozoa, which were followed by further technical adaptations to these specific cells ([Fulwyler 1977](#), [Dean et al. 1978](#), [Stovel et al. 1978](#), [Pinkel et al. 1982](#), [Garner et al. 1983](#), [Johnson & Pinkel 1986](#), [Johnson et al. 1987a](#), [1987b](#), [Johnson & Clarke 1988](#)). The first pre-sexed offspring using this technique were born in 1988 ([Morrell et al. 1988](#)). In principle, the sorting technique is based on quantitative flow cytometry, where spermatozoa, afore co-incubated and labelled with the DNA fluorescent dye Hoechst 33342 ([Johnson & Pinkel 1986](#)), are hydrodynamically focused in a discontinuous droplet stream, which passes an u.v. laser beam. The orthogonal set-up of two detectors requires a precise orientation of the sperm head in front of the laser to resolve the small quantitative DNA difference between X- and Y-chromosome bearing spermatozoa. Before droplets disrupt from the discontinuous stream, the last hanging drop is charged according to the DNA content of the encased spermatozoon, which then passes an electrostatic field (3000 V), and droplets are deflected according to their charge. Sorted cells are pushed into a collection medium from where they are distributed to further preservation steps ([Johnson & Welch 1999b](#)). This set-up allows identification and selection of individual spermatozoa with sort purity above 90% of the desired characteristics ([Johnson & Welch 1999b](#)). Fine-tuning of the sorter hardware and acquisition software optimisation has been reported to allow a maximum sort rate of 8000 cells/s under ideal conditions ([Sharpe & Evans 2009](#)).

This is equivalent to two conventional bovine AI doses produced per hour, or 14 doses if 2.1 million spermatozoa are used for insemination ([Seidel et al. 1999](#)). The numerical limits are even more obvious for sex-sorted porcine sperm and prevent commercial applications for this species because at least 50 million spermatozoa are required to achieve a pregnancy employing deep intra-uterine AI ([Rath et al. 2003](#), [Grossfeld et al. 2005](#)). In horses, [Lindsey et al. \(2002\)](#) reported reasonable pregnancy results after hysteroscopic insemination with five million sperm.

During the sorting process, several stress factors have been identified that originate from four major sources: i) shear forces acting during the hydrodynamic focusing and passage through the injection nozzle; ii) the combination of the fluorescent dye bis-benzimide (Hoechst 33342) and cell exposure to certain wavelengths of the u.v. light spectrum; iii) repeated electrical doping corresponding to sperm DNA content and iv) the subsequent passage through the electrostatic deflection field. In addition to the sorting process itself, further impact on sperm quality is related to pre- and post-sort processes such as co-incubation with the fluorescent dye at slightly below body temperature and preservation at low temperature until insemination respectively.

Correct labelling of the condensed sperm chromatin is a prerequisite to identify DNA size differences of <4%. Hoechst 33342 (bis-benzimide) was found to be suitable for sperm DNA labelling as it passes the intact sperm membranes and preferentially binds to the AT-rich regions in the minor groove of the DNA helix (for review, see [Rath & Johnson \(2008\)](#)). While Hoechst 33342 is apparently not genotoxic, it is known to be mutagenic to cells and may induce disturbances in embryo development. Moreover, the fate of Hoechst dye transported by the spermatozoon into the oocyte and thereby into the embryo and offspring is almost unknown ([Garner 2009](#)).

Since introduction, the sperm sexing technique has undergone several modifications in order to increase efficiency as well as its separation purity. Major improvements, including the use of a bevelled needle and an orientation nozzle tip with double elliptic torsions that promote the alignment of spermatozoa in front of the laser, were developed ([Rens et al. 1998](#)). These modifications resulted in a fourfold increase in the number of sorted spermatozoa produced per unit of time. Inserted into a high-speed flow cytometer, sort rates of 12–15 million spermatozoa per hour became a reality and were the prerequisite for commercial application ([Johnson et al. 1999](#)). While this 'HISON' orientating nozzle ([Rens et al. 1998](#)) had a double torsional elliptic shape, most commercial sorters today work with a further refined single torsion nozzle (Cytonozzle; XY, Inc., Fort Collins, CO, USA) for better sperm orientation. Recently, we developed an updated version of the double torsional nozzle with an improved internal

geometry in order to optimise the efficiency of sperm orientation. Moreover, the hydrodynamic focussing is amplified by the shape of the injection tube, which has a spatule-like shape with phased edges.

Since then, only minor technical modifications have been made to further improve the rate and purity of sorting. Attempts to minimise any adverse effects of the laser excitation on spermatozoa and to reduce the intensity from 150 mW to as low as 25 mW failed to improve fertility after AI in heifers (Schenk & Seidel 2007) and after porcine embryo production (Guthrie *et al.* 2002). However, a positive effect on fertility was shown when the hydrodynamic pressure of the droplet stream was reduced from 50 to 40 psi (Schenk & Seidel 2007, Schenk *et al.* 2009). Moreover, smaller technical adaptations of the general flow system such as reduced photomultiplier signal noise (Sharpe & Evans 2009) and faster digital computing have also been implemented to improve the efficiency of sorting (Beckman-Coulter, MoFlo XDP, Krefeld, Germany). Software developments include a zoomed presentation, rotation of events and signal tracking to compensate for laser fluctuations (Beckman-Coulter Summit 4.0 and further).

Recent advances in sex-sorting strategies

Labelling the sex of sperm with nanogold: gold nanoparticle design strategy

A completely different approach focuses on a qualitative identification of sex-related differences in sperm. Numerous ineffective efforts were made in the past to bulk-separate spermatozoa by physical methods and qualitative surface markers but failed to be used on a wider scale (Cran & Johnson 1996). A well-known difference of haploid spermatozoa is their difference in DNA sequences of X- and Y-chromosomes. *In vitro* methods such as fluorescent *in situ* hybridisation are able to distinguish spermatozoa based on this information, but it requires the disintegration of the sperm head (Kawarasaki *et al.* 1998). Here we report on the status of our current stage of research employing functionalised gold nanoparticles (AuNPs) to identify Y-chromosome-specific sequences in morphologically and functionally intact sperm. Three major aspects have to be taken in account: i) penetration of functionalised AuNP through the sperm membranes, ii) non-invasive coupling of a specific DNA probe with the intact DNA double strand by triplex binding and iii) recognition of the sex-specific signal pattern to sort the sperm population.

Delivering the nanogold label: cellular internalisation of AuNPs

Interactions of NPs with cellular membranes are highly complex and depend on several intrinsic and extrinsic factors (Nel *et al.* 2009) that determine particle

behaviour at the 'nano-bio-interface': The particle as such influences cellular uptake by its particular chemical composition as well as by steric aspects such as size, shape and ligands. The medium actuates the particle's electrochemical properties by providing pH, ionic strength and temperature (Gao *et al.* 2005, Chithrani *et al.* 2006, Chithrani & Chan 2007, Jiang *et al.* 2008, Arvizo *et al.* 2010, Zhang *et al.* 2010). Furthermore, NP uptake differs among cell types due to specific membrane fluidity, surface charge and functional molecules attached to the outer cell membrane. Such effects can be nicely exemplified when comparing, for instance, the influence of ligands on internalisation. While primary ligand-free AuNPs entered the cells by an unknown non-endosomal uptake mechanism (Salmaso *et al.* 2009, Taylor *et al.* 2010), AuNPs with ordered arrangements of hydrophilic and hydrophobic functional groups were internalised by membrane wrapping (Verma *et al.* 2008), and AuNP with citrate ligands underwent endocytosis (Shukla *et al.* 2000).

To increase the efficiency of NP uptake viral vectors (Everts *et al.* 2006), dendrimers (Shi *et al.* 2007) as well as peptides containing protein transduction domains (cell penetrating peptides (CPPs)) were used (Green & Loewenstein 1988, Derossi *et al.* 1994, Lundberg & Johansson 2002). Especially, the employment of CPPs showed promising results (Tkachenko *et al.* 2004, Nativo *et al.* 2008, Mandal *et al.* 2009). Nevertheless, successful internalisation depended strongly on the cargo itself as well as on peptide concentration (Stewart *et al.* 2008, Mandal *et al.* 2009). Some of them, such as penetratin, led to a tremendously enhanced uptake of AuNPs, but only into endosomes (Petersen *et al.* 2011). However, modification of AuNPs with Tat peptide, derived from the human immunodeficiency virus type-1, and also with a peptide named nucleus localisation signal caused the particles to directly enter into the cytosol and even into the nucleus (Tkachenko *et al.* 2004, de la Fuente & Berry 2005, Berry *et al.* 2007).

Anchoring the nanogold label: principle of signal recognition

In addition to a membrane-penetration mediator, a nucleic acid probe is required to be conjugated to the AuNP for specific accumulation at the Y-chromosome. Therefore, such bivalent NPs will have to carry two different functional biomolecules at their surface, covalently bound via thiol linkers. To accomplish DNA hybridisation in non-denatured, chromatin triplex formation as described by Hoogsteen (1963) is necessary. As triplex hybridisation with DNA probes is rather weak, new DNA derivatives, such as locked nucleic acids (LNA) and peptide nucleic acids, were developed to form more stable bindings (Johnson & Fresco 1999a, Buchini & Leumann 2003, Seidman & Glazer 2003). *In vitro* AuNP-conjugated LNA probes formed stable triplexes in

solution (McKenzie *et al.* 2008). Even *in situ* triplex hybridisation without prior denaturation of the DNA could be achieved if suitable probes and protocols were used (Johnson & Fresco 1999a, Schwarz-Finsterle *et al.* 2007). The detection principle is based on the fact that AuNPs that are aggregated or accumulated, for example due to binding of probes to highly repetitive DNA sequences, exhibit a bathochromic shift of the peak maximum as well as a significant increase of the extinction in the bathochromic regime of the spectrum (Jain *et al.* 2007). The detection limit for AuNP aggregate quantification was 35 fg, which is equivalent to a single aggregate with a diameter of 60 nm (Klein *et al.* 2010; Fig. 1).

Sperm toxicity of AuNPs

The question whether AuNP may cause toxic effects on mammalian cells is a matter of concern. In particular, detrimental effects on germ cells may become obvious in the following generation. After fusion of the gametes, a series of reprogramming events is activated, which includes DNA methylation, protamine exchange, histone marks, embryonic genome activation and degradation of maternal and paternal transcripts and proteins (Bermejo-Alvarez *et al.* 2008, Kues *et al.* 2008a). A well-orchestrated succession of these events is required to allow normal development, potentially making gametes and early embryos vulnerable to NPs, which may accumulate in the reproductive tract or

which are intentionally added to spermatozoa *in vitro* (Bonde 2010, Ema *et al.* 2010, Schrand *et al.* 2010).

Information on the cytotoxicity of AuNPs is highly heterogeneous, which is mainly caused by the application to different cell lines and differently synthesised NPs; also experimental conditions are often not comparable. Only few studies investigated consequences of NP exposure on gamete and embryo development. AuNP are assumed to be relatively inert and less toxic than for example silver NPs (AgNPs). *In vivo*, an accumulation of AuNP was found in testes, as well as in liver and spleen of rats, which received i.v. injections of 20 nm AuNP. Thus, 20 nm AuNP seem to be able to cross the blood testis barrier (Balasubramanian *et al.* 2010). To address the size dependency of NPs, rats were injected with AuNP with diameters ranging from 10 to 250 nm (De Jong *et al.* 2008). In that study, the 10 nm AuNP showed the most widespread distribution in different organs, including the testis. However, only a relative small percentage of 0.2% of the total AuNP accumulated in the testis.

Few studies assessed the effects of NPs, and in particular AuNP, in IVF systems. As fertilisation comprises a number of successive steps, including capacitation, acrosome reaction, penetration of the zona pellucida, membrane fusion and cortical reaction, even subtle interferences may result in large consequences. A recent study in cattle supports the notion that concentrations of AuNP above 50 μM affect both motility of spermatozoa and fertilisation rates (Taylor *et al.* 2012).

Figure 1 Nanoparticle-based sperm sorting. Optical detection principle based on the shift of the plasmon resonance peak upon regional accumulation of gold nanoparticles. (1) Design of a bivalent nano-bioconjugate with a cell penetrating peptide (CPP) and a hybridising nucleic acid (NA). (2) Cell penetration and distribution by diffusion. (3) Selective triplex formation at the Y chromosome causes regioselective aggregation. (4) Aggregation causes a decrease of the interparticle distance Δd and results in a bathochromic shift of the peak maximum.

Another study demonstrated the occurrence of sublethal effects after incubation with 250 μM AuNPs ranging in size from 3 to 50 nm in zebra fish embryos (Bar-Ilan *et al.* 2009). Besides these acute effects, whether chronic long-term effects may be initiated remains to be tested. So far only few studies assessed the toxicity of NP on spermatozoa of farm animals. The recent development of transgenic pigs with fluorophore-loaded spermatozoa (Garrels *et al.* 2011) may be instrumental for establishment of direct comparative tests.

Droplet charging and deflection

In addition to mechanical effects (De Ambrogi *et al.* 2006), repeated electric charging and electrostatic deflection (3000 V), which are necessary to sort sperm populations, are responsible for the reduced lifespan of sorted spermatozoa observed in most species (Spinaci *et al.* 2006, 2010, Rath & Johnson 2008, Rath *et al.* 2009b). The electrostatic voltage is similar to that used for electroporation, causing a reversible transformation of membrane pores. Gagne *et al.* (1991) showed that electroporation of bovine spermatozoa reduced their *in vitro* fertility by about 30% after exposure to 500 V. Similarly, electroporation induced the acrosome reaction in the sperm membranes of human spermatozoa (Tomkins & Houghton 1988). These findings were confirmed indirectly by Maxwell & Johnson (1997) who found capacitation-like patterns in the sperm membrane after sex sorting.

Exposure of cells to an electrostatic field, as it is used to separate the two sperm populations flow cytometrically, is known to induce formation of reactive oxygen species (ROS; Sauer *et al.* 2005), and it causes membrane damages to spermatozoa (Alvarez *et al.* 1987, Aitken *et al.* 1989, Leahy *et al.* 2010). Other than a physiological level of ROS, necessary for hyperactivation, capacitation and acrosome reaction *in vitro* (de Lamirande *et al.* 1997), excessive presence of ROS directly seems to affect sperm tail integrity (Klinc & Rath 2007, Klinc *et al.* 2007). For stallion sperm, Baumber *et al.* (2000) and for human sperm, Shi *et al.* (2012) indicated that a decrease of the mitochondrial membrane potential caused by ROS is correlated with decreasing motility.

In order to detect ultrastructural changes caused to spermatozoa during the sorting process, bull sperm samples were collected at different steps during their passage through the flow cytometer. Our transmission electron microscopic analyses focused on mitochondria in the midpiece, which like glycolytic processes are thought to provide energy for motility in bull spermatozoa (Storey 2008, Piomboni *et al.* 2012). Sorted spermatozoa revealed a broad range of ultrastructural changes including among others gross disturbances of the helical arrangement of mitochondria and various degrees of mitochondrial swelling. However, the most

remarkable ultrastructural finding was a significant switch from an orthodox to a condensed mitochondrial conformation, the latter being almost absent in native sperm (J Michl, University of Muenster, Germany; personal communication). Condensed mitochondria demonstrated a clear centre, composed of enlarged intracristal spaces, which was circumscribed by a rim of margined condensed matrix (Fig. 2).

Ultrastructurally similar, if not identical changes from orthodox to condensed configurations of midpiece mitochondria have been noted under very different circumstances and conditions. Rahmy & Ayoub (2002) reported that goat spermatozoa develop condensed mitochondria, referred to as 'mitochondria with distorted cristae' by the authors, after *in vitro* application of cobra venom. According to Rahmy & Ayoub (2002), these ultrastructural alterations of the midpiece mitochondria may contribute to the observed reduction in sperm motility, viability and fertilising ability. Likewise, rapid cooling of ram spermatozoa results in the appearance of condensed mitochondria as well as in the irreversible reduction of sperm motility (Simpson *et al.* 1986). It has been clearly shown by Simpson *et al.* (1987) that even subtle influences can lead to a switch from orthodox to condensed mitochondrial forms. The authors used the ionophorous antibiotic A23187 to induce an increased uptake of calcium and oxygen in ram spermatozoa. As a result, mitochondria were significantly transformed from an orthodox to a condensed conformation at pH 8.0, but not at pH 7.0 (Simpson *et al.* 1987). Correspondingly, in mice deficient for the calcium extrusion pump membrane calcium ATPase 4 (PMCA4), intracellular calcium overload promotes an increase in the number of condensed sperm mitochondria, which failed to achieve hyperactivated motility (Okunade *et al.* 2004). Oscillatory ultrastructural modifications take place during rat spermatogenesis, transforming mitochondria from orthodox to condensed forms in spermatocytes and spermatids up to the early maturation phase (André 1962, De Martino *et al.* 1979). These condensed mitochondria, which require activin A from Sertoli cells for maintenance (Meinhardt *et al.* 2000), reverted to an 'orthodox-like' appearance during further maturation (De Martino *et al.* 1979).

It is assumed that transformation from an orthodox to a condensed conformation during sex sorting may influence energy production and motility of bull spermatozoa (J Michl 2012, personal communication). Our hypothesis is substantiated by recent evidence that ultrastructurally altered midpiece mitochondria among other mitochondria with dilated intermembrane spaces are associated with human asthenozoospermia (Pelliccione *et al.* 2011).

To avoid repeated charging and electrostatic stress during sex sorting, a new deflection method was developed (patent WO 2010/149739). The electrostatic deflection of the sperm droplet was replaced by laser

Figure 2 Midpiece mitochondria of bull spermatozoa transform from an orthodox to a condensed configuration upon sex sorting. Transmission electron micrographs show unsorted controls (A and C) and bulk sorted samples (B and D). (A) Orthodox mitochondria (m) reveal lamellar cristae (c) and an electron translucent matrix. (B) Condensed mitochondria (some marked by asterisks) demonstrate enlarged intracristal spaces and a ring-like peripheral condensation of electron dense matrix. (C and D) While orthodox mitochondria predominate in unsorted controls (C), a high ratio of condensed mitochondria was found in sorted sperm samples (D). ax, axoneme; df, outer dense fibre; n, nucleus; pm, plasma membrane. Scale bars = 200 µm (A and B) or 2 µm (C and D).

irradiation of the droplet flow. The laser does not kill sperm with unwanted sex characteristics but deflects the droplet stream into the waste. Desired sperm pass the system without any deflection force. The emitted laser light from a DPSS Er+YAG laser (MIRPac, Laser Components, Olching, Germany) was used to generate recoil in the droplet by laser-based evaporation. About 70% of the laser light was absorbed according to the Lambert Beers law by the water droplet within the first micrometre, causing droplet evaporation. Consequently, a steam jet formation resulted in an acceleration of the droplet in the direction of the beam propagation. Owing to the high absorption coefficient of the liquid at this wavelength, damage of the spermatozoa by thermal interaction with the laser is not to be expected.

Laser induced evaporation on a droplet stream resulted in a droplet deflection of 2.8° as depicted in Fig. 3.

Degree of motility and morphological integrity using phase contrast microscopy were compared 0 and 6 h after sorting of bovine spermatozoa in a sperm sorter assembled with either the usual electrostatic deflection system, or after implementation of the laser-based deflection system.

The results of six replicates indicate a significant difference ($P < 0.05$) for sperm motility between the two

techniques (Fig. 4). Comparison of both parameters with non-sorted sperm of the same ejaculate showed no differences between laser deflected and unsorted sperm, whereas electrostatic spermatozoa lost 17% of motility within 6 h after sorting, confirming the advantages of the laser-based deflection system.

Recently, a promising patent was granted that replaces the orthogonal laser set-up with epifluorescence, a technique originally described by Van Dilla *et al.* (1977). When combined with a second laser system that kills sperm of the unwanted sex (Microbix, LumiSort, Mississauga, ON, Canada), such a machine may provide an alternative method for high speed sorting. Similarly, parallel sorting based on microchip technologies might avoid the complex orientation of spermatozoa and provide much higher rates of sorting.

Recent biological improvements

Cattle

The fertilising ability of sorted spermatozoa is highly related to their motility and membrane integrity. Sperm motility requires ATP synthesis. As spermatozoa are relatively poor generators of energy, their preservation

Figure 3 Deflection of the droplet stream by laser induced photo vapourisation. The left depiction shows an undeflected droplet stream from a sorter nozzle. The right image represents the deflection of the droplet stream by laser induced photo vaporisation with an Er^{+3} -YAG laser at an emission of 1 W cw at 2.94 μm wavelength.

aims to minimise their energy consumption. This is normally accomplished by storage at a low temperature. As it would be almost impossible to run a flow cytometer under such conditions, a reversible, chemical inhibition of motility could be an alternative. Sodium fluoride (NaF) at low concentrations is able to reversibly stop the movement of spermatozoa (Zakrzewska & Udala 2006) as it inhibits the activity of critical enzymes for ATP generation, such as alkaline phosphatases, ATPase, endolases and dehydrogenases (Chinoy *et al.* 1991, Zakrzewska *et al.* 2002, Liu *et al.* 2008, Sun *et al.* 2010). These enzymes play an important role in glycolysis, cell respiration, metabolism and protein syntheses and thereby are essential for sperm motility (Sun *et al.* 2010). The presence of NaF in the sample fluid improves the post-sort/post-thaw maintenance of sperm motility in cattle (Klinc 2005, Moench-Tegeder 2008, 2011, Rath *et al.* 2009a). This is important at the time of insemination. In a modified protocol to process spermatozoa before, during and after sorting (Sexcess; Rath *et al.* 2009b), the sample fluid is supplemented with NaF. Figure 5 shows the motility pattern of sex-sorted spermatozoa during a 6-h thermo-tolerance test,

indicating that no significant differences exist between sorted, using Sexcess and non-sorted frozen/thawed spermatozoa, whereas sorted spermatozoa treated with standard protocols lose their motility much faster within the first 3 h after thawing. Similar effects are also seen for membrane integrity (Moench-Tegeder 2008, Rath *et al.* 2009b). In an initial field trial, Klinc (2005) showed that pregnancy rates equal to AI with unsorted frozen/thawed spermatozoa can be obtained from the Sexcess protocol. In a larger set-up with 2300 AI on 19 farms in Saxony, Germany acceptable pregnancy rates were confirmed when semen was sorted accordingly. However, results in this trial indicated a bull-specific effect (Moench-Tegeder 2011). An interesting side effect seen in this field study was the increased number of stillbirth (40%) in male calves originating from spermatozoa sorted for the X-chromosomal sperm population. Higher incidence of stillbirth in male calves were reported by DeJarnette *et al.* (2009) and Norman *et al.* (2010), whereas the stillbirth rate among female calves was not affected by sorting. Whether the effect is related to an accumulation of male spermatozoa with aneuploidy or whether epigenetic reasons may cause stillbirth requires further studies.

AI strategies with sex-sorted sperm

Conception rates to AI in cattle are related to sire, semen processing, sperm dosage and service number. For economic reasons, the number of spermatozoa in sorted samples is reduced to about two million sperm per dose, and this results in a pregnancy depression of 15–20% compared with unsorted spermatozoa (DeJarnette *et al.* 2011). Bull-related dilution effects may apply independently from sorting stress (Den Daas *et al.* 1998), but this remains unproven as DeJarnette *et al.* (2011) found that even the insemination of high numbers of sex-sorted spermatozoa (ten million) led to reduced pregnancy rates compared with unsorted spermatozoa inseminated at the same dose.

Even higher doses of spermatozoa are normally required for hormonally superovulated cows. The successful use of sexed spermatozoa in bovine multi-ovulation–embryo transfer (MOET) programmes critically depends on the AI dose; two million spermatozoa, as used for regular AI, are insufficient (Schenk *et al.* 2006). The success after depositing higher doses of 10 to 20 million spermatozoa seems to be dependent on the sire or MOET conditions. Whereas Schenk *et al.* (2006) obtained lower fertilisation rates after AI with 20 million sex-sorted spermatozoa compared with controls, another study showed comparable fertility among groups when two inseminations were performed in 12-h intervals with five million sex-sorted spermatozoa (Hayakawa *et al.* 2009).

In human reproduction, gamete intra-fallopian transfer (GIFT) is an accepted tool to produce

Figure 4 Deflection of the droplet stream by laser induced photo vapourisation. Comparison of motility and morphology with non-sorted sperm of the same ejaculate showed no differences between laser deflected and unsorted sperm, whereas electrostatic spermatozoa lost 17% of motility within 6 h after sorting, confirming the advantages of the laser-based deflection system.

pregnancies. For this method, instead of IVF, male and female gametes are transferred together into the oviduct. This has been tested successfully with sexed spermatozoa in pigs (Rath *et al.* 1994). Sperm intra-fallopian transfer replaces the intrauterine insemination with the deposition of spermatozoa directly into the oviduct. The new technique allows a significant reduction in the number of sorted cells required per insemination. Grossfeld *et al.* (2011) described a newly developed device for non-surgical transfer of sperm preparations in small volumes (30 μ l, 10^5 sperm) directly into the bovine oviduct. In the first field trials, 41.2% of the cows became pregnant using this method. Such a technique has the potential to have a significant impact on the efficiency of utilisation of sex-sorted spermatozoa.

IVF with sorted spermatozoa

IVF is a routine technique that requires very low numbers of spermatozoa for embryo production. Therefore, it seems obvious to use sex-sorted spermatozoa in IVF. An early report on bovine IVF with sex-sorted spermatozoa, Cran *et al.* (1993) reported birth of the first calves where the sex was determined before fertilisation. Cleavage rates were similar to unsorted controls, but blastocyst development was diminished after IVF with sexed spermatozoa, presumably due to capacitation-like reorganisation of the sperm membranes (Merton *et al.* 1997, Lu *et al.* 1999). Later reports indicated negative effects of sorting on sperm integrity but neither cleavage rate nor blastocyst rates were affected if sperm were centrifuged with a Percoll gradient (Carvalho *et al.* 2010). Similar results were seen by Xu *et al.* (2006) after IVF with sex-sorted sperm and vitrification. Subsequent embryo transfer revealed no developmental differences of control groups to non-sexed IVF groups nor to *in vivo* produced embryos.

However, in comparison with their counterparts derived from unsorted spermatozoa, bovine IVF embryos derived from sex-sorted spermatozoa display a reduction in the relative abundance of developmentally important genes like glucose transporter 3 (Glut3) and glucose-6-phosphate dehydrogenase (G6PD; Morton *et al.* 2007a, 2007b), which may be deleterious to the developmental competence of embryos. In other studies cleavage rates after IVF with sex-sorted spermatozoa were 30% below those with unsorted spermatozoa from the same ejaculate. In this study, blastocyst formation on day 8 was 30–40% lower than for the controls (Bermejo-Alvarez *et al.* 2008), and the number of cell cycles were reduced (Beyhan *et al.* 1999). Additionally, significant sex related differences in polyA mRNA abundance were observed in day 7 blastocysts for the genes glutathione S-transferase M3 (GSTM3), DNA (cytosine-5)-methyltransferase 3A (DNMT3A) and progesterone receptor membrane component 1 (PGRMC1). The resulting embryos exhibited a delayed onset of the first cleavage (Bermejo-Alvarez *et al.* 2010).

IVF with sex-sorted spermatozoa also affects the ultrastructure of blastocysts. In particular, the mitochondria, rough endoplasmic reticulum and nuclear envelope showed deviations (Palma *et al.* 2008). Additionally, the authors showed a positive correlation of the percentage of progressively moving, sorted spermatozoa, and blastocyst development. IVF results are also affected by sperm concentration, which needs to be higher than for IVF with unsorted sperm, and by the hydrodynamic pressure used for sorting (Barcelo-Fimbres *et al.* 2011). Although the sorting process has significant effects on sperm viability, and bull effects are obvious, compromised DNA does not affect reduced fertility as most non-viable spermatozoa, and those with aneuploidies are out-gated from sorting by parallel

labelling with Hoechst 33342 and food dye #40 (Warner Jenkinson, St Louis, MO, USA; Johnson & Welch 1999b). Similarly, bisulphide sequencing of Nellore bull spermatozoa DNA indicated that the sorting process by flow cytometry does not affect the overall DNA methylation patterns of the insulin-like growth factor 2 (*IGF2*) and *IGF2* receptor (*IGF2R*) genes in sperm, although individual variation in methylation patterns among bulls was observed (Carvalho *et al.* 2012).

Figure 5 Thermo-tolerance test for motility and acrosome integrity of sex sorted, frozen/thawed spermatozoa using different sperm treatments.

Sex-sorted porcine spermatozoa

For the swine industry, sex-sorted spermatozoa would have major applications, for example to avoid castration. However, because of the high number of sperm required for AI, the flow cytometric method is far too slow and inefficient to satisfy biological and commercial demands. Theoretically, the number of spermatozoa necessary for AI can be reduced from three billion to ten million if semen is deposited directly in front of the utero-tubal junction (Krueger & Rath 2000), whereby uterine mechanisms of sperm selection are circumvented (Schuberth *et al.* 2008, Taylor *et al.* 2008, 2009a, 2009b, 2009c). Accordingly, a suitable insemination instrument has been developed for deep intrauterine AI (Martinez *et al.* 2001, Rath *et al.* 2003, Vazquez *et al.* 2003, Grossfeld *et al.* 2005), and pregnancies were obtained when only 50–100 million sorted sperm were inseminated into the tip of the uterine horn. Further reduction could be obtained after laparoscopic insemination into the oviduct (Vazquez *et al.* 2005).

For limited applications, embryo transfer may be an alternative to AI. The first IVF pig embryos derived via sexed sperm were produced in 1993 (Rath *et al.* 1993) with the first pregnancies attained 3 years later (Rath *et al.* 1997, 1999, Abeydeera *et al.* 1998). Despite this success, the system never reached a commercially applicable standard, as the litter size was low compared with the unsorted controls.

In vitro sexed spermatozoa have a tendency for polyspermic oocyte penetration. An alternative is to utilise ICSI, a highly efficient method using only a single sperm cell that is mechanically introduced into the ooplasm (Probst & Rath 2003). The limitations of this technique are the expensive equipment required, and the need for surgical transfer of the resulting early stage embryos into the oviduct. With current laboratory protocols, later stage embryos are difficult to obtain *in vitro* after ICSI.

For practical reasons, GIFT into the oviduct may have advantages over IVF and has been demonstrated in pigs (Rath *et al.* 1994) in combination with surgical intervention. However, to use GIFT as a broader application, much emphasis has to be focussed on the development of non-surgical access to the oviduct. First developmental steps have been demonstrated (Vazquez *et al.* 2008).

Sex-sorted ovine spermatozoa

The use of sexed spermatozoa has genetic, management and financial benefits for sheep production enterprises, whether they are dairy, wool and/or meat. The use of IVF and ICSI is generally not commercially relevant in these industries, so sex-sorting research in sheep

has focused on delivery of sexed, frozen semen by laparoscopic intrauterine AI.

Early experiments showed that sex-sorted, frozen-thawed ram spermatozoa were functionally compromised to a similar extent to that of cattle, with reduced fertility following AI when compared with non-sorted, frozen controls (Hollinshead *et al.* 2002). *In vitro* assessment of sex-sorted ram spermatozoa showed a population of cells with reduced post-thaw motility and longevity as well as a tendency towards premature capacitation (Hollinshead *et al.* 2003). Combining these findings suggested that sex-sorted ram spermatozoa had a reduced fertilising lifespan, which would explain the aforementioned decrease in fertility. In subsequent field experiments, these fertility problems were shown to be partly ameliorated by insemination of larger numbers of sexed cells (Hollinshead *et al.* 2003), but this was an unpalatable solution considering the commercial imperative to minimise the number of sexed spermatozoa per AI dose. Fortunately, with increased stability of the flow cytometer and modifications to the cryopreservation process of the sexed cells, the function and fertility of sexed ram spermatozoa dramatically improved.

It has since been established that sex-sorted ram spermatozoa break the dogma that sex sorting by flow cytometry has a negative impact on the function and fertility of spermatozoa from all species (de Graaf *et al.* 2009). Ram spermatozoa, which have been sex sorted, exhibit higher motility, viability, acrosome integrity and mitochondrial activity than non-sorted controls (de Graaf *et al.* 2006). These *in vitro* results are supported by *in vivo* studies demonstrating that sex-sorted ram spermatozoa result in similar or superior fertilisation/lambing percentages to non-sorted controls, when artificially inseminated into non-superovulated ewes in very low numbers (de Graaf *et al.* 2007c, Beilby *et al.* 2009; one million motile spermatozoa/ewe) or when sorted from previously frozen samples and re-frozen (de Graaf *et al.* 2007b); known as 'reverse sorting' or into superovulated animals used in MOET programs (de Graaf *et al.* 2007a). It appears that the sex-sorting process selects a highly functional sub-population of spermatozoa from the ejaculate, which results in sexed ram spermatozoa demonstrating a superior fertilising lifespan inside of the female reproductive tract compared with unsorted spermatozoa from the same ejaculate (Beilby *et al.* 2009). This beneficial effect of sorting is also observed when sex-sorted ram spermatozoa are used for IVF (Beilby *et al.* 2010).

While there is no apparent (negative) effect on fertility, ram spermatozoa are not left unchanged by the process of sex sorting by flow cytometry. Sex-sorted ram spermatozoa exhibit reduced velocity characteristics and cervical mucus penetration (de Graaf *et al.* 2006) as well as increased susceptibility to oxidative damage by hydrogen peroxide compared with their unsorted counterparts (Leahy *et al.* 2010). In addition, ovine

embryos fertilised *in vivo* with sex-sorted spermatozoa display down-regulation of *G6PD* and *SLC2A3* gene transcripts, but with no apparent effect on embryo development (Beilby *et al.* 2011). The importance of these changes are easy to overlook given the lack of an effect on fertility, but they perhaps shed some light on potential sub lethal damage that occurs during the sorting process that may be of more significance in other species with a reduced tolerance to the stressors of flow cytometry or less supportive sorting protocols.

Whatever the case, the recent fertility successes with sheep are an encouragement to investigators working to improve the fertility of sexed spermatozoa in other species and make sex pre-selection technology a commercially viable and effective reproductive management option for the sheep industry.

Reverse sorting

The previous section touched on a procedure known as 'reverse sorting', which has become popular in recent times as it allows previously frozen spermatozoa to be separated for sex. This technique is useful for males that are located a great distance from a sex-sorting site or which are deceased and thus unable to provide fresh ejaculates for sex sorting. The modern 'reverse sorting' technique overcomes the resolution and staining problems observed by early investigators (Stap *et al.* 1998) by washing frozen spermatozoa through a density gradient and thereby removing the dead cells, glycerol and egg yolk, which may interfere with the staining process or negatively impact on the physiology of the remaining viable cells (O'Brien *et al.* 2003, Underwood *et al.* 2009a).

The first pre-sexed offspring produced from frozen-thawed, sex-sorted re-frozen-thawed spermatozoa were achieved in sheep following IVF and embryo transfer (ET) (Hollinshead *et al.* 2004) and some years later using AI (de Graaf *et al.* 2007b). Notably, in the latter study, frozen-thawed, sex-sorted, re-frozen-thawed ram spermatozoa had similar fertility to sex-sorted and even non-sorted controls. These *in vivo* findings were supported by *in vitro* data, which showed that frozen-sorted-refrozen ram spermatozoa, while slightly inferior to spermatozoa sex-sorted from fresh samples, still had higher motility, acrosome integrity, viability and mitochondrial activity than frozen-thawed controls (de Graaf *et al.* 2006). As with normal sex-sorted samples, frozen-sorted-refrozen spermatozoa displayed reduced velocity characteristics and ability to penetrate artificial cervical mucus, but the *in vivo* findings of de Graaf *et al.* (2007b) suggest that these functional alterations have a minimal bearing on fertility.

Unfortunately, similar success with reverse sorting has not been shared by cattle. Reverse sorted re-frozen bull spermatozoa display poorer motility and viability *in vitro*, particularly after incubation, when compared

with frozen–thawed controls (Underwood *et al.* 2009b). While pre-sexed calves have been born following AI of frozen–sorted–refrozen bull spermatozoa, fertility is very low (Underwood *et al.* 2010b) and characterised by pregnancy loss (Underwood *et al.* 2010a). Greater success has been achieved with the use of frozen–sorted–refrozen bull spermatozoa in IVP systems where cleavage and blastocyst production have been comparable to that obtained with sex-sorted frozen spermatozoa and non-sorted frozen controls (Underwood *et al.* 2010c).

The reason for the varied success of the ‘reverse sorting’ technique between sheep and cattle is no doubt related to the species variation in fertility of sex-sorted spermatozoa mentioned earlier in this review. The reason for this difference between the species is the source of some debate (de Graaf *et al.* 2009) and has yet to be determined. A greater understanding of this issue would likely bring improvements to the fertility of sex-sorted re-frozen bull spermatozoa, and perhaps further development of this technique in other species. To date, only the bottlenose dolphin (O’Brien *et al.* 2009) has joined cattle and sheep in the production of pre-sexed offspring from frozen–sorted–refrozen spermatozoa, but preliminary development of sorting protocols continues in a number of other wildlife and domestic species.

Sex-sorted equine spermatozoa

The limitations of equine AI with sex-sorted spermatozoa are similar to those in pigs. Normal AI in mares requires 0.5 to 1 billion spermatozoa. Buchanan *et al.* (2000) reported the birth of the first foal after non-surgical AI with sex-sorted spermatozoa. Two methods helped to reduce the insemination dose. A non-surgical method was described by Buchanan *et al.* (2000), who introduced a flexible AI catheter deep into the uterine horn. More effective was the hysteroscopic insemination, where spermatozoa are deposited directly onto the uterine papilla, the entrance of the utero-tubal junction (Rigby *et al.* 2001). The success seems to be dose dependent as Morris *et al.* (2000) found an increasing pregnancy rate from 29% (0.5 million spermatozoa) to 75% (five million spermatozoa). Higher dosages did not result in higher pregnancy rates. Sortability varies among stallions and ejaculates. Post-thaw survival of spermatozoa depends on the extenders used for sorting and freezing (Clulow *et al.* 2008, Gibb *et al.* 2011, Balao da Silva *et al.* 2012). A good method to rank stallions and predict their usability for sorting and freezing is the microscopic determination of the percentage of dead sperm (Clulow *et al.* 2009).

A modified freezing protocol significantly improved the post-thaw quality of sex-sorted spermatozoa (Heer 2007). The author sorted equine spermatozoa in the presence of different antioxidants and pre-selected spermatozoa after collection by density gradient

centrifugation. In consequence, a significantly higher percentage of morphologically normal spermatozoa and viable, non-acrosome reacted spermatozoa as well as a significantly lower percentage of dead spermatozoa were obtained after sorting and freezing/thawing compared with samples that were prepared in the conventional way.

Conclusions and outlook

Despite more than 30 years of development, commercial operations that utilise sperm sorting by flow cytometry to pre-select the sex of offspring remain largely restricted to applications in cattle. In other farm animals, only marginal numbers of offspring have been born, and commercial availability of the sorting technology remains limited. Even though the speed of sorting has greatly increased during this time period, each individual spermatozoon still needs to be assessed and sorted accordingly. In consequence, the sperm dosage for AI in all species has to be reduced, which has negative impacts on fertility and has contributed greatly to the limited introduction of sexed spermatozoa into the breeding market.

Several improvements have recently made sperm sorting more efficient and less harmful for spermatozoa. The future use of inert AuNPs and a harmless deflection method are two specific modifications, which show great promise to improve the longevity and fertilising capacity of sorted spermatozoa. Besides the technical improvements, several biological adaptations have been developed and have increased the sort efficiency and post-sort sperm quality. In combination with other techniques such as IVF, ICSI or intra-tubal sperm transfer, it is very likely that sexed spermatozoa will be used in those species where higher numbers of spermatozoa are usually required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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